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Key Words:	bacterial infections, primary ciliary dyskinesia, lung disease, biofilm, mucociliary clearance

Primary Ciliary Dyskinesia Ciliated Airway Cells Show Increased Susceptibility to *Haemophilus influenzae* Biofilm Formation

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Abstract

Nontypeable *Haemophilus influenzae* (NTHi) is the most common pathogen in primary ciliary dyskinesia (PCD) patients. We hypothesized that abnormal ciliary motility and low airway nitric oxide (NO) levels on airway epithelial cells from PCD patients might be permissive for NTHi colonization and biofilm development. We used a primary epithelial cell co-culture model to investigate NTHi infection.

Primary airway epithelial cells from PCD and non-PCD patients were differentiated to ciliation using air-liquid interface culture and then co-cultured with NTHi. NTHi adherence was greater on PCD epithelial cells compared to non-PCD cells ($P<0.05$) and the distribution of NTHi on PCD epithelium showed more aggregated NTHi in biofilms ($P<0.001$). Apart from defective ciliary motility, PCD cells did not significantly differ from non-PCD epithelial cells in the degree of ciliation and epithelial integrity or in cytokine, LL-37 and NO production. Treatment of PCD epithelia using exogenous NO and antibiotic significantly reduced NTHi viability in biofilms compared to antibiotic treatment alone.

Impaired ciliary function was the primary defect in PCD airway epithelium underlying susceptibility to NTHi biofilm development compared with non-PCD epithelium. Although NO responses were similar, use of targeted NO with antibiotics enhanced killing of NTHi in biofilms, suggesting a novel therapeutic approach.

Introduction

Motile cilia in the airway contribute to mucociliary clearance (MCC), which is fundamental for protecting the host from respiratory infection. Primary ciliary dyskinesia (PCD) is a genetically heterogeneous condition characterised by abnormal ciliary function with compromised MCC. Patients are characterised by recurrent and chronic sinopulmonary infection, chronic otitis media (OM) and progressive suppurative lung disease from a young age [1].

Non-typeable *Haemophilus influenzae* (NTHi) is a Gram-negative coccobacillus and, with pneumococcus, the most common bacterial pathogen isolated from the upper and lower

respiratory tract of pediatric patients with PCD [2] and chronic OM [3]. NTHi biofilms are present *in vivo* [4], [5] and *ex vivo* on adenoid epithelium, or middle ear epithelial biopsies from children with OM [6], [7].

Biofilms are self-organising aggregates of bacteria enveloped in an exopolymeric matrix associated with the respiratory mucosal surface [8], [9], [10] and are present in chronic upper and lower respiratory tract infections in OM [6], cystic fibrosis (CF) [11], chronic rhinosinusitis [12] and non-CF bronchiectasis [13]. Biofilm development contributes to the pathophysiology of chronic suppurative respiratory diseases by increasing bacterial recalcitrance to antibiotic therapy and host innate immune responses [14], [15]. Although the effects of abnormal ciliary function on respiratory infections have been studied in animal models of bacterial-virus co-infection [16], [17], bacterial infection has not been widely studied in PCD.

For reasons that are not understood, patients with PCD have low airway nitric oxide (NO) levels compared to non-PCD patients [18], [19]. NO, produced by host epithelial cells and innate immune cells, can be low in response to infection with some respiratory bacteria [20], [21]. We previously observed no difference in the mean NO levels of uninfected primary cultured PCD and non-PCD epithelia, consistent with baseline NO levels reported by others [20], [22]. However we found PCD epithelial cells significantly increased NO production in response to either stimulation with pro-inflammatory cytokines or to NTHi infection, similar to non-PCD cells [22]. Given the susceptibility of children with PCD to airway infection with NTHi, we hypothesized that epithelial cells from patients with PCD might be permissive for increased NTHi adherence and subsequent biofilm development. To address this hypothesis, we developed a human primary co-culture model using ciliated PCD or non-PCD airway epithelial cells, differentiated at an air-liquid interface (ALI) and infected with a PCD clinical NTHi isolate. In the present study, we used the ALI-epithelial cell NTHi co-culture model [22] to more extensively investigate: 1) airway cell responses to NTHi, including NO production, and 2) the ability of a biofilm-targeted NO-donor to augment antibiotic treatment of NTHi.

Materials and Methods

Study participants

We recruited 15 patients with a diagnosis of PCD, according to the 2009 European guidelines [23], 10 healthy volunteers and 9 patients with respiratory symptoms not caused by PCD (Supplementary Table 1). We have previously published details of the diagnostic methods undertaken at our national PCD Centre [24]. The study gained UK National Health Service Research Ethics approval (06/Q1702/109 and 08/H0502/126) and we obtained written, informed consent.

Culture and biofilm development by clinical NTHi isolates from PCD patients

NTHi isolates from 4 paediatric PCD patients (HI1-HI4) were sub-cultured from frozen-stocks [25] and verified by Gram stain, V and X diagnostic disc testing, and polymerase chain reaction [6]. NTHi was cultured in brain heart infusion medium supplemented with hemin and nicotinamide adenine dinucleotide (sBHI) at 37^{o}C to OD_{600} 0.1 (~2x10^{8} CFUs/mL for all strains tested) [25]. Biofilm growth was determined *in vitro* in triplicate experiments by crystal violet (CV) staining and colony forming unit (CFU) counts on chocolate blood agar (CBA) [26], [27] and by scanning electron microscopy (SEM) and confocal microscopy using fluorescence *in situ* hybridization (FISH) [7], [28].

Epithelial cell and NTHi co-culture model

Detailed information regarding epithelial cell recovery and subsequent analysis is available in the supplementary material and has been published elsewhere. Briefly, primary human nasal epithelial cells were cultured at an ALI until differentiated and ciliated [29], [30]. We used ALI-cultured cells within 1 month. NTHi was co-cultured on the apical surface of epithelial cells from 5 PCD and 9 non-PCD subjects and compared with 5 non-infected non-PCD controls. We inoculated approximately 1x10^{8} CFUs/mL in 500 µL antibiotic-free ALI-medium (multiplicity of infection 100) onto ALI-cultured cells on transwell membranes. Co-cultures were incubated at 37^{o}C/5% CO_2 for 72 hours with daily medium changes and

washed with Hanks' Balanced Salt Solution (HBSS) to remove non-adherent bacteria prior to analyses [25].

Trans-epithelial electrical resistance (TEER) was measured daily and background subtracted to confirm epithelial integrity (EVOM 2 epithelial voltohmmeter, World Precision Instruments, US) [31]. Ciliary beat frequency (CBF) and the percentage area of motile to non-motile cilia on ALI-cultured cells before and during NTHi co-culture was measured by whole field Fast Fourier Transform (FFT) analysis (ImageJ https://imagej.net/ with an in-house written plugin) of high-speed video microscopy videos. Briefly, samples were imaged at 37^{o}C using an inverted Olympus IX71 microscope with x40 objective lens (Olympus, UK) (1 field = 90 x 95 µm) and a PC2 Photron FASTCAM high-speed video recorded ciliary movement at 250 frames per second (fps) [29].

Characterisation of NTHi on ciliated airway cells

Bacteria and epithelial cells were scraped from transwell membranes into sBHI, serially diluted, plated onto CBA and incubated at 37 ^{o}C/5% CO_2 before CFU enumeration (triplicate experiments). We also identified NTHi on ALI-culture samples using confocal microscopy by fluorescence *in situ* hybridisation (FISH) with the Hinf 16S probe [6], [7] and β-tubulin in cilia co-labelled by immunofluorescence [32]. Consecutive fields across the membrane diameter were imaged using an SP5 confocal laser scanning microscope (Leica Microsystems, UK) and NTHi volume per field was calculated by Volocity 3D image analysis software (version 6.0.1, PerkinElmer, UK). Selected transwell membranes were removed and processed and imaged using an FEI Quanta 200 Scanning Electron Microscope (FEI, NL) [25], [28].

Cytokine and LL-37 responses to NTHi infection

Baso-lateral medium from ALI-cultures was frozen before inoculation and then daily following co-culture with NTHi. We measured FGF-β, G-CSF, GM-CSF, IL-6, IL-1Rα, MCP-1, MIP-1α, TNF-α and VEGF by Fluorokine® Human MultiAnalyte Profiling (MAP) assay with Bio-Plex® 200 Analyser detection (R&D systems®, UK) in supernatants (50 µL per 96-well in duplicate) (see supplemental methods) [33]. Interleukin-8 was measured separately by

human IL-8 DuoSet ELISA kit (DY208, R&D systems®, UK) and HRP substrate was detected by ThermoMax Microplate Reader (Molecular Devices, US) as per manufacturer's instructions.

Apical supernatants (100 µL PBS) from ALI-cultured cells were frozen before co-culture and then daily following NTHi inoculation. Samples were dotted onto nitrocellulose membranes and immunolabelled overnight (4 ^{o}C) with 1:1,000 rabbit anti-LL-37 antibody (AB64892, Abcam®) followed by 1:10,000 horseradish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody. After washes Immuno-star™ Luminol chemiluminescent substrate was detected using a VersaDoc^{TM} Imager and quantified by Quantity One®v4.6.9 software (Bio-Rad Laboratories, UK). LL-37 concentrations (duplicate experiments) were compared to a standard curve from serially diluted recombinant LL-37 peptide (AB140725, Abcam®).

Nitric oxide production by airway epithelial cells and treatment with exogenous NO

Transwell membranes of live ALI-cultured epithelial cells were treated with 10 µM 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM) to assess the presence of NO [34]. Immunofluorescence labelling was used to localize NO synthase (NOS) (neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) isoenzymes in fixed epithelial cell cultures [32] using confocal microscopy.

