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Grigg, Jonathan; Walters, Haydn; Sohal, Sukhwinder Singh; Wood-Baker, Richard; Reid, David W.; Xu, Cang-Bao; Edvinsson, Lars; Morissette, Mathieu C.; Staempfli, Martin R.; Kirwan, Michael; Koh, Lee; Suri, Reetika; Mushtaq, Naseem

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LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

**Cigarette smoke and platelet activating factor receptor-dependent
adhesion of *Streptococcus pneumoniae* to lower airway cells**

Jonathan Grigg¹, E Haydn Walters², Sukhwinder Singh Sohal², Iain Dickson¹,
Michael Kirwan¹, Cang-Bao Xu³, Lars Edvinsson³, Naseem Mushtaq¹

¹ Centre for Paediatrics, Blizard Institute, Barts and the London School of
Medicine and Dentistry, Queen Mary University London, London, E1 2AT, UK

² NHMRC National Centre for Research Excellence in Chronic Respiratory
Disease, Menzies Research Institute, 17 Liverpool St, Hobart, 7000, Australia.

³ Division of Experimental Vascular Research, Institute of Clinical Science in
Lund, Lund University, Lund, Sweden

Correspondence to:

Professor Jonathan Grigg

Blizard Institute,

Barts and the London School of Medicine and Dentistry,

Queen Mary University London,

4 Newark Street

London E1 2AT, UK

E mail; j.grigg@qmul.ac.uk

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Abstract.

Background

Active smoking and passive exposure to environmental tobacco smoke is associated with increased risk of invasive pneumococcal disease, including pneumonia. The mechanism for this association is unclear. Adhesion of *S. pneumoniae* is mediated by the receptor for platelet activating factor (PAFR) expressed on lower airway cells. In this study, we sought to assess PAFR expression in smokers' airways, and model the effect of cigarette smoke extract (CSE) on pneumococcal adhesion to A549 cells *in vitro*.

Methods

Expression of PAFR was assessed by immunostaining and image analysis of bronchial biopsies from healthy active-smokers and never-smokers.

Pneumococcal adhesion *in vitro* was assessed using A549 cells exposed to cigarette smoke extract (CSE $\leq 1\%$) and expressed as colony forming units (CFU)/mL. The functional role of PAFR was determined using the specific receptor blocker CV-3988, and PAFR transcript level by quantitative RT-PCR.

Results

No PAFR expression was found in 11/11 never-smokers. PAFR expression was found in 6/16 active smokers (Chi square $P < 0.001$). CSE $\geq 0.5\%$ increased pneumococcal adhesion to A549 cells ($P < 0.01$ vs. control). CSE 0.5% increased PAFR transcript level ($P < 0.05$) and stimulated-adhesion was abrogated by co-incubation with the PAFR receptor blocker.

Conclusion.

Cigarette smoke upregulates pneumococcal adhesion to lower airway cells via a PAFR-dependent mechanism. Since we have previously reported that fossil-fuel pollution upregulates pneumococcal adhesion via PAFR, these findings suggest that there are common mechanisms underlying vulnerability to pneumonia associated with emissions from combustion fuels/products.

BACKGROUND

Epidemiological studies suggest that exposure to cigarette smoke is a major risk factor for infection by *Streptococcus pneumoniae* (pneumococcus). Nuorti *et al*¹ reported that for active smokers, the adjusted risk for invasive pneumococcal disease is 4.1 (95% confidence interval; 2.4 to 7.3), and 2.5 (1.2 to 5.1) for adults exposed to environmental tobacco smoke (ETS). Suzuki *et al*² reported that ETS was associated with paediatric hospital admissions with pneumonia (adjusted OR 1.55, 1.25 to 1.92), and estimated that 28% of pneumonia in Vietnamese children is attributable to ETS exposure. Since *S. pneumoniae* is the major cause of pneumonia³, these data suggest that vulnerability to pneumococcal pneumonia is increased by both active and passive exposure to cigarette smoke. Biological plausibility for this association is provided by animal models. For example, chronic exposure of mice to cigarette smoke followed by pneumococcal infection, increases morbidity and colony forming unit counts (CFU) of pneumococci in lung tissue⁴.

To date, the mechanism whereby cigarette smoke increases vulnerability to pneumococcal pneumonia is unclear. A prerequisite step for the development of pneumonia is the ability of bacteria to adhere to lower airway cells³.

