The mucus bundles responsible for airway cleaning are retained in cystic fibrosis and by cholinergic stimulation

Anna Ermund[1], Lauren N. Meiss[1], Brendan Dolan[1], Andrea Bähr[2], Nikolai Klymiuk[2] and Gunnar C. Hansson[1]

Affiliations: [1]Dept of Medical Biochemistry, University of Gothenburg, Gothenburg, Sweden. [2]Institute of Molecular Animal Breeding and Biotechnology, Gene Center, Ludwig-Maximilians-University Munich, Munich, Germany.

Correspondence: Gunnar C. Hansson, Dept of Medical Biochemistry, University of Gothenburg, Box 440, 40530 Gothenburg, Sweden. E-mail: gunnar.hansson@medkem.gu.se

@ERSpublications
ACh uncouples airway surface liquid transport from transport of mucus bundles and lack of CFTR inhibits bundle movement http://ow.ly/Izbm30kb2F4

Cite this article as: Ermund A, Meiss LN, Dolan B, *et al*. The mucus bundles responsible for airway cleaning are retained in cystic fibrosis and by cholinergic stimulation. *Eur Respir J* 2018; 52: 1800457 [https://doi.org/10.1183/13993003.00457-2018].

ABSTRACT The beneficial effect of anticholinergic therapy for chronic lung diseases such as chronic obstructive pulmonary disease (COPD) is well documented, although cholinergic stimulation paradoxically inhibits liquid absorption, increases ciliary beat frequency and increases airway surface liquid transport.

Using pig tracheobronchial explants, we quantified basal mucus transport before as well as after incubation with the clinically used antimuscarinic compound ipratropium bromide (Atrovent) and stimulation with acetylcholine.

As expected, surface liquid transport was increased by acetylcholine and carbachol. In contrast, the mucus bundles secreted from the submucosal glands normally transported on the cilia were stopped from moving by acetylcholine, an effect inhibited by ipratropium bromide. Interestingly, in pigs lacking a functional cystic fibrosis (CF) transmembrane conductance regulator (CFTR) channel, the mucus bundles were almost immobile. As in wild-type pigs, CF surface liquid transport increased after carbachol stimulation. The stagnant CF mucus bundles were trapped on the tracheal surface attached to the surface goblet cells. *Pseudomonas aeruginosa* bacteria were moved by the mucus bundles in wild-type but not CF pigs.

Acetylcholine thus uncouples airway surface liquid transport from transport of the surface mucus bundles as the bundles are dynamically inhibited by acetylcholine and the CFTR channel, explaining initiation of CF and COPD, and opening novel therapeutic windows.

This article has supplementary material available from erj.ersjournals.com

Received: March 05 2018 | Accepted after revision: May 17 2018

Introduction

Airway mucus obstruction is common to many chronic lung diseases such as asthma, chronic obstructive pulmonary disease (COPD) as well as cystic fibrosis (CF) and contributes substantially to airflow limitations [1]. This is caused by increased vagal tone and increased levels of acetylcholine (ACh) that cause increased airway smooth muscle tone. In addition, ACh also causes submucosal gland secretion, increased ciliary beat frequency and inhibits air surface liquid absorption [2, 3]. Treating airway diseases with muscarinic antagonists to block parasympathetic signalling is an old tradition, but it was not until the introduction of nonabsorbable atropine derivatives such as ipratropium that this became an accepted therapy, especially for COPD. However, the beneficial effect of anticholinergic treatment, except for the muscle-relaxing effect, is paradoxically partly opposite to what could be considered physiologically beneficial.