ALI-cultured epithelial cell-NTHi co-cultures (72 hour) were washed with HBSS to remove non-adherent bacteria [25] and then treated with either 4 mg/mL azithromycin, 50 µM cephalosporin-3'-diazeniumdiolate NO donor prodrug PYRRO-C3D, or with a combination of both azithromycin and PYRRO-C3D for 2 hours. All treatments were prepared in HBSS with 1% DMSO (also used as the untreated control), and NTHi CFUs were enumerated on CBA as above (n=4) [25].

Statistical methods

Student t and Mann Whitney U tests were performed using Graphpad Prism® software (version 6.0a, 2012, Graphpad Software Inc.). The Sidak-Bonferroni method was used to adapt P values for multiple comparison t-tests. $P<0.05$ demonstrated statistical significance.

Results

Study participants

Demographic and clinical details of the 15 PCD and 19 non-PCD participants are presented in Table 1. All PCD patients exhibited abnormal ciliary function (see Supplementary Table 1).

Biofilm development by clinical NTHi isolates from PCD patients

We assessed the ability of 4 PCD NTHi isolates to form biofilms by standard assays (CV, CFU counts) and by SEM and confocal microscopy using the Hinf-specific 16S FISH probe (supplementary methods) (Figure 1A-F). Clinical isolates (HI3 and HI4), from children who repeatedly cultured NTHi over 4-years, demonstrated biofilm development with significantly greater CV staining of exopolymeric matrix compared with isolates from children culturing NTHi for the first time (HI1 and HI2) (Figure 1A). However CFU counts were not significantly different between isolates (Figure 1B). Isolate HI4 was used for future experiments.

Epithelial cell and NTHi co-culture

NTHi adhered to ciliated airway epithelial cells from both PCD and non-PCD patients. At 72 hours, however, there was significantly more viable NTHi $3.8x10^6$ CFUs ($\pm 7.5x10^5$) on PCD epithelia compared to $2.0x10^6$ CFUs ($\pm 4.8x10^5$) on non-PCD epithelia ($P<0.05$) (Figure 2A), in agreement with SEM data (Figure 2B and C). PCD cells harboured discrete adherent aggregates of bacterial cells (biofilms) (Figure 2B) in contrast to the singularly distributed NTHi bacterial cells on non-PCD epithelial cells (Figure 2C).

We imaged co-cultures by confocal microscopy using a combination of FISH to label NTHi and an anti-β-tubulin antibody to label cilia. Orthogonal section image analysis of confocal microscope Z-stacks showed that the mean (±SEM) NTHi biomass volume (per field of view) was 4 times greater on PCD ALI-cultured epithelia (710 $\mu m^3 \pm 150$) compared to non-PCD primary cell ALI-cultures (160 $\mu m^3 \pm 20$) ($P<0.001$) (Figure 2D). Aggregated NTHi were

evident in biofilms on PCD primary cell epithelia that had dysfunctional or static cilia (Figure 2E). In contrast there was less NTHi labelling on non-PCD epithelia with normally functioning cilia (Figure 2F).

PCD cells showed either static (n=4) or slow dyskinetic (n=1) cilia. The CBF of non-PCD epithelial cells remained within normal ranges (11-20 Hz) throughout the 72 hours of NTHi co-culture indicating that NTHi did not impair ciliary motility (Figure 3A). Ciliation of ALI-cultured epithelial cells was comparable between PCD and non-PCD, as observed by light microscopy and SEM (Figure 3B). The percentage area of motile cilia per field of view on non-PCD ALI-cultured cells did not change during the co-culture period on non-PCD airway cells (Figure 3C). TEER was used to measure epithelial cell barrier function before and after apical NTHi inoculation onto ciliated ALI-cultured primary airway cells. The TEER plateau reached prior to NTHi infection was the same for PCD and non-PCD cells and was maintained for 72 hours in co-culture, demonstrating epithelial integrity (p values ranged from 0.35 to 0.93) (Figure 3D).

Cytokine and LL-37 responses to NTHi infection

The mean (±SEM) concentration of cationic LL-37 peptide secreted from epithelial cell ALI-cultures peaked 24 h following NTHi inoculation, where an 8.8 fold (±2.9) increase in PCD and 3.8 fold (±0.6) increase in non-PCD samples was observed, but values remained within group variation at baseline and after inoculation (Figure 4A and B). The levels of FGF-β, G-CSF, GM-CSF, VEGF or IL-1Ra, IL-6, IL-8, MCP-1, MIP-1α and TNF-α in basolateral medium from PCD or non-PCD cells, sampled before or daily during 72 hours of NTHi co-culture (Figure 4C) did not significantly differ, indicating that PCD airway cells produced cytokines and chemokines comparable to non-PCD cells. These data indicate that PCD and non-PCD epithelial cells produce similar defence responses to NTHi.

Nitric oxide production by airway epithelial cells and treatment with exogenous NO

Airway levels of NO are extremely low in patients with PCD [18],[19]. However, we found using immunofluorescence labelling of epithelial cells that the nNOS, iNOS and eNOS

isoenzymes are present in both ciliated PCD and non-PCD cells (Figure 5). Additionally, we detected NO in PCD and non-PCD ALI-culture samples using DAF-FM, which strongly fluoresces in the presence of NO (Figure 5).

We have previously shown that NO modulates NTHi metabolism *in vitro* [25]. Therefore we tested if exogenous NO treatment impacted NTHi growing on ciliated PCD epithelial cells. Epithelial cell-NTHi co-cultures were treated with the cephalosporin-3'-diazeniumdiolate NO donor prodrug, PYRRO-C3D, which releases NO following activation by β-lactamases [25]. We found that 4 mg/mL azithromycin treatment, which is in excess of the planktonic minimum inhibitory concentration (1 μg/mL), failed to kill biofilm NTHi on PCD epithelial cells. In contrast, when PYRRO-C3D was used alongside azithromycin, NTHi viability decreased nearly 3-logs (Figure 6C). These results suggest that NTHi in biofilms on PCD ciliated epithelial cells are tolerant of antibiotic treatment and that tolerance was abrogated by β-lactamase-mediated delivery of NO from the prodrug to NTHi.

Discussion

Our principal finding was that NTHi formed adherent biofilms on ALI-cultured PCD ciliated airway cells significantly more than on non-PCD controls over 72 hours of co-culture, with the only attributable difference being ciliary dysfunction of PCD samples. Since host-pathogen interactions have not been widely studied in the context of PCD, these results are novel. NTHi is prevalent in PCD and other diseases in children associated with impaired mucociliary clearance, such as CF [35] and chronic OM [36]. Whilst NTHi biofilm development features in the pathophysiology of chronic or recurrent respiratory infections in both CF and chronic OM [6], [4, 13], little is known about NTHi infection in PCD. We hypothesized that abnormal ciliary motility on airway epithelial cells from PCD patients might be permissive for NTHi colonization and biofilm development. To our knowledge, our polarized airway epithelial ALI-culture infection model is the first to examine the effects of NTHi on primary ciliated airway cells from PCD patients and non-PCD donors. Biofilm

development was previously demonstrated with NTHi isolates from young CF patients using Calu-3 cell co-cultures [11]. However, because Calu-3 cells do not ciliate, this model did not assess the role of cilia in NTHi adherence and biofilm development [37].

We compared PCD and non-PCD epithelial cell responses, including secreted inflammatory and antimicrobial proteins and epithelial integrity, ciliation and ciliary function following NTHi co-culture. Cytokine production, including pro-inflammatory cytokines and chemokines involved in leukocyte recruitment, and secreted levels of the antimicrobial peptide LL-37, were commensurable between PCD and non-PCD co-cultures. Epithelial integrity was also comparable in airway epithelial cells co-cultured with NTHi. Although all PCD ALI-cultured epithelial cells had dysfunctional ciliary movement, in contrast to the normally functioning non-PCD epithelial cells (see supplementary table), the total ciliation pre-infection was similar, as demonstrated by SEM and immuno-fluorescent labelling of β-tubulin on cilia using confocal microscopy. Importantly, NTHi infection did not adversely affect normal ciliary function (CBF and percentage area of motile cilia) on non-PCD ALI-cultured airway cells over the experimental period. Taken together, we found the only difference between PCD and non-PCD was in ciliary motility, supporting the hypothesis that abnormal ciliary function in PCD increases the risk of NTHi biofilm development.

PCD patients exhibit abnormally low airway (particularly nasal) NO levels, the causes of which are still being debated [18]. Previously it was found that ALI-cultured PCD primary epithelial cells fail to increase NO levels early after infection with *S. pneumoniae*, suggesting that NO biosynthesis and iNOS induction was abnormal in PCD epithelial cells [20]. In contrast, the work herein extends our previous study and demonstrates that NO biosynthesis is similar in PCD and non-PCD epithelial cells, both constitutively and following NTHi infection [22]. We have now also assessed NO levels using complementary methods and show that PCD and non-PCD cells exhibit comparable NO biosynthesis and NOS isoenzyme expression that co-localises with the cilia marker β-tubulin, during co-culture. These studies support our previous data showing that PCD epithelial cells are capable of biosynthesising NO. Primary epithelial cell NO responses may, however, vary with the type of bacteria, and notably the PCD NTHi isolate did not induce the cytotoxic effects observed with *S. pneumoniae* [38].

Our results indicate that NTHi is present in matrix-enclosed aggregates adherent to polarised ciliated PCD epithelial cells [8]. Biofilm development is well known to reduce the efficacy of antibiotic therapy. Our data indicate that NTHi biofilms on airway epithelial cell cultures were tolerant of azithromycin treatment that easily kills planktonic NTHi [25], as it was ineffective in reducing NTHi viability. In contrast, co-administration of azithromycin with the novel pro-drug PYRRO-C3D, designed to target NO release specifically to bacteria following β-lactamase activation, reduced NTHi viability by nearly 3-logs on PCD epithelial cells. These results support recent studies with *S. pneumoniae* [28] and NTHi [25] showing that NO plays a role in modulating metabolic activity in these respiratory bacteria, resulting in increased antibiotic sensitivity. Importantly, our airway epithelial cell co-culture model permitted the translational investigation of anti-NTHi biofilm treatments in a physiologically relevant context with human primary cells. These results suggest that adjunctive exogenous NO therapy administered in combination with antibiotics to treat infections warrants further investigation.