Pneumococcal adhesion to lower airway cells is enhanced by heterogeneous stimuli including; acid⁵, respiratory viral infection⁶, and interleukin-1 (IL) α ⁷. For clinically relevant strains of *S. pneumoniae*, adhesion to lower airway cells is achieved by phosphorylcholine in the bacterial cell wall interacting with the receptor for platelet activating factor (PAFR) expressed on the surface of host

cells^{5 6 7}. Normal internalisation of PAFR subsequently transports pneumococci through the cell wall into the cytoplasm⁷. Recently, we reported that carbonaceous particulate matter (PM) from urban air pollution increases adhesion of *S pneumoniae* to lower airway cells *in vitro*⁸. We therefore hypothesised that cigarette smoke upregulates pneumococcal adhesion to lower airway cells via a PAFR-dependent mechanism. In this study, we sought to assess PAFR expression in bronchial epithelial cells from healthy adult smokers, and model the effect of cigarette smoke on pneumococcal adhesion using cultured human airway cells.

METHODS

Subjects

Active smokers without clinical lung function impairment and healthy never-smokers underwent bronchoscopy and bronchial biopsy. All subjects gave written, informed consent approved by the Human Research Ethics Committee (Tasmania) Network (Approval number: H0007017). Potential participants were interviewed and examined by a respiratory physician. Subjects with a history suggestive of asthma, other respiratory disorders and uncontrolled comorbidities were excluded. Non-smokers had no history of respiratory illness or smoking. For normal smokers the inclusion criteria were; a minimum 10 pack-year history of cigarette smoking with spirometry within normal limits ($FEV_1 > 80\%$ of predicted, and $FEV_1/FVC > 70\%$). Bronchoscopies were performed as previously described⁹. Endobronchial biopsies were taken from subsegmental carinae of the right lower lobe of each patient, using alligator forceps (FB-15C; Olympus, Tokyo, Japan).

Immunostaining

Bronchial biopsies were fixed in 4% neutral buffered formalin for 2 h and subsequently processed into paraffin through graded alcohol and xylene using a Leica ASP 200 tissue processor. Sections were cut at 3 μ m from individual paraffin blocks, stained with haematoxylin and eosin and morphologically assessed for immunostaining. Following removal of paraffin and hydration to water, immunostaining for PAFR was done using anti-PAFR monoclonal antibody

(11A4, clone 21, Catalogue No. 160600, 1/80 dilution, for 1 h at 20 °C with no heat retrieval). In each case, the primary antibody was replaced using a species-appropriate IgG₁ (Dakocytomation, Denmark X0931 clone DAK-G01) at equivalent dilutions and conditions as negative controls. Endogenous peroxidase blocking was done with 3% hydrogen peroxide in milli-ro water for 15 min at 20 °C to eliminate unspecific staining but no serum block was necessary. Bound antibodies were elaborated using Peroxidase-labeled Envision + (Dakocytomation, Denmark cat. no. K4001) and liquid DAB + (Dakocytomation, Denmark cat. no. K3468).

Biopsy analysis

Computer-assisted image analysis was performed (Leica DM 2500 microscope, Microsystems, Germany), with a Spot insight 12 digital camera and Image Pro V5.1 (Media Cybernetics, USA) software. Expression of PAFR was measured on randomised and coded slides an operator thus blinded to smoking status and expressed as either; i) percentage of epithelium stained for PAFR, or ii) the proportion of subjects exhibiting any PAFR staining of bronchial epithelium.

Cell culture

A549, a human type II pneumocyte cell line, was obtained from Sigma Aldrich (Poole, Dorset UK) and maintained in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum and 1% L-glutamine/penicillin-streptomycin (Lonza Ltd, Basel, Switzerland). The virulent type 2 *S. pneumoniae* encapsulated strain D39

(NCTC 7466) was purchased from the National Collection of Type Cultures (Central Public Health Laboratory, London, UK) and grown to mid-log phase (Optical Density 600=0.5) prior to adding to airway epithelial cells at $200 \mu\text{L} \times 10^8$ mid-logarithmic phase.