The respiratory tract is kept essentially free from inhaled particles and bacteria by the mucociliary clearance system. The constantly beating cilia generate an escalator transporting mucus cephalically to the larynx. There has, however, been some confusion as the organisation of the mucus system differs between commonly used small experimental animals and higher species. Mice essentially lack submucosal glands, whereas humans and pigs have numerous glands [2]. Submucosal glands produce the MUC5B mucin that forms linear polymers [4]. In the submucosal glands, the most peripheral cells generate a chloride- and bicarbonate-rich fluid that passes by the MUCB-secreting cells, and by this unfolds and pulls out the MUC5B polymers [5, 6]. During duct passage these polymers form thick mucus bundles (25–30 µm) that are made up of more than 1000 polymers. The bundles appear from the gland openings and then move in or on top of the 7–10 µm deep airway surface liquid (ASL) with a mean velocity of 0.3 $\mu m \cdot min^{-1}$ [2, 6]. The ASL moves faster (3–5 $\mu m \cdot min^{-1}$) than the bundles, a discrepancy we suggest to be caused by the mucus bundles being anchored to the surface goblet cells [6]. We have now studied mucus bundle transport on live tracheal tissue from normal newborn piglets and piglets lacking the CF transmembrane conductance regulator (CFTR) channel, as in CF, and can show that ACh blocks mucus bundle movement, and thus we give an additional explanation for the beneficial effect of ipratropium bromide (Atrovent), where in CF the mucus bundles were essentially immobile.

Material and methods

Piglets and airway preparation

All animal procedures were performed according to the German Animal Welfare Act with permission of the local regulatory authority. Breeding of CF and wild-type (WT) littermate piglets was performed as described previously [7]. Births were induced, and airways were dissected and shipped as described previously within 24 h of birth [6]. For further methods, see the supplementary material.

Statistical analysis

All statistical tests were performed using Prism version 7.02 (GraphPad, La Jolla, CA, USA) and all data are represented as mean with standard error of the mean. Each time-lapse recording was considered as one replicate. Differences were assessed with the two-tailed Mann–Whitney U-test to compare two groups and the Kruskal–Wallis test followed by Dunn's multiple comparisons test was employed for multiple comparisons as appropriate; $p<0.05$ was defined as significant. No statistical methods were used to predetermine sample size and no randomisation was employed. No animals were excluded from the analyses and because the morphology of CF piglet trachea is distinct, the experiments could not be performed blinded to the observer.

Results

Cholinergic inhibition by ipratropium bromide promotes mucus bundle transport

Mucus bundles from submucosal glands clean the airways by sweeping over the surface to collect debris and bacteria [6]. The movement of Alcian blue-stained mucus bundles can be studied in explant piglet airways mounted in a temperature-controlled chamber [6]. The uneven movement of the Alcian blue-stained mucus bundles is best illustrated by movies (supplementary movie S1). Cholinergic signalling has been claimed to be increased in lung diseases such as COPD [8], asthma [9] and CF [10]. To mimic this situation we added the metabolically more stable ACh analogue carbachol (Cch) to the chamber and studied mucus bundle transport in WT piglet trachea. Surprisingly, the transport of mucus bundles was essentially stopped by Cch (figure 1a). To exclude off-target effects, we next tested the endogenous transmitter ACh on the transport of Alcian blue-stained mucus bundles and found that ACh exerted the same effect as Cch. The ACh-stopped mucus was mobilised again by the nonselective muscarinic receptor antagonist ipratropium bromide. Interestingly, the ipratropium bromide effect lasted only 45 min as the transport returned to ACh levels 45 min after ipratropium removal (figure 1b). Ipratropium bromide did