This study has some limitations. First, because PCD cases are rare (≈1:10,000), the co-culture experiments are challenging to replicate in high numbers. Secondly, we examined a limited number of clinical NTHi isolates in co-cultures. Thirdly, our studies focused on epithelial cells and did not investigate other innate cell responses that are important in host defences to NTHi, or may contribute to disease progression. Despite these limitations, our studies suggest that NTHi biofilms play a role in the chronicity of respiratory infections in PCD and provide hypothesis-driven research questions for further work assessing mechanisms of pathogenesis.

In summary, this study reports key observations regarding NTHi infection of primary ciliated epithelial cells from PCD patients. Firstly, abnormal ciliary function in PCD appears to be more permissive for NTHi biofilm development than non-PCD airway cells that have normal motility. Secondly, ALI-cultured primary airway cells from PCD patients appear to be competent in several defensive responses following NTHi infection, exhibiting comparable cytokine and LL-37 production with non-PCD airway cells. Thirdly, although nasal NO levels are abnormally low in most cases of PCD, ALI-cultured airway cells from PCD patients

produced similar levels of NO following NTHi co-culture compared to non-PCD cells. Fourthly, this study demonstrates that our co-culture model provided a robust assay to investigate anti-infective treatments. Finally, we show that targeted exogenous NO enhanced antibiotic killing of NTHi in biofilms growing on PCD epithelia, suggesting a novel therapeutic approach for NTHi infection in PCD.

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Figure legends

Figure 1: Biofilm formation by PCD NTHi isolates (HI1-4). Bar charts show the mean (±SEM) optical densities (OD_{600}) of crystal violet staining (A) and mean (±SEM) log biofilm colony forming unit counts ($CFUs/cm^2$) (B) for PCD-clinical NTHi isolates (HI1-4) grown for 72 hours (*$p<0.05$). Scanning electron micrographs (x4000) (C, D) highlight bacterial aggregates and exopolymeric matrix for HI1 and HI4. Confocal micrographs (E, F) depict representative maximum projections (of Z-stacks) and orthogonal sections (x/z right and y/z bottom images) of 72 h HI1 and HI4 biofilms hybridized with the CY3-labelled *H. influenzae*-specific FISH probe (green). Scale bars = 10 µm (C, D) 40 µm (E, F).

Figure 2: Characterisation of NTHi co-cultured with air-liquid interface (ALI) differentiated and ciliated airway epithelial cells. Graph A depicts the mean (±SEM) log biofilm $CFUs/cm^2$ after 72 h of NTHi co-culture on ALI ciliated PCD (n=5), and non-PCD (n=9) epithelial cells (*$p<0.05$). Representative scanning electron micrographs (x4000) (B and C) demonstrate increased bacterial aggregates and exopolymeric matrix formation (arrows) on PCD ALI-cultured ciliated epithelial cells in contrast to non-PCD cells. NTHi co-cultured on ALI ciliated PCD and non-PCD epithelial cells were labelled for confocal analysis. Bacterial cells were labelled with the CY3-*H. influenzae*-specific FISH probe (green), cilia were labelled with anti-β-tubulin antibody (red) and nuclei were counterstained with SYTOX blue (blue). Graph D shows the mean (±SEM) biofilm volume (FISH probe labelling) per field of view (determined by Volocity software analysis of confocal microscopy z-stacks) on PCD versus non-PCD ciliated ALI-cultures (*$p<0.05$). Confocal micrographs E and F are represented as 3D opacity views of ciliated PCD (E), and non-PCD (F) cells co-cultured for 72 h with NTHi. Scale bars = 20 µm.

Figure 3: Comparison of percent ciliation, ciliary function and trans-epithelial electrical resistance (TEER) in PCD and non-PCD ALI-cultured airway epithelial cells, and characterisation of the effect of NTHi co-culture on cilia. Graph A demonstrates mean (±SEM) CBF (in Hz) of PCD (n=4 static cilia and n=1 slow dyskinetic cilia) and non-PCD (n=9) measured before (baseline) and daily during 72 h of NTHi co-culture, compared to uninfected non-PCD controls (n=5). The normal CBF range is 11-20 Hz at the Southampton

PCD Centre and infection did not modify CBF in any group (A). Graph B shows the mean (±SEM) percentage ciliation corresponding to coverage of β-tubulin immuno-labelled cilia on differentiated ALI-cultures. Ten randomly selected microscopic fields of view were compared per sample and there was no difference in the percentage ciliation between PCD (n=5) and non-PCD (n=5) samples. Graph C demonstrates the mean percentage ciliation (±SEM) calculated from whole field Fast Fourier Transform (FFT) analysis of ciliary beat frequency (CBF) where the ratio of motile to not-motile pixels (15360 data points per total field of view from a video recorded at a resolution of 512x480; analysis box size = 4x4 pixels). The mean (±SEM) percentage ciliation of non-PCD (n=8) ALI-cultured cells before (baseline) and daily after NTHi co-culture did not change over 72 hours. Graph D shows the mean (±SEM) TEER in Ohms/cm^2 of NTHi-infected PCD (n=5) and non-PCD (n=9), measured before (baseline) and daily during co-culture for 72 h was compared to uninfected non-PCD controls (n=5). TEER remained stable and was not significantly different between groups.

Figure 4: PCD and non-PCD epithelial cell LL-37 and cytokine responses to NTHi infection. Dot and Whisker plots A and B demonstrate the median (and interquartile range) concentration of cationic the antimicrobial peptide, cathelicidin LL-37 (ng/ml), measured in the apical supernatants of ALI-cultured epithelial cells from non-PCD (A) and PCD (B) patients, before (baseline) or daily after NTHi co-culture for 72 hours. Line graph C depicts mean (±SEM) concentrations (pg/ml) of 10 cytokines: basic fibroblast growth factor (FGF-β), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage-colony stimulating factor GM-CSF), Interleukin (IL)-6, IL-8, IL-1receptor antagonist (IL-1ra), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), tumour necrosis factor (TNF-α) and vascular endothelial growth factor (VEGF) in the basolateral medium of PCD (n=5) and non-PCD (n=9) epithelial cell ALI cultures before (baseline) and daily after NTHi co-culture for 72 hours. (*p<0.05, **p<0.01, ***p<0.001).

Figure 5: Nitric Oxide Synthase (NOS) isoenzymes and Nitric Oxide (NO) were compared between ciliated PCD and non-PCD airway epithelial cells cultured at ALI. Representative micrographs of ALI-differentiated epithelial cells show fluorescent labelling with antibodies to neuronal NOS (A, D), inducible NOS (B, E) and endothelial NOS (C, F) and an Alexafluor488

conjugated secondary antibody (green) in non-PCD (A-C) and PCD (D-F) subjects (n=3 repeats per group). As a negative control primary antibodies were omitted and confirmed that there was no non-specific secondary antibody labelling in non-PCD ALI-cultured epithelium (G). Representative micrographs show the presence of NO by DAF-FM labelling (green) in 1 non-PCD (H) and in 2 PCD (I, J) subjects (n=4 non-PCD and n=3 PCD subjects were tested in total). There was no appreciable difference in NOS isoenzyme or DAF-FM labelling between non-PCD and PCD ALI-cultures. Very low background auto-fluorescence can be seen in an untreated human bronchial epithelial cell line (16HBE) negative control (K). Cell nuclei were stained with Hoechst (red) (B, C, E-G) and cilia were co-labelled with an anti-β-tubulin antibody (shown in red) (A, D). Merged maximum projections from confocal (A, D, H-K) or epifluorescent (B, C, E-G) microscope images using 100x or 40x objectives (40 μm scale bars).

Figure 6: Treatment of PCD ALI-differentiated airway epithelial cells with antibiotic and NO donor prodrug. The graph shows mean (±SEM) log biofilm colony forming unit counts (CFUs/cm^2) for adherent (biofilm) NTHi-co-cultured for 72 hours on PCD airway epithelial cells that were cultured and ciliated at ALI (n=4). Co-cultures were incubated for 2 hours either, without (control) or with, NO donor prodrug PYRRO-C3D (50 μM), 4 mg/mL azithromycin, or both. NTHi viability was decreased nearly 3 logs by PYRRO-C3D/azithromycin treatment. (*$p<0.05$).