Cigarette smoke extract

Cigarette smoke extract in filter material was prepared at the Institute of Clinical Science Lund, (Sweden), as previously described ¹⁰. Briefly, 3 cigarettes (0.8 mg nicotine per cigarette; Marlboro®, Philip Morris USA, Pittsburgh, PA, USA) were “smoked” by a water aspirator, and cigarette smoke aspirated through a cotton wool filter. Extract of cigarette smoke (CSE) was obtained by vortexing the cotton wool filter in 1 ml DMSO (CSE 100%) ¹⁰. We previously determined that the nicotine content of CSE was 0.1 to 0.15 mg/mL ¹⁰. CSE was subsequently diluted in medium and used in the range 0.025% to 1%. DMSO *per se* at these concentrations did not alter pneumococcal adhesion.

Adhesion

Adhesion of pneumococci to A549 cells was assessed using a standard bacterial adhesion assay ^{7 11 8}. Briefly, airway cells at $2 \times 10^5/\text{mL}$ were seeded in 24-well plates (Costar, Sigma Aldrich Poole, Dorset, UK). CSE (0.025 to 1%) was added to cell monolayers and incubated for 4 h at 37°C. CSE was removed by washing twice with Dulbecco's Modified Eagle Medium (Invitrogen Ltd, Paisley, UK) and pneumococci added and incubated for 2 h ⁵. Cell monolayers were washed five

times and cells removed from the tissue culture plate by trypsin-EDTA, and lysed with ice cold sterile distilled water for 10 min. Serial lysate dilutions were plated for quantitative bacterial culture and assessment of colony forming unit count (CFU/mL) ¹². The role of PAFR in mediating adhesion was assessed by co-incubation with a PAFR antagonist. A stock solution of the specific PAFR antagonist CV-3988 ¹³ (Enzo Life Sciences, Plymouth Meeting, PA) was prepared at 1 mM in ethanol then diluted in medium to a final concentration of 10 μ M. We established that CSE did not stimulate pneumococcal proliferation *per se* (data not shown).

Cell viability

Cell viability by lactate dehydrogenase (LDH) release was done according to the manufacturer's protocol (Sigma-Aldrich). A total cell lysis control (total LDH) was included. Optical density of LDH was assessed by spectroscopy. Cytotoxicity was assessed by the MTT (3-(4,-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium) assay ¹⁴. This assay assesses the conversion of the MTT reagent to formazan which then accumulates in healthy cells. Briefly, 20 μ L MTT (5 mg/mL, Sigma Aldrich) in PBS was added to A549 cells, and incubated for 1 h. Medium was removed and 100 μ L of dimethyl sulfoxide (DMSO, Sigma Aldrich) added for 30 min. Absorbance (optical density (OD) units) was assessed at 550 nm.

PAFR transcript

Transcript level of PAFR was assessed by quantitative (q) RT-PCR. Briefly, 2 x 10⁵ A549 cells were harvested for each sample and RNA was prepared using the RNeasy mini kit (Qiagen, Crawley, UK). Random hexamers (Qiagen) and M-MLV reverse transcriptase (Invitrogen, Paisley, UK) were used to synthesise first strand cDNA according to manufacturer's instructions. mRNA transcript level of PAFR and the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined by quantitative real-time PCR using an ABI 7500 real-time PCR system (Applied Biosystems, Warrington, UK) with TaqMan primer and probe sets HS00265399_S1 (PAFR), and Hs99999905_m1 (GAPDH) respectively. mRNA transcript level was normalized to GAPDH, and the relative change in expression between samples calculated using the comparative C_T method according to the manufacturer's instructions (Applied Biosystems).

Statistical analysis

Normally distributed data are summarised as mean (standard error of the mean; SEM) and analysed using GraphPad Prism version 5.03 (GraphPad Software Inc., La Jolla, CA), with analyses done by unpaired t test. For non-normally distributed data, comparisons were done by Mann Whitney and Chi-Square tests, and data summarised as median (range, 25th, 75th centile). Cell culture data were validated by at least 3 separate experiments done at different times. For mRNA transcript number, the mean of raw data of control samples (n=5) was assigned 100% and the SEM converted to a percentage of the raw value. A *P* value of <0.05 was considered significant.

RESULTS

Bronchial biopsies for immunostaining were obtained from 16 healthy active-smokers and 11 never-smokers (Table 1). There was no difference in FEV₁/FVC ratio between the 2 groups, although FEV₁ was lower in active-smokers ($P<0.01$, Table 1). Six (38%) smokers exhibited extensive epithelial PAFR immunostaining (Fig 1A). There was no significant difference in age, pack-years, or lung function between PAFR-positive and PAFR-negative active smokers. PAFR staining of bronchial cells was not present in never-smokers (Chi-Square, never-smokers vs. smokers, $P<0.001$, Fig 1B). The median PAFR immunostaining was therefore higher in smokers compared with never-smokers (0 (0 to 0.464) vs. 0 (0 to 0) units, $P<0.05$).