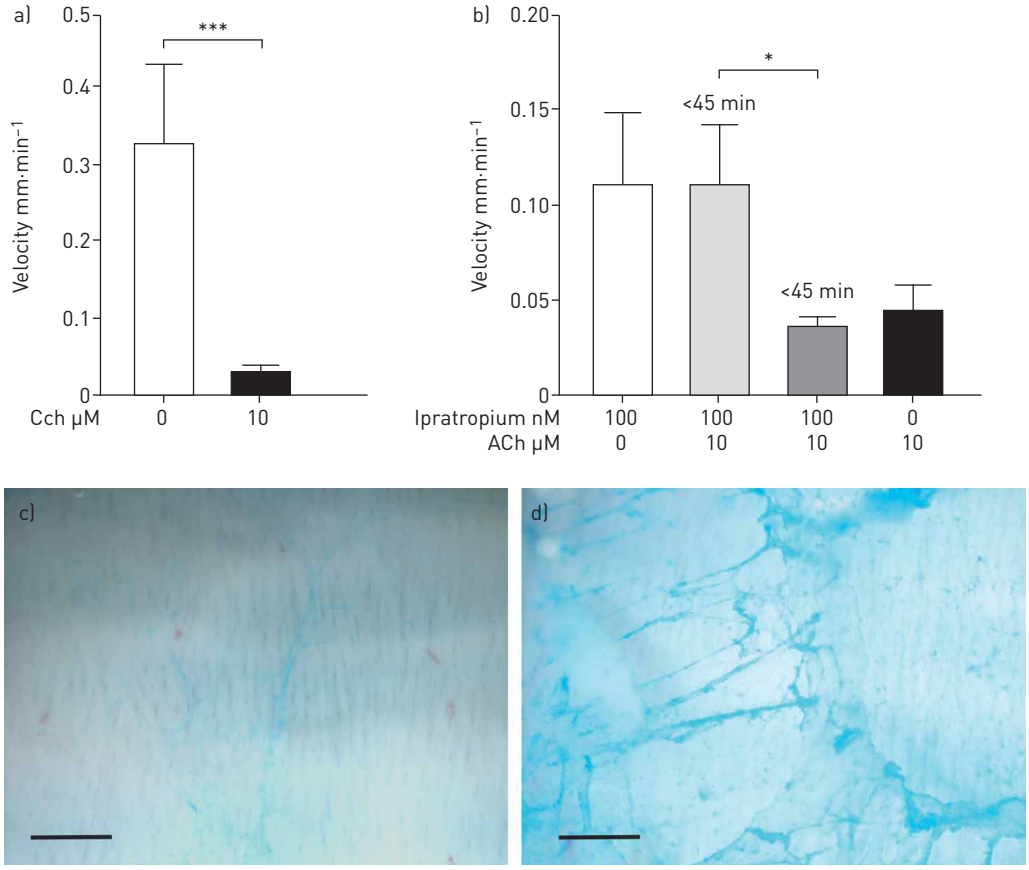

FIGURE 1 Cholinergic inhibition promotes mucus transport. Cch: carbachol; WT: wild-type; ACh: acetylcholine. a) Alcian blue-stained mucus bundles essentially stopped moving after Cch stimulation. n=45 WT control and n=12 Cch time-lapse recordings; ***: p=0.0001. b) Ipratropium bromide reversed the decreased movement induced by ACh. n=7 ipratropium, n=5 ipratropium+ACh <45 min, n=9 ipratropium+ACh >45 min and n=8 ACh time-lapse recordings; *: p=0.03. Data analysed by the two-tailed Mann–Whitney U-test. c) Live submerged WT piglet trachea pre-incubated with 100 nM ipratropium bromide for 1 h and stained with Alcian blue. d) WT piglet trachea incubated with ipratropium bromide after stimulation with ACh. Scale bar: 1 mm.

not inhibit the formation of Alcian blue-stained bundles either before or after ACh stimulation (figure 1c and d, respectively).

Mucociliary transport, often referred to as mucus transport, is traditionally visualised by the movement of particles on the mucosal surface [11]. Similar to others [12], we used 40 nm carboxylated fluorescent beads added to piglet airway preparations. As shown previously [6], Alcian blue and beads stained distinct material in WT piglet trachea (supplementary figure S1 and supplementary movie S2).

The Alcian blue bundles exited the submucosal gland openings (figure 2a, green arrow), and the carboxylated beads gathered in dots on the epithelial surface and sometimes in linear patterns (figure 2a). The Alcian blue bundles and beads did not stain the same shapes (supplementary figure S2). To further address these differences, the velocities of the beads and bundles were compared, showing that the beads moved at higher velocity than the Alcian blue-stained bundles (figure 2b). Furthermore, adding Cch caused the beads to move even faster, whereas the Alcian blue-stained mucus bundles further slowed down within 5 min of Cch application (figure 2b). The fluorescent beads thus responded to Cch stimulation by an increased transport as previously observed for mucociliary clearance of what has been called mucus and corresponds to the ASL. To avoid confusion, we suggest using "ASL" for the liquid directly transported by the cilia. The bead-gathering strands are in the ASL and their velocity is given as that of the ASL.