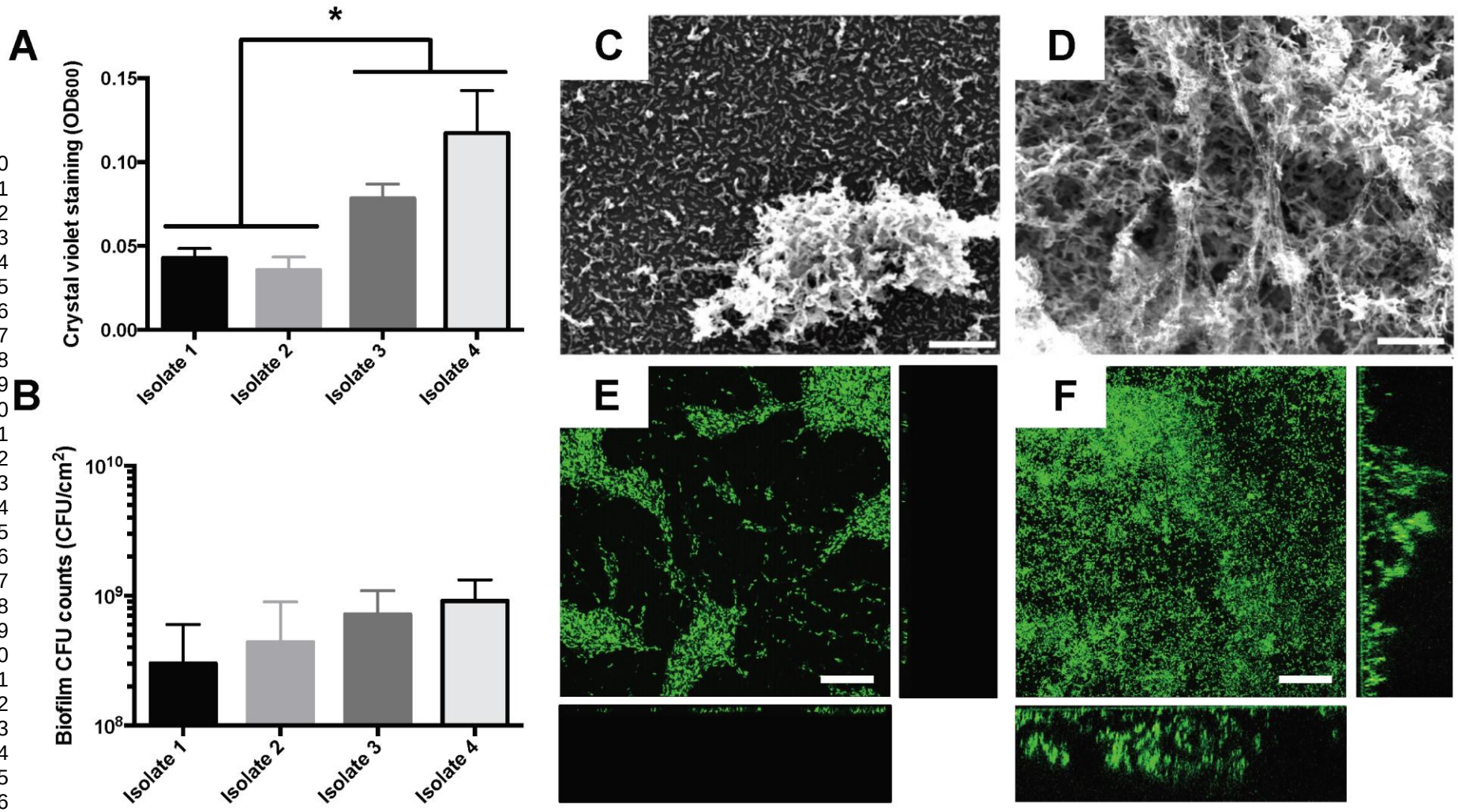

A
*
Crystal violet staining (OD600)
0.15
0.10
0.05
0.00
Isolate 1
Isolate 2
Isolate 3
Isolate 4
B
Biofilm CFU counts (CFU/cm2)
10^10
10^9
10^8
Isolate 1
Isolate 2
Isolate 3
Isolate 4
C
D
E
F

Figure 1

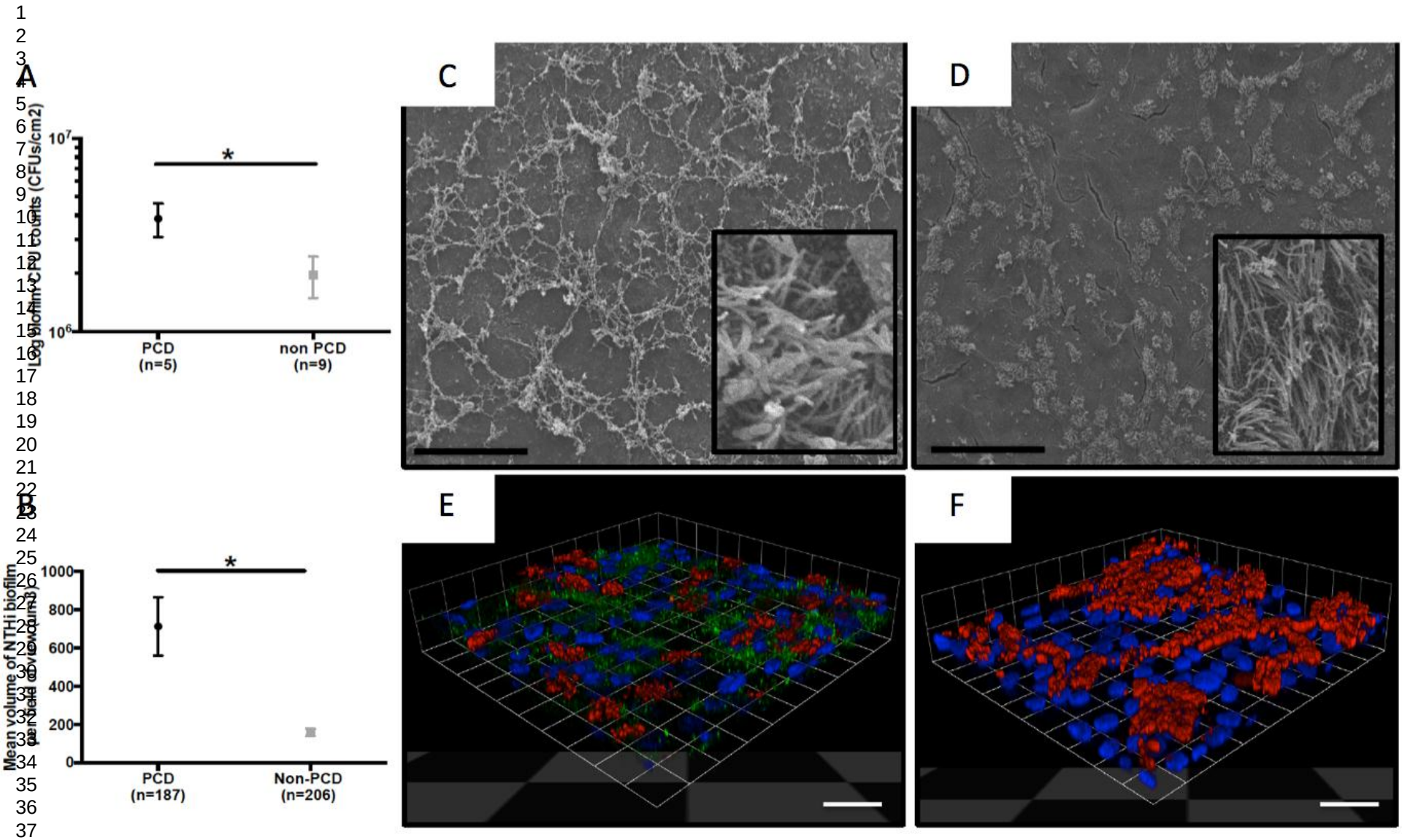
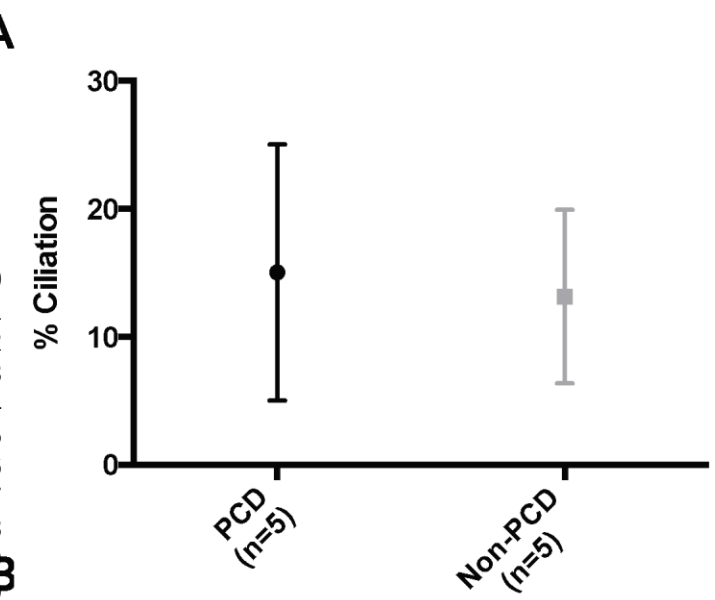
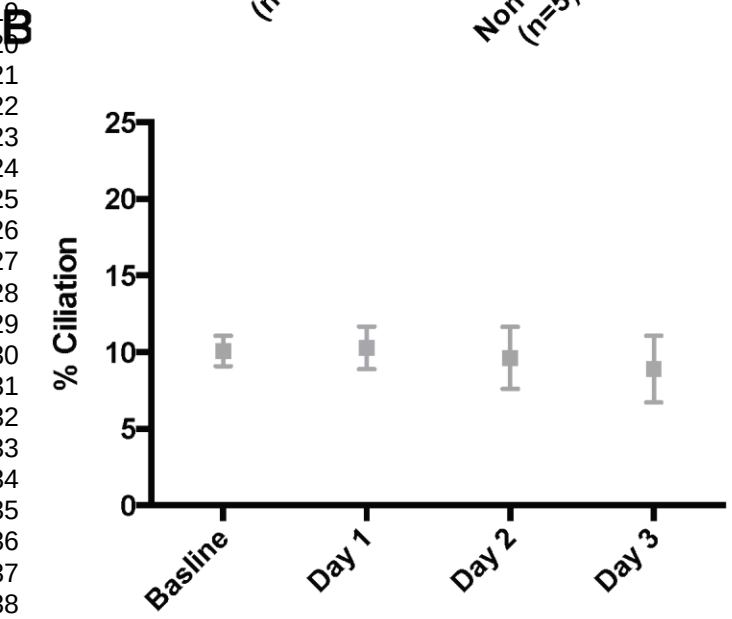
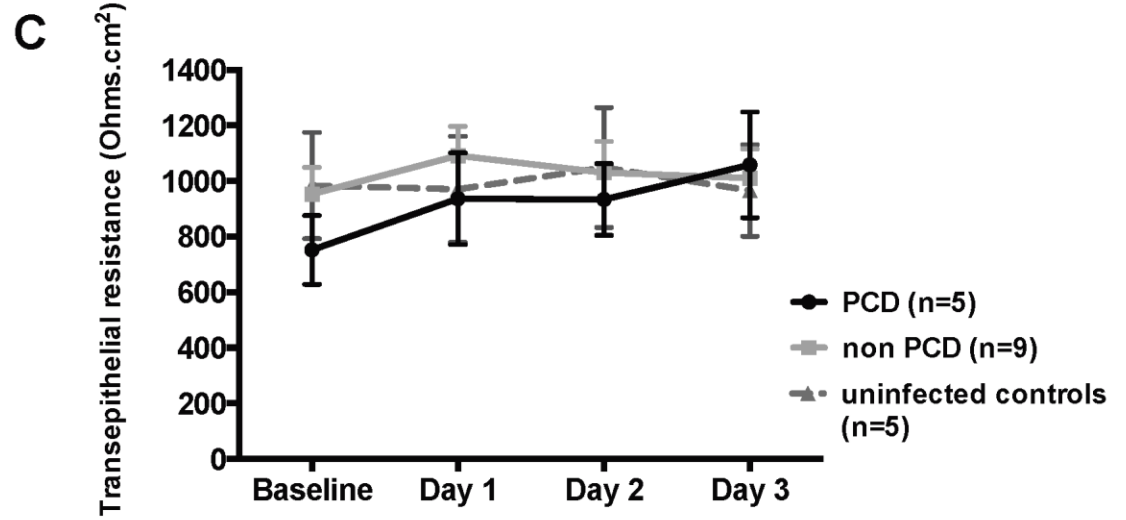