Cigarette smoke extract up to 1% was not cytotoxic to A549 cells assessed by the MTT assay (Fig 2), and did not result in deposition of black particulate matter on cultured cells (Fig 3 A,B). Exposure of A549 cells to CSE 1%, but not 0.5%, induced a modest increase in LDH release ($P<0.01$ vs. medium control, Fig 4).

Cigarette smoke extract at 0.5%, and 1.0%, increased pneumococcal adhesion to A549 cells ($P<0.01$ vs. control, Fig 5). Co-incubation with the specific PAFR antagonist CV3988 abrogated the CSE-stimulated increase in pneumococcal adhesion ($P<0.001$ CSE 0.5%+CV3988 vs. CSE 0.5%, Fig 6), but had no effect on basal pneumococcal adhesion to A549 cells (data not shown).

DISCUSSION

In this study we found that PAFR, a host receptor co-opted by pneumococci to adhere to, and infect, lower airway cells, was not expressed on bronchial epithelial cells from never-smokers, but was present in a proportion of active smokers. Using an *in vitro* adhesion assay we also found upregulation of pneumococcal adhesion by CSE in cultured airway epithelial cells. Pneumococcal adhesion stimulated by CSE was blocked by a PAFR antagonist.

To date, whether there is constitutive PAFR expression in human lower airway cells is unclear. On one hand, Ishizuka *et al*⁵ reported PAFR expression in cultured ciliated tracheal epithelial cells obtained post-mortem. Shirasaki *et al*¹⁵, on the other hand, found no expression of PAFR mRNA in airway epithelium in lung tissue obtained from heart-lung transplant donors. In the present study, we found no evidence of PAFR expression in never-smokers. Expression of PAFR by bronchial epithelial cells was present in a proportion of smokers - suggesting that cigarette smoke upregulates this receptor. It is however unclear why PAFR positivity was found in only a proportion of smokers. The kinetics of PAFR expression *in vivo* are unknown. We speculate that if upregulation of PAFR by cigarette smoke is transient, then not all smokers will exhibit PAFR positivity. The time from last cigarette to bronchial biopsy was not recorded, we cannot therefore assess whether PAFR positivity was limited to those subjects who smoked on the day of the biopsy. Whether PAFR expression in smokers is associated with increased pneumococcal adhesion *in vivo* is also unknown. However, previous studies suggest that the airway cells of smokers have an

increased capacity to support pneumococcal adhesion. Riise *et al* ¹⁶, using fixed cells from bronchial biopsies, found a non-significant trend for increased adhesion of *S. pneumoniae* to airway cells of smokers with chronic bronchitis. El Ahmer *et al* ¹⁷ reported increased binding of *S. pneumoniae* to buccal epithelial cells from active smokers compared with non-smokers. More direct evidence for a role of PAFR in pneumococcal infection is provided by animal models. For example, vulnerability to pneumococcal pneumonia in rabbits exposed to exogenous IL-1 is a direct result of increased PAFR expression ⁷. In addition, increased pulmonary PAFR expression in mice after influenza virus infection is associated with increased vulnerability to subsequent pneumococcal pneumonia ¹⁸, and knockout of PAFR in this model reduces disease severity and mortality ¹⁸.

We chose to assess pneumococcal adhesion in vitro using an alveolar cell line since the alveoli are a major site for the development of pneumonia ¹⁹. Cigarette smoke extract is reported to induce significant cytotoxicity in airway cells ²⁰, but we excluded cytotoxicity as a mechanism for increased adhesion. In contrast, CSE-exposed cells released moderate levels of LDH, indicating a degree of cellular stress - a finding compatible with a previous studies showing that CS stimulates both oxidative stress and DNA synthesis in A549 cells ²¹.