Immunostaining for MUC5AC and MUC5B mucins illustrated that the main mucin in the glands is MUC5B, whereas the surface goblet cells contain mainly MUC5AC (figure 2c). Mucus bundles exiting submucosal glands were previously identified in WT piglet airways with scanning electron microscopy

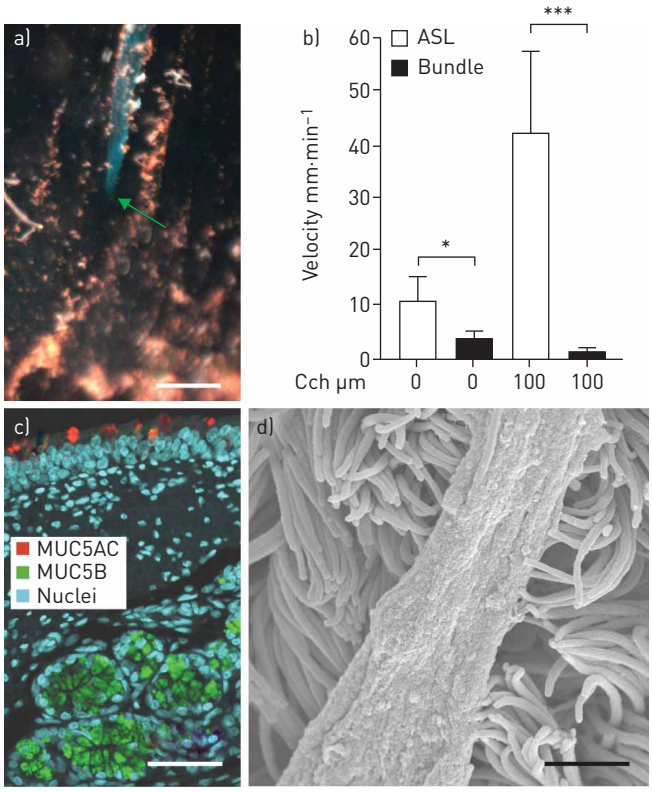

FIGURE 2 Submucosal glands secrete mucus bundles stained by Alcian blue. ASL: airway surface liquid; Cch: carbachol; WT: wild-type. a) Alcian blue-stained bundle (blue) from a submucosal gland and beads (orange). The green arrow points to the gland opening. Scale bar: 250 µm. b) Velocity of bead-collecting strands ("ASL") and Alcian blue-stained bundles ("Bundle") without or with 100 µM Cch. The beads moved faster than Alcian blue-stained bundles in both cases. n=41 Alcian blue, n=9 beads, n=7 Alcian blue+Cch and n=9 beads+Cch time-lapse recordings; *: p=0.03, for no Cch; ***: p=0.0003, for 100 µM Cch. Data analysed by the two-tailed Mann–Whitney U-test. c) MUC5B mucin was expressed mainly in the submucosal glands and to a minor extent on the surface goblet cells, whereas MUC5AC mucin was expressed exclusively in the airway surface goblet cells in WT piglet trachea. Scale bar: 50 µm. d) Bundle on the cilia observed using scanning electron microscopy in human trachea. Scale bar: 2 µm. Experiments in (a) and (c) were performed on at least n=4 animals.

(SEM) [6] and, as humans also have numerous submucosal glands, we found the same type of mucus bundles on the human airway surface (figure 2d).

Mucus bundles are immobile in CF trachea

CF is a disease caused by a nonfunctional CFTR, and is characterised by decreased mucociliary clearance and mucus accumulation. When the transport velocity of Alcian blue-stained mucus bundles in neonatal WT and CF piglet tracheas was compared, a dramatic impairment in mucus transport was observed in CF airways (figure 3a). Time-lapse recordings of Alcian blue-stained CF tracheas revealed a very slow transport (supplementary movie S3). To study whether ipratropium bromide could abrogate the decreased velocity in CF, similar to what was observed in WT, we measured Alcian blue-stained bundle velocity after ipratropium bromide treatment. Pre-incubation with ipratropium had no direct effect in CF (figure 3b). Addition of ACh further arrested bundle movement in CF and pre-incubation with ipratropium had no rescuing effect. A similar pattern of bundles was noted on CF as on WT trachea after ipratropium (figure 3c), but ACh stimulation did not cause additional mucus secretion, in contrast to WT trachea (figure 3d).