A
Log biofilm CFU counts (CFUs/cm2)
10^7
10^6
*
PCD (n=5)
non PCD (n=9)
B
Mean volume of NTHi biofilm per field of view (μm3)
1000
800
600
400
200
0
*
PCD (n=187)
Non-PCD (n=206)
C
D
E
F

Figure 2


A
% Ciliation
30
20
10
0
PCD (n=5)
Non-PCD (n=5)


B
% Ciliation
25
20
15
10
5
0
Basline
Day 1
Day 2
Day 3


C
Transepithelial resistance (Ohms.cm2)
1400
1200
1000
800
600
400
200
0
Baseline
Day 1
Day 2
Day 3
PCD (n=5)
non PCD (n=9)
uninfected controls (n=5)

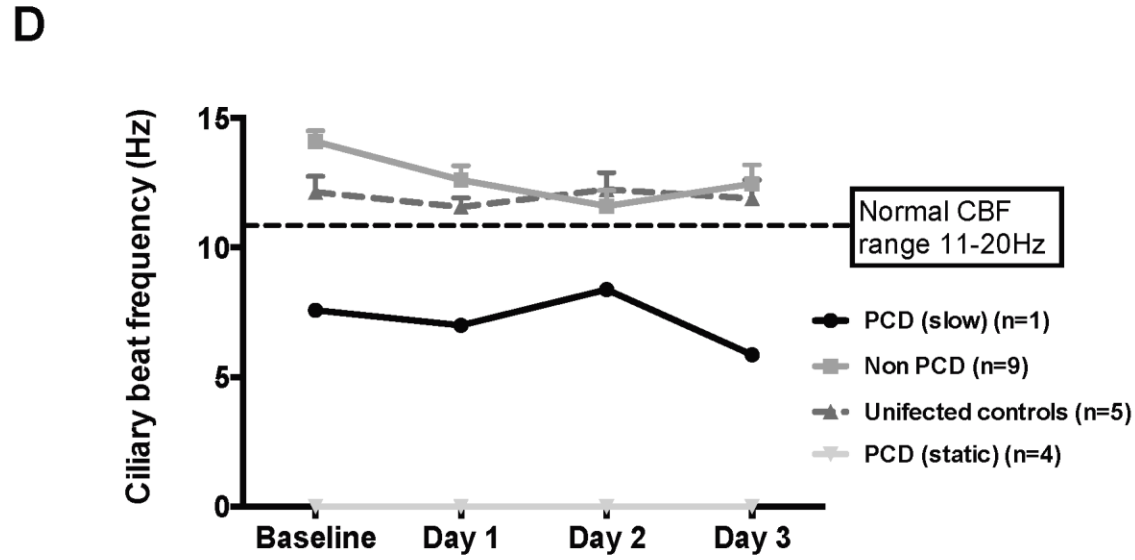

D
Ciliary beat frequency (Hz)
15
10
5
0
Baseline
Day 1
Day 2
Day 3
Normal CBF range 11-20Hz
PCD (slow) (n=1)
Non PCD (n=9)
Unifected controls (n=5)
PCD (static) (n=4)

Figure 3

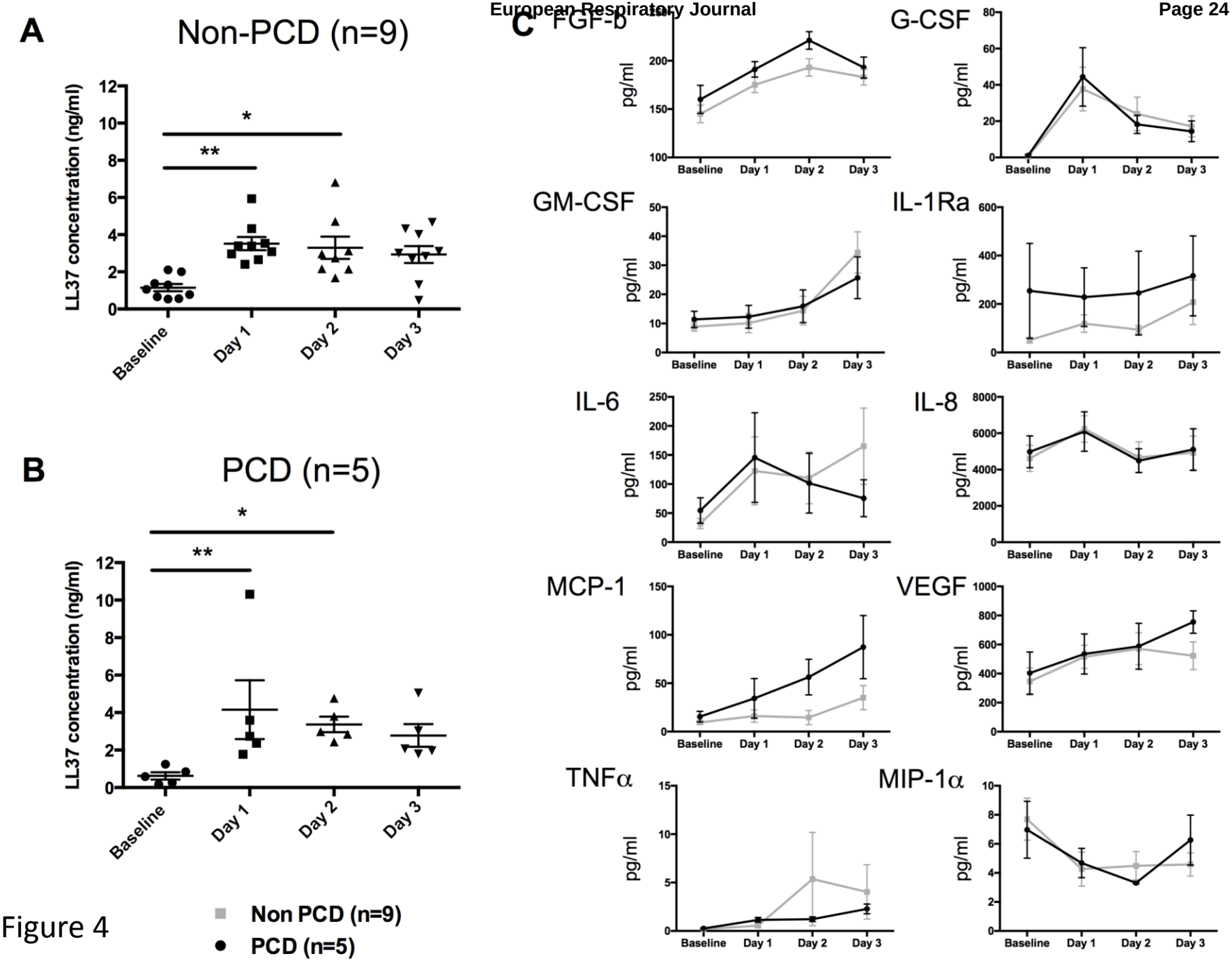

A
Non-PCD (n=9)
LL37 concentration (ng/ml)
Baseline
Day 1
Day 2
Day 3
B
PCD (n=5)
Non PCD (n=9)
PCD (n=5)
C
FGF-b
G-CSF
GM-CSF
IL-1Ra
IL-6
IL-8
MCP-1
VEGF
TNFα
MIP-1α
pg/ml