The increased PAFR mRNA transcript level and attenuation of adhesion by the specific PAFR antagonist CV3988 strongly suggests a major role for PAFR in modulating CSE-stimulated pneumococcal adhesion. We did not determine

which component (s) of CSE is responsible for increased adhesion- since cigarette smoke is a mixture of thousands of potentially bioactive chemical compounds - including carbonaceous particulate matter, nicotine, alkaloids, metals, nitrosamines, polyaromatic hydrocarbons, aromatic amines, volatile organic compounds ²², and endotoxin ²³. All combustion sources release inhalable aggregates of carbonaceous nanoparticles (black carbon), and there is strong evidence that black carbon is associated with adverse health effects ²⁴. Indeed, we previously found that carbonaceous particles from urban areas in both the UK and Ghana stimulate pneumococcal adhesion to A549 cells ⁸. Undiluted CSE (100%) extract was a black tarry liquid, but when diluted to 1% it did not increase the optical density of the medium. Furthermore, in contrast with our previous study ⁸, black particulate matter was not visible on CSE-stimulated cells (4 h) under light microscopy. The mediator of CSE-stimulated adhesion is therefore unlikely to be black carbon, and more likely to be a water-soluble compound. Some evidence for this is provided by Sohn *et al* ²⁵, who assessed the effect of 3 tobacco compounds on 1152 immune response -related genes in A549 cells using an array analysis. Compared with control cells, nicotine up-regulated 39 of the 1152 genes - one of which was PAFR ²⁵.

There are limitations to the present study. First, airway epithelial cells in the lung are covered by an anti-oxidant and surfactant-rich layer of epithelial lining fluid ²⁶. Second, there are other factors that may contribute to cigarette smoke-induced vulnerability to bacterial infection. For example, cigarette smoke attenuates human β defensin-2 production ²⁷, and CSE reduces complement-

mediated phagocytosis of *S. pneumoniae* by murine airway macrophages⁴. Third, cigarette smoke in aqueous solution does not fully reflect the complex behavior and dose of chemicals depositing on cells *in vivo* - where initial deposition is followed by evaporation then diffusion back to the cell surface²⁸. Finally, although we have shown that PAFR has a major role in CSE-stimulated adhesion - we did not measure PAFR expression directly. We used flow cytometry in an attempt to assess PAFR in A549 cells, but found that CSE increased cellular auto-fluorescence in a dose-dependent manner and were therefore unable to detect specific immunostaining (data not shown).

In conclusion, we have found evidence of increased expression of PAFR in the bronchial epithelium of active smokers, and have shown that CSE upregulates pneumococcal adhesion to a lower airway cell line *in vitro* via PAFR. Since we previously found that PM₁₀ also upregulates pneumococcal adhesion via PAFR⁸, there may be common mechanisms underlying increased risk of bacterial pneumonia resulting from exposure to emissions from a range of combustion fuels/products.

LEGEND FOR FIGURES

Fig 1 A: Section of a bronchial biopsy from one of the 6/16 active smokers showing positive immunostaining of epithelial cells for platelet activating factor receptor (PAFR). PAFR staining is brown and is most pronounced in the apical part of the bronchial epithelium (arrowed). **B;** bronchial biopsy representative of all 11 never-smokers showing no significant PAFR staining. Sections are imaged by light microscopy.

Fig MTT. Conversion of MTT by A459 cells stimulated with cigarette smoke extract (CSE) for 4 h. Optical density (OD) of the formazin product is detected by spectroscopy at 550 nm. CSE does not attenuate in the ability of cells to convert MTT, indicating no significant cytotoxicity.

Fig Cells. Light microscopy (x100) of A549 cells cultured for 4 h in either medium alone (A), or 1% cigarette smoke extract (CSE) 1% (B). CSE-treated cells do not show significant deposition of black particulate matter (PM), indicating that the concentration of carbonaceous PM in 1% CSE is very low.

Fig LDH. Release of lactate dehydrogenase (LDH) by A549 cells stimulated with CSE for 4 h. Compared with LDH release after total cells lysis (total LDH), there is a modest increase of LDH by cells stimulated by 1% CSE (* $P < 0.01$ vs. medium control).

Fig. Stimulation. Effect of cigarette smoke extract (CSE) on the adhesion of *S. pneumoniae* D39 to A549 cells. Cells were cultured with CSE for 4 h, then infected with *S. pneumoniae* for 2 h. Adherence of bacteria was determined by quantitative culture and is expressed as colony forming unit count (CFU)/mL. Incubation with CSE at either 0.5% or 1.0%, increases CFU count (* $P < 0.05$ vs. medium control, by t test). Data are represented as mean and SEM ($n \geq 5$) and are representative of >3 separate experiments.