SEM micrographs of CF tracheas showed long bundles exiting submucosal glands (figure 4a, yellow arrow), immunostaining MUC5B in submucosal glands and MUC5AC in surface goblet cells (figure 4b), similar to WT. As in WT tracheas, we studied the transport of fluorescent beads and Alcian blue-stained mucus bundles. The beads collected in similar shapes as in WT and moved toward the larynx (supplementary movie S4). When the images were superimposed, it was clear that the beads did not overlap with the Alcian blue staining (supplementary movie S5). Just as in WT, there was a tendency for the beads to move faster after Cch stimulation, whereas the movement of Alcian blue-stained bundles

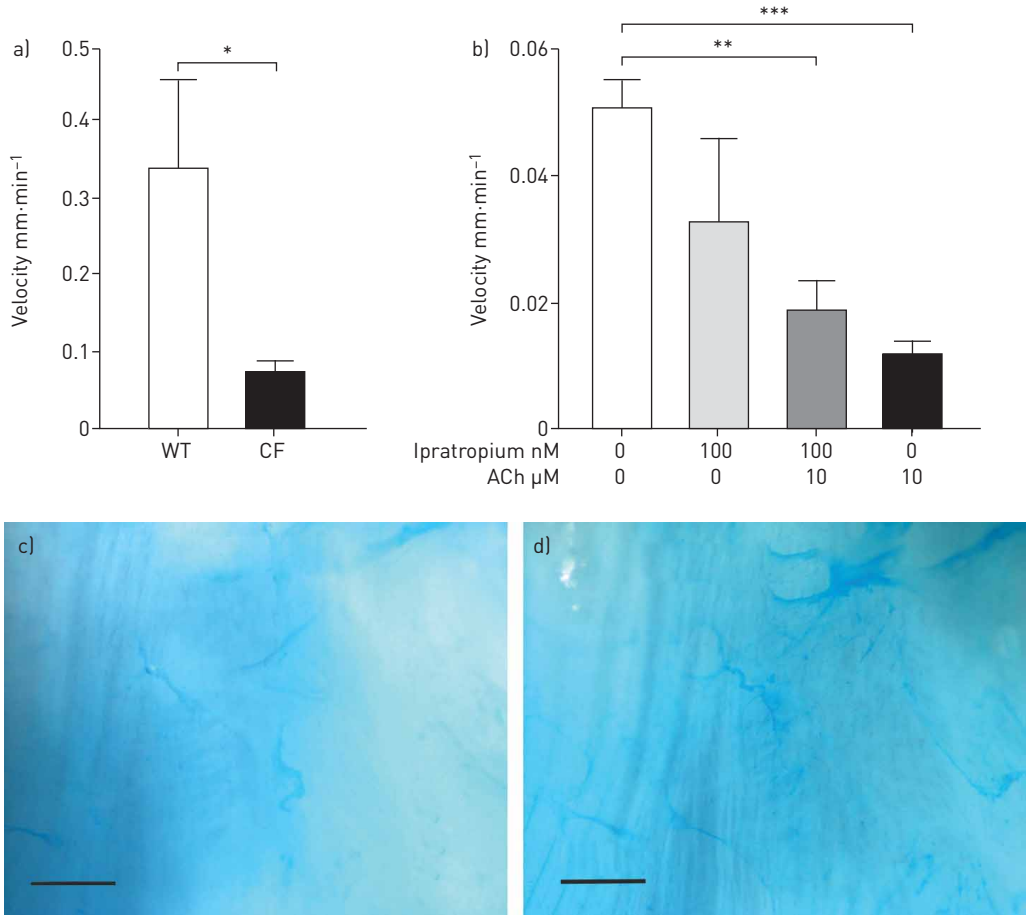

FIGURE 3 Mucus bundles are immobile in cystic fibrosis (CF) piglet tracheas. WT: wild-type; ACh: acetylcholine. a) Comparison of transport velocity for Alcian blue-stained bundles in WT and CF piglet trachea. n=39 WT and n=32 CF; *: p=0.03. b) In CF, ACh stimulation decreased the transport velocity of Alcian blue-stained bundles, even after pre-incubation with ipratropium bromide. n=18 CF control, n=7 ipratropium, n=7 ipratropium+ACh <45 min and n=7 ACh time-lapse recordings; **: p=0.0003, CF control compared with ACh; ***: p=0.0034, CF control compared with ipratropium+ACh <45 min; no difference between ipratropium +ACh <45 min and ACh alone, and no difference between control and ipratropium alone. Data analysed by the Kruskal–Wallis multiple comparisons test with Dunn's multiple comparisons test. c) CF piglet trachea stained with Alcian blue after pre-incubation with 100 nM ipratropium bromide for 1 h. d) CF trachea incubated with ipratropium bromide, stained with Alcian blue and stimulated with ACh. Scale bar: 1 mm.

slowed down (figure 4c). Thus, the ASL and mucus bundles also had opposite responses in CF, although the velocities were considerably lower.

MUC5B mucus bundles from submucosal glands are retained by MUC5AC from surface goblet cells in CF

The Alcian blue-stained bundles had a core of MUC5B stained with the *Lotus tetragonolobus* lectin (LTL) [6] in both WT and CF (figure 5a and c). Live WT piglet trachea mucus bundles stained with fluorescein-labelled LTL, but the fluorescent beads did not bind to the LTL-positive mucus bundles as they appeared directly from the submucosal glands (figure 5b and d). Thus, the bundles exited the gland without binding beads, but at later time-points the mucus bundles collected beads, suggesting that the material that collected the beads later also gathered on the bundles. Thus, the mucus bundles stained with Alcian blue or LTL were distinct from the fluorescent beads, similar to WT.

The thickness of the Alcian blue-stained bundles was similar in WT and CF (figure 6a). The bundle thickness estimated from LTL staining was also similar, with a tendency to be thinner in CF (figure 6b). It has been suggested that the mucus bundles were trapped in the glands in CF [12], a phenomenon that might result in fewer mucus bundles in CF. When the total volume of lectin-stained bundles was measured, no major differences were observed (figure 6c).