Figure 4

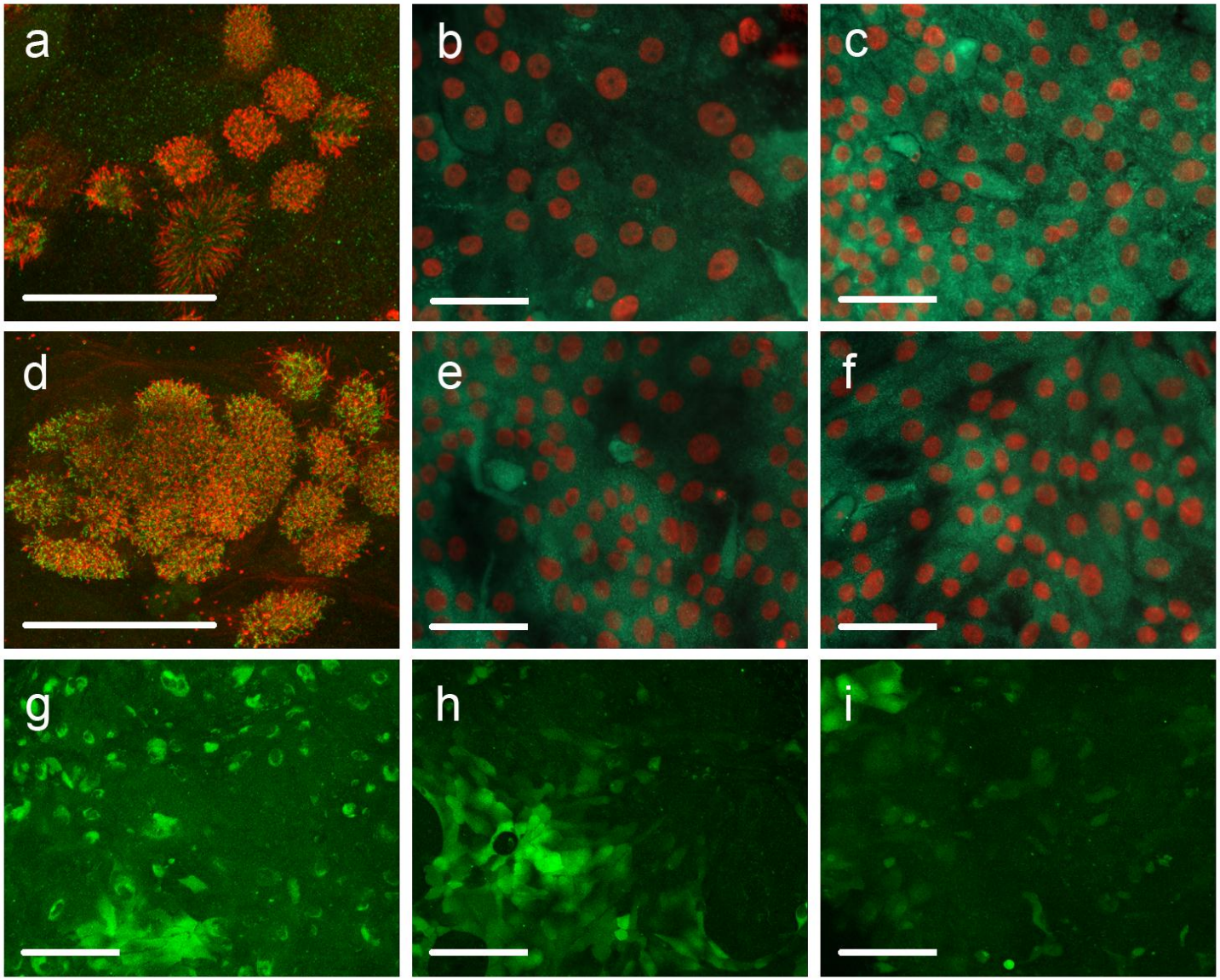


Figure 5

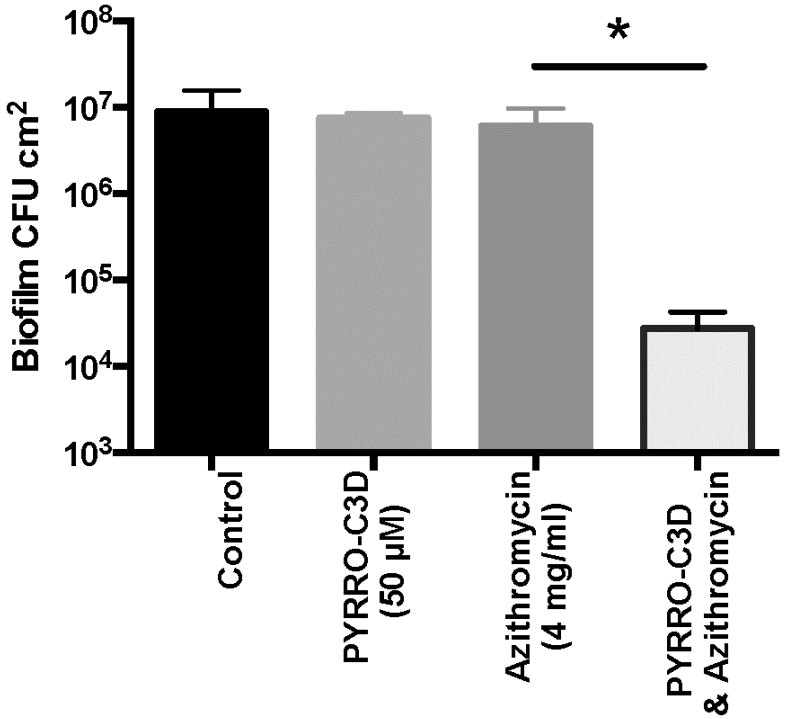

Biofilm CFU cm2
10^8
10^7
10^6
10^5
10^4
10^3
*
Control
PYRRO-C3D (50 µM)
Azithromycin (4 mg/ml)
PYRRO-C3D & Azithromycin

Figure 6

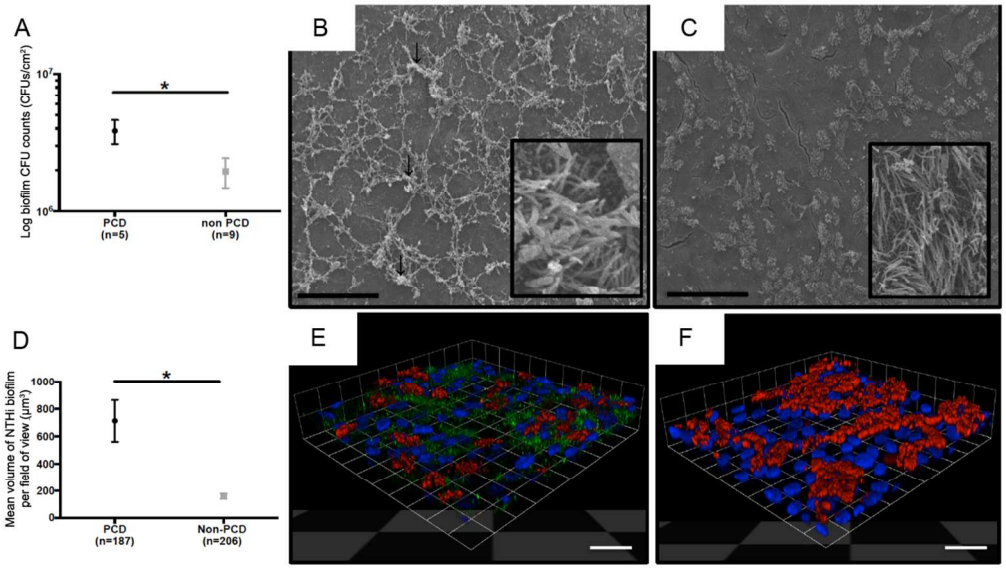
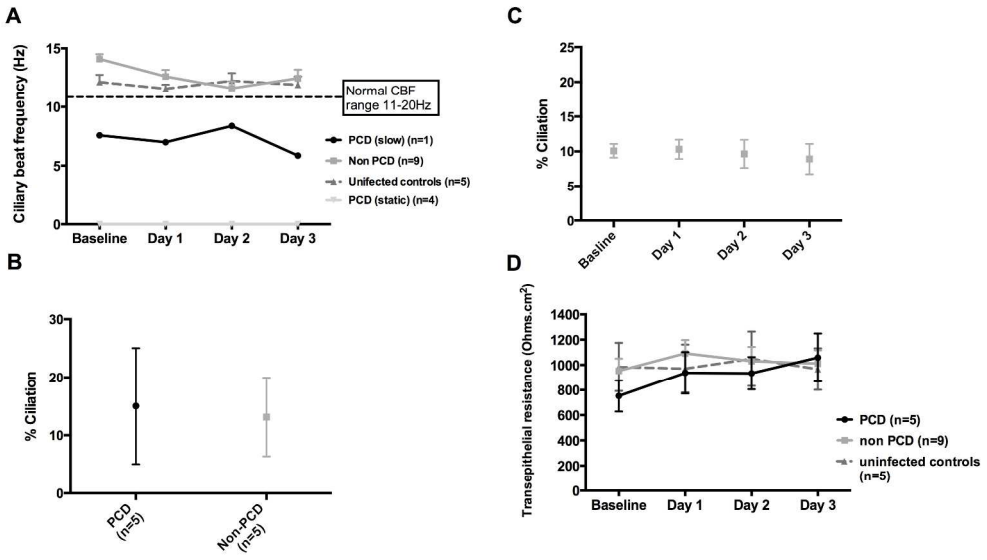


Figure 2: Characterisation of NTHi co-cultured with air-liquid interface (ALI) differentiated and ciliated airway epithelial cells. Graph A depicts the mean (±SEM) log biofilm CFUs/cm2 after 72 h of NTHi co-culture on ALI ciliated PCD (n=5), and non-PCD (n=9) epithelial cells (*p<0.05). Representative scanning electron micrographs (x4000) (B and C) demonstrate increased bacterial aggregates and exopolymeric matrix formation (arrows) on PCD ALI-cultured ciliated epithelial cells in contrast to non-PCD cells. NTHi co-cultured on ALI ciliated PCD and non-PCD epithelial cells were labelled for confocal analysis. Bacterial cells were labelled with the CY3-H. influenzae-specific FISH probe (green), cilia were labelled with anti-β-tubulin antibody (red) and nuclei were counterstained with SYTOX blue (blue). Graph D shows the mean (±SEM) biofilm volume (FISH probe labelling) per field of view (determined by Volocity software analysis of confocal microscopy z-stacks) on PCD versus non-PCD ciliated ALI-cultures (*p<0.05). Confocal micrographs E and F are represented as 3D opacity views of ciliated PCD (E), and non-PCD (F) cells co-cultured for 72 h with NTHi. Scale bars = 20 µm.