Fig Blocker. Effect of the PAFR blocker CV3988 (10 μ M) on the adhesion of *S. pneumoniae* to A549 cells stimulated with 0.5% cigarette smoke extract (CSE). The amount of adherent bacteria is expressed as colony forming units/mL (CFU). Blocking PAFR by co-incubation with CV3988 attenuates CSE-stimulated increase in CFU count. * $P < 0.001$ vs. CSE without PAFR blocker, by t-test. Data are represented as mean and SEM ($n = 5$), and are representative of >3 separate experiments

Table 1 Demographics and lung function data for adults undergoing bronchoscopy and bronchial biopsy.

	Active-smokers	Never-smokers
n	16	11
Male/Female	12/4	4/7
Age (yr)	50 (30 to 66)	54 (41 to 68)
Smoking (pack yr)	32 (10 to 57)	0
FEV ₁ (% predicted)*	99 (78 to 125)**	114 (113 to 125)
FEV ₁ /FVC ratio (% predicted)*	77 (70 to 96)	82 (71 to 88)

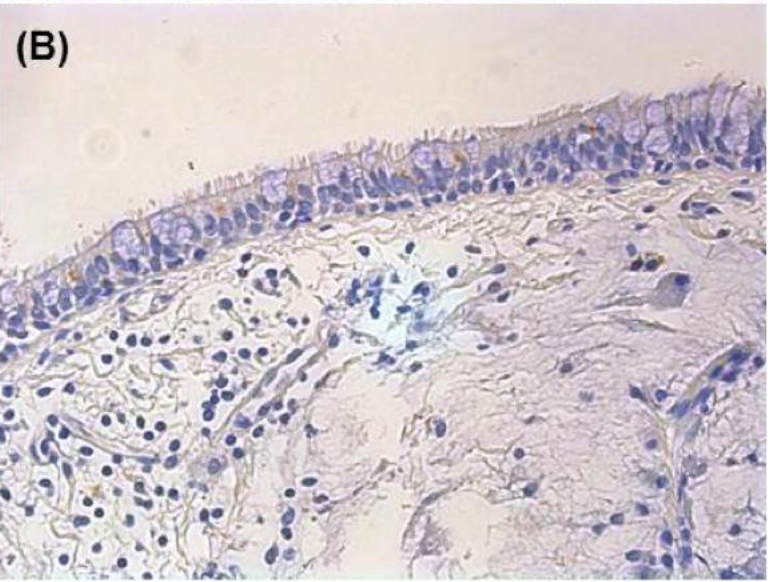
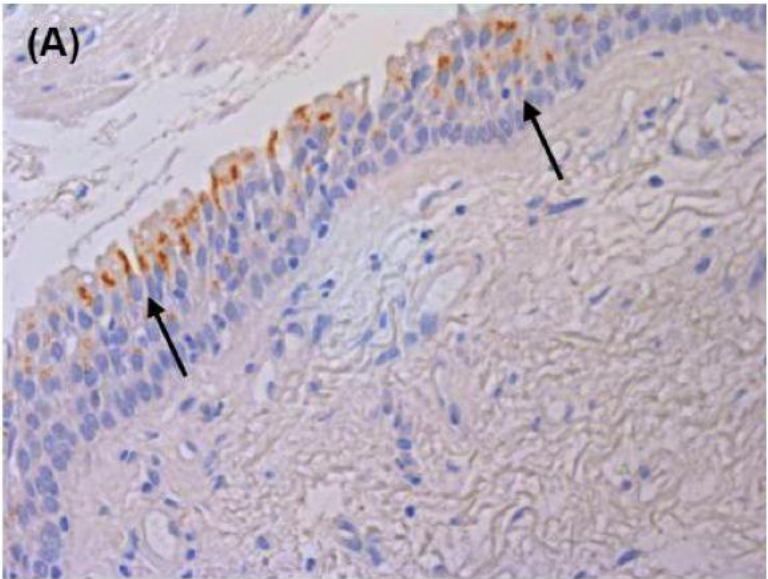
Data expressed as median and range. FEV₁; forced expiratory volume in 1 sec, FVC; forced vital capacity.