115x66mm (300 x 300 DPI)



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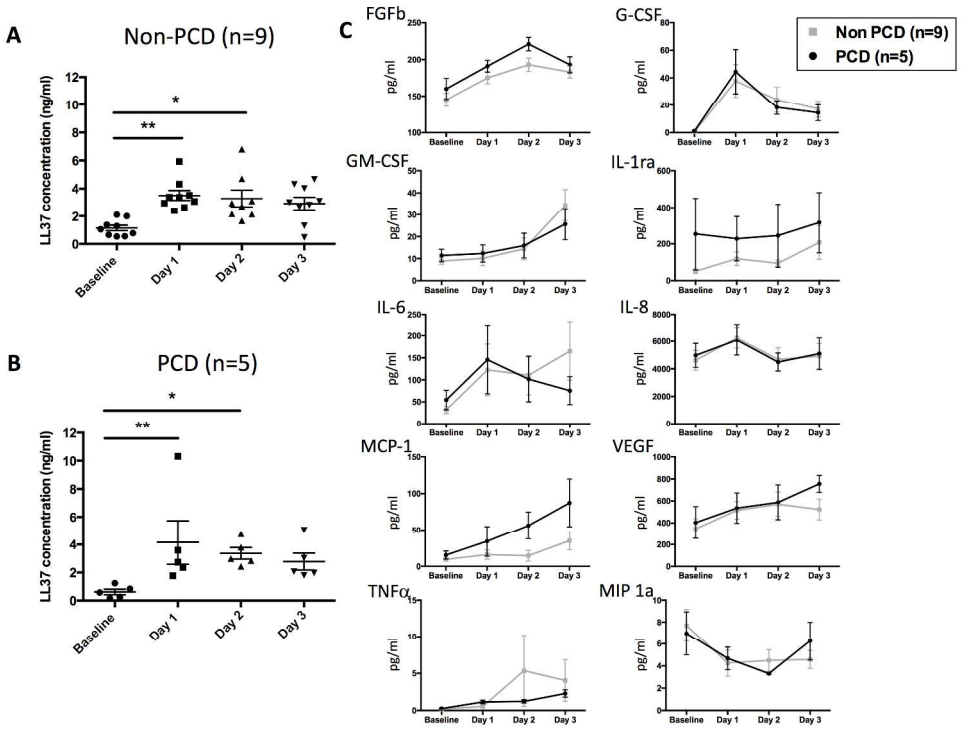

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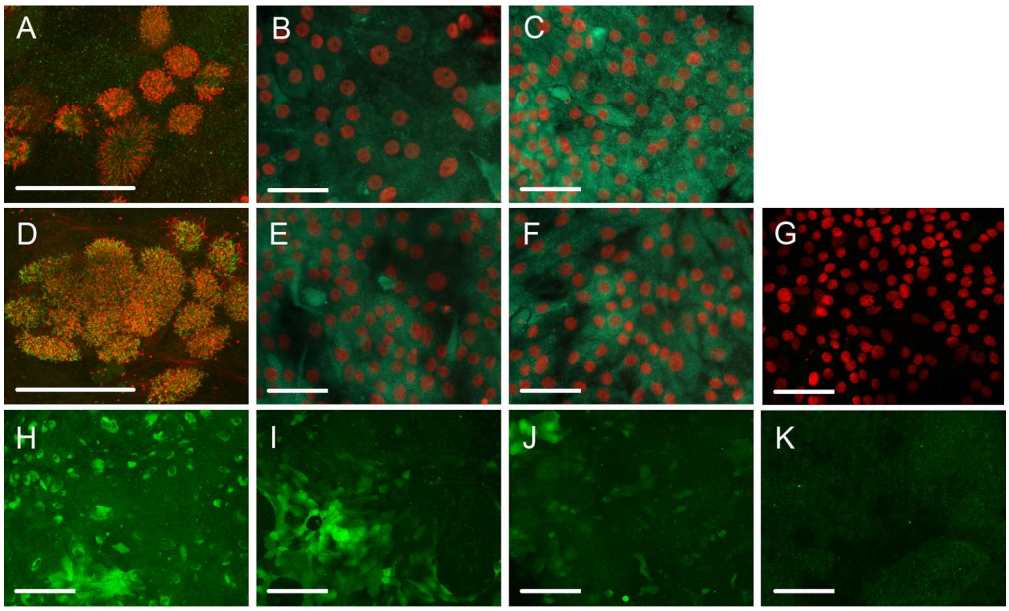

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163x98mm (300 x 300 DPI)

Supplementary materials and methods

Primary airway epithelial cell sampling

The patients and healthy samples were obtained between 2012 and 2015. Information on the PCD and respiratory control patients and healthy volunteers is summarised in Supplementary Table 1. The clinical history of PCD patients is summarised in Table 1. We performed 2 nasal brushings per PCD patient or healthy volunteer to obtain primary human airway epithelial cells, using 2 mm diameter cytology brushes (ConMed, Linvatec, UK). Brushes were placed into 1.5 ml Medium 199 (Gibco, Life Technologies, UK) and cells dislodged by vigorous pipetting. Cells were pelleted by centrifugation at 1,400 rpm (using a Jouan CR312 centrifuge) for 5 minutes.

Air-liquid interface culture

Costar™ culture-ware (Fisher Scientific, ThermoFisher Scientific, UK) was pre-coated with 300 mg/ml PureCol (Nutacon, NL) before use. Cells were re-suspended in 1 ml bronchial epithelial growth medium (BEGM) supplemented with Clonetics™ SingleQuots (Lonza, UK) and additional 50 Units/ml penicillin and 50 µg/ml streptomycin (Gibco, Life Technologies, UK), and 20 Units/ml nystatin (Sigma-Aldrich, UK) and seeded into wells of 12-well plates. Cultures were incubated at 37^{o}C (5% CO2, 100% relative humidity) and fed three times per week. At approximately 80% confluence the cells were passaged in 0.25% trypsin-EDTA solution (5 minutes at 37^{o}C), pelleted by centrifugation and resuspended in 5 ml BEGM into a T-25 flask. The cells were passaged again, at approximately 80% confluence, before the pellet was re-suspended in 1 ml air-liquid interface medium (ALI medium) containing a 1:1 ratio of BEBM:Dulbecco's modified eagle medium (Gibco, Life Technologies, UK) supplemented with growth Clonetics™ SingleQuots (using only 1/5th volume of the hEGF solution), antibiotics and anti-fungal additives. Cells (~500,000 per 0.5 ml) were seeded submerged into 12-well Costar Transwell inserts and when confluent taken to an air-liquid interface (ALI). At ALI the cells were fed baso-laterally with ALI medium, supplemented with additional 100 nM retinol (Sigma-Aldrich, UK) to promote basal epithelial cell differentiation

to a ciliated columnar cell phenotype (approximately 3 weeks). ALI-culture ciliation was confirmed by light microscopy and ALI-cultures used when cells were ciliated maximally (variable coverage). Polarized ciliated epithelial cells from PCD patients or non-PCD volunteers were cultured for two days at ALI in ALI-medium without antibiotic or anti-fungal treatment prior to NTHi co-culture.

Characterisation of NTHi on ciliated airway cells

Fluorescence in situ hybridization (*16S rRNA FISH*) was performed as previously described using the *H. influenzae* 16S ribosomal probe sequence: Hinf Cy3 labeled (Integrated DNA Technologies Inc, Lueven, Belgium) 5'- CCGCACTTTCATCTCCG-3' (16S [185-202]). ALI-cultured specimens were fixed with fresh 4% paraformaldehyde in HBSS followed by washes with HBSS and HBSS-ethanol (1:1), with subsequent 3-minute incubations in 80% and 100% ethanol. Cilia on epithelial cells were specifically immuno-labelled using an anti-β-tubulin antibody so that the localisation of FISH-labelled NTHi could be judged in proximity to cilia. Samples were rinsed with sterile PBS and blocked with (20% Newborn calf serum in DMEM, with 10% BSA and 0.05% Triton X) for 30 min, rinsed, and incubated with the T5293 anti-β-tubulin monoclonal antibody (Sigma-Aldrich, UK) (diluted 1:2,000 in blocking buffer) to specifically label cilia. Samples were washed 3 times before incubation for 90 minutes with the secondary Alexa Fluor® 594 chicken anti-mouse IgG antibody (A21201, Molecular Probes®, Life Technologies, UK). Epithelial cell nuclei were counterstained for 30 minutes with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Life Technologies, UK) diluted 1:360 in 1x PBS before being washed in 1xPBS and mounted on glass slides. Samples were analysed by confocal microscopy (SP5, Leica Microsystems, UK) at x400 magnification and every consecutive field of view was assessed across the width of the membrane. The volume of adherent NTHi bacterial micro-colonies on the epithelial cell layers were calculated by Volocity 3D image analysis software (version 6.0.1, PerkinElmer, UK).

Cytokine and LL-37 responses to NTHi infection

Baso-lateral medium from ALI-cultures was frozen before inoculation and daily following co-culture with NTHi. We measured basic fibroblast growth factor (FGF-b), granulocyte- and granulocyte-macrophage- colony stimulating factors (G-CSF, GM-CSF), Interleukins- (IL) -6

and -8, IL-1receptor antagonist (IL-1ra), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), tumor necrosis factor (TNF-α) and vascular endothelial growth factor (VEGF) by Fluorokine® Human MultiAnalyte Profiling (MAP) assay. Cytokine specific antibodies were pre-coated on colour-coded polystyrene beads and incubated with thawed supernatants, blank controls and standards (50 µl per 96-well in duplicate) for 3 hours. After washing away unbound sample, a cocktail of biotinylated antibodies specific to each cytokine was added for 1 hour, washed, and a streptavidin-phycoerythrin (streptavidin-PE) conjugate was added for 30 minutes to bind to the biotinylated detection antibodies. All incubations were done at room temperature and in the dark on a horizontal orbital shaker (550 rpm). Unbound streptavidin-PE was finally washed away before analysis with a Bio-Plex® 200 Analyser (R&D systems®, UK). Lasers detected bead type and PE signal, directly proportional to the amount of cytokine bound [33]. Interleukin-8 was measured separately by human IL-8 DuoSet ELISA kit (DY208, R&D systems®, UK). Basolateral supernatants were diluted 1:5 with 1% bovine serum albumin in 1x phosphate buffered saline (1% BSA/PBS) and 50 µl incubated at room temperature for 2 hours in duplicate wells of a 96-well plate (pre-coated overnight at room temperature with 4 µg/ml monoclonal anti-human IL-8 antibody, washed and blocked in 1% BSA/PBS for 1 hour at room temperature prior to use) with blank controls and standards. After washing samples, controls and standards were incubated for 2 hours at room temperature with 20 ng/ml biotinylated goat anti-human IL-8, washed and further incubated for 45 minutes with 50 µl streptavidin-horseradish peroxidase (HRP). An HRP substrate (50 µl 1:1 solution of H_2O_2 :tetramethybenzidine) was added for 20 minutes and the reaction was stopped by addition of 50 µl 2N H_2SO_4. Light emissions (at a wavelength of 450 nm) were detected in wells by a ThermoMax Microplate Reader (Molecular Devices, US) and mean IL-8 concentration was determined from a standard curve.

Nitric oxide production by airway epithelial cells

Transwell membranes of live ALI-cultured epithelial cells were treated with 10 µM 4-amino-5-methylamino-2',7'-difluororescein diacetate (DAF-FM) [34], which was added directly into the basolateral medium (at 37^{o}C) of Transwells inserts and imaged by live confocal microscopy (SP5 TCS Confocal Microscope, Leica Microsystems UK) to assess the presence of NO. DAF-FM diacetate is weakly fluorescent until the diacetate is cleaved in the presence

of NO to produce highly fluorescence DAF-FM. Images were collected within an hour of exposure to DAF-FM diacetate.

Immunofluorescence labelling was used to localise NO synthase (NOS) isoenzymes, (neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) in fixed epithelial cell cultures [32] using confocal microscopy. Transwell membranes sections containing cells were fixed in ice cold methanol for 10 minutes, blocked for 30 minutes in 1% BSA/PBS containing additional 0.05% triton X-100 (blocking buffer). Calbiochem rabbit anti-human polyclonal NOS antibodies (Merck, UK) to nNOS (1414–1434), iNOS (1131–1144) and eNOS (599–913) were diluted 1:200 (from 1 mg/ml stock) in blocking buffer and incubated with samples for 90 minutes at room temperature. Excess unbound antibody was washed away before incubating samples in the dark for a further 90 minutes at room temperature with a secondary Alexa488 conjugated goat anti-rabbit antibody (Invitrogen, UK) diluted 1:500 (from 2 mg/ml stock). We have previously demonstrated that nNOS co-localised with β-tubulin labelling in cilia [32]. Therefore for nNOS labelled samples, cilia were co-localised with a T5293 monoclonal anti-human β-tubulin antibody (Sigma-Aldrich, UK) diluted 1:2000, followed by an Alexa594 conjugated chicken anti-mouse antibody (Molecular Probes, Life Technologies, UK) diluted 1:500 (from 2 mg/ml stock) [32]. Cell nuclei were stained for 30 minutes with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) diluted 1:360 in 1x PBS (Molecular Probes, Life Technologies, UK). Slides were mounted under coverslips with Mowiol. Fluorescence labelling was imaged by confocal microscopy.

Table 1: Demographics of the PCD, respiratory control and healthy participants.

	PCD	Non-PCD	
		Respiratory Control	Healthy
Number of subjects	**15**	**9**	**10**
Male n (%)	**10 (66.7)**	**5 (55.6)**	**3 (30)**
Median age in years (IQR)	**16.85 (19.89)**	**2.9 (4.87)**	**32.55 (14.96)**
Pulmonary symptoms n (%)	**15 (100)**	**8 (88.9)**	**0**
Rhinosinusitis n (%)	**14 (93.3)**	**7 (77.8)**	**0**
Situs abnormality n (%)	**9 (60)**	**1 (11.1)$^{\Delta}$**	**0**
Footnote: $^{\Delta}$ normal diagnostic tests (see Supplementary Table 1, participant 018)			

Supplementary Table 1: Diagnostic results for PCD and respiratory control and healthy participants. We have indicated which experiments the participants were recruited to (NTHi isolation, co-culture (*Biofilm CFUs, SEM, FISH, TEER, Cytokines, LL-37*), % ciliation by beta-tubulin immunofluorescence (IF) or Fast Fourier Transform (FFT) analysis, nitric oxide synthase (NOS) isoenzyme IF, DAF-FM labelling of nitric oxide, PYRRO-CD3 treatment).

	PCD or Respiratory control or Healthy	Age (Years)	Situs	nNO (nL/min)	CBF (normal range 11-20 Hz)	CBP	TEM defect	Genotype	Experiment(s)
001	**PCD**	**8**	**Dextrocardia**	**9.6**	**0**	**Immotile**	**ODA & IDA**	**ZMYND10**	**NTHi isolation**
002	**PCD**	**15**	**Heterotaxy**	**6**	**0**	**Immotile**	**ODA & IDA**	**DYX1C1**	**NTHi isolation**
003	**PCD**	**10**	**Normal**	**15**	**N/A**	**Predominantly immotile (some residual movement)**	**ODA**	**DNAH5**	**NTHi isolation**
004	**PCD**	**5**	**Dextrocardia**	**3**	**0**	**Immotile**	**ODA**	**DNAH5**	**NTHi isolation**
005	**PCD**	**27.97**	**Normal**	**77.4**	**8.67**	**Asynchronous stiff cilia on 2 separate occasions, also consistent after ALI-culture**	**Normal**	**Unknown**	**Co-culture, % ciliation (by beta-tubulin IF)**
006	**PCD**	**23.05**	**Dextrocardia**	**7.98**	**N/A**	**Predominantly immotile with some stiff hyperfreq. cilia (reduced amplitude)**	**Normal**	**DNAH11**	**Co-culture, PYRRO-C3D**
007	**PCD**	**14.8**	**Normal**	**19.5**	**0**	**Immotile**	**ODA**	**DNAH5**	**Co-culture, NOS isoenzymes by IF**
008	**PCD**	**16.85**	**Normal**	**25.8**	**0**	**Immotile**	**ODA**	**DNAH5**	**Co-culture**
009	**PCD**	**21.61**	**Dextrocardia**	**12.9**	**N/A**	**Predominantly**	**ODA**	**DNAI1**	**Co-culture**

						Immotile (some residual movement)			NOS isoenzymes by IF
010	PCD	47.35	Dextrocardia	12	0	Immotile	ODA	DNAH5	NOS isoenzymes by IF, % ciliation (by beta-tubulin IF)
011	PCD	29.70	Normal	12.6	N/A	Predominantly stiff moving asynchronous cilia and others immotile	Normal	HYDIN	% ciliation (by beta-tubulin IF), DAF-FM, PYRRO-C3D
012	PCD	7.51	Dextrocardia	3	0	Immotile	ODA	DNAH5	% ciliation (by beta-tubulin IF), DAF-FM
013	PCD	0.50	Dextrocardia	ND	0	Immotile	ODA	Unknown	% ciliation (by beta-tubulin IF), DAF-FM
014	PCD	27.89	Normal	1.6	N/A	Predominantly stiff moving asynchronous cilia and others immotile	Normal	HYDIN	PYRRO-C3D
015	PCD	20.45	Dextrocardia	7.32	N/A	Predominantly stiff moving asynchronous cilia and others immotile	Normal	DNAH11	PYRRO-C3D
016	Respiratory control	46.18	Normal	277.5	18.47	Normal	Normal	ND	Co-culture
017	Respiratory control	2.30	Normal	ND	12.2	Normal	Normal	ND	NOS isoenzymes by IF
018	Respiratory control	1.49	Dextrocardia	ND	15	Normal	ND	ND	NOS isoenzymes by IF
019	Respiratory control	1.80	Normal	ND	15.6	Normal	ND	ND	NOS isoenzymes by IF, % ciliation (by beta-tubulin IF)
020	Respiratory control	3.02	Normal	52.8	15.5	Normal	ND	ND	% ciliation (by beta-tubulin IF)
021	Respiratory control	0.19	Unknown	ND	14.56	Normal	Normal	ND	% ciliation (by beta-tubulin IF)
022	Respiratory control	2.91	Normal	ND	15.7	Normal	Normal	ND	% ciliation (by beta-tubulin IF), DAF-FM
023	Respiratory control	2.98	Unknown	ND	18.7	Normal	Normal	ND	% ciliation (by beta-tubulin IF)

024	Respiratory control	10.01	Unknown	ND	14.3	Normal	Normal	ND	DAF-FM
025	Healthy	40.67	ND	ND	ND	ND	ND	ND	Co-culture, % ciliation (by FFT analysis)
026	Healthy	29.88	ND	ND	ND	ND	ND	ND	Co-culture, % ciliation (by FFT analysis)
027	Healthy	39.47	ND	ND	ND	ND	ND	ND	Co-culture, % ciliation (by FFT analysis)
028	Healthy	22.73	ND	ND	ND	ND	ND	ND	Co-culture, % ciliation (by FFT analysis)
029	Healthy	33.27	ND	ND	ND	ND	ND	ND	Co-culture, % ciliation (by FFT analysis)
030	Healthy	25.04	ND	ND	ND	ND	ND	ND	Co-culture, % ciliation (by FFT analysis)
031	Healthy	33.92	ND	ND	ND	ND	ND	ND	Co-culture, % ciliation (by FFT analysis)
032	Healthy	23.38	ND	ND	ND	ND	ND	ND	Co-culture, % ciliation (by FFT analysis)
033	Healthy	39.93	ND	ND	ND	ND	ND	ND	DAF-FM
034	Healthy	31.82	ND	ND	ND	ND	ND	ND	DAF-FM

Key: nasal nitric oxide (nNO); ciliary beat frequency (CBF); ciliary beat pattern (CBP); transmission electron microscopy (TEM); ND = test not done, N/A = test none applicable (e.g. CBF result was irrelevant due to variable CBP); air-liquid interface (ALI) culture

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