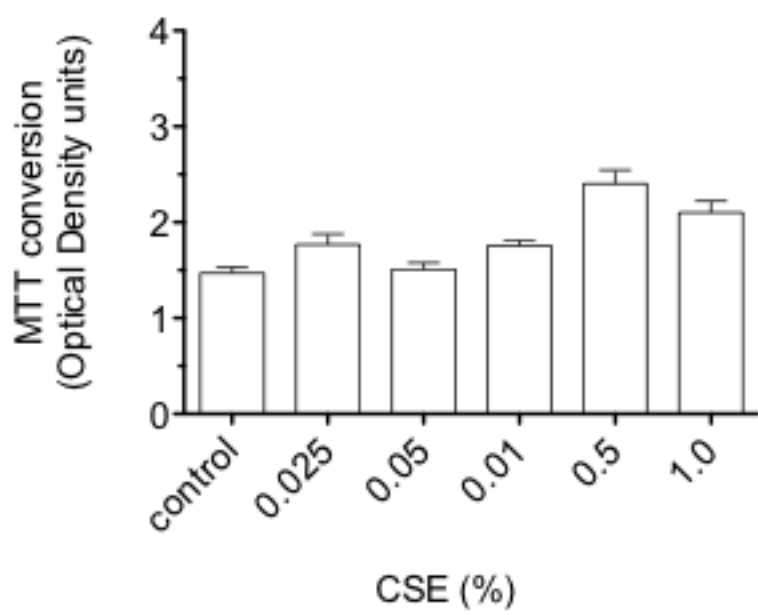
* after 400µg inhaled salbutamol, ** $P < 0.01$ vs. never -smokers.

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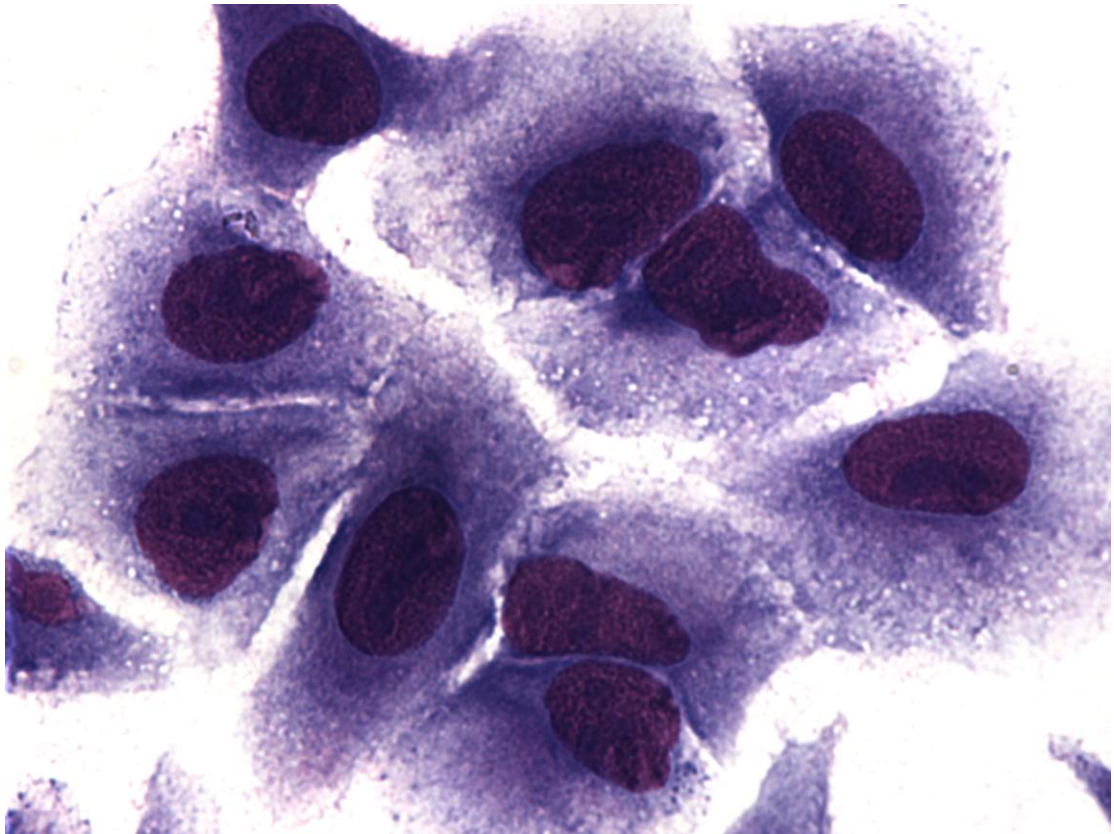
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