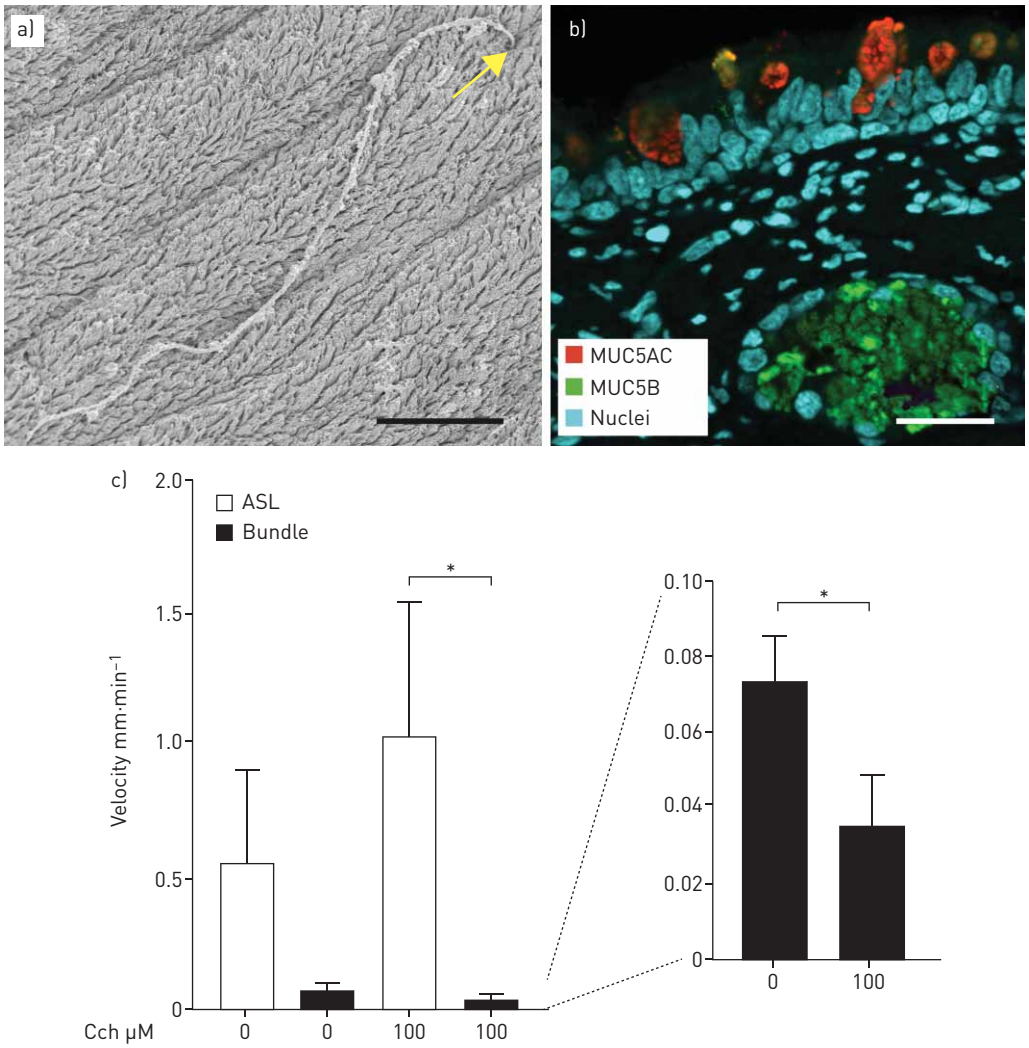

FIGURE 4 Submucosal glands in cystic fibrosis (CF) piglets secrete Alcian blue-stained mucus bundles. WT: wild-type; ASL: airway surface liquid; Cch: carbachol. a) Bundles formed in CF piglet trachea visualised by scanning electron microscopy. A submucosal gland opening is indicated by the yellow arrow. Scale bar: 50 µm. b) Similar expression pattern in CF piglet trachea as in WT. Scale bar: 25 µm. Experiments in (a) and (b) were performed on at least n=4 animals. c) Velocity of bead-collecting strands ("ASL") and Alcian blue-stained bundles ("Bundle") without or with 100 µM Cch. The beads moved faster than Alcian blue-stained bundles. The beads moved faster than Alcian blue-stained bundles when stimulated with 100 µM Cch; *: p=0.03. Inset: enlargement focused on Alcian blue bundle movement; *p=0.043. n=34 Alcian blue, n=8 beads, n=9 Alcian blue+Cch and n=13 beads+Cch. Data analysed by the two-tailed Mann–Whitney U-test.

We have previously demonstrated that bicarbonate in sufficient amounts is necessary for normal detachment of the mucus in the small intestine and that removal of serosal bicarbonate caused the WT intestine to mimic that of CF [13]. When bicarbonate was removed from the buffer on WT tracheal explants stained with Alcian blue, the transport was as slow as in CF (figure 6d), emphasising the importance for bicarbonate and functional CFTR.

Mucus bundles are retained by MUC5AC from surface goblet cells in CF

To explore how bundle transport was slowed in CF, we stained MUC5B bundles with LTL (green) and MUC5AC mucin from the surface goblet cells with UEA1 lectin (red) (figures 2c and 4b). In WT (figure 7a) and CF (figure 7c), the bundles consisted of a core of MUC5B coated with MUC5AC [6]. The contact points between the bundles and the goblet cells could be observed in confocal images at higher magnification (figure 7a′ and c′) and in SEM images (figure 7b and d). In both WT and CF, the MUC5AC mucin appeared from the tracheal surface goblet cells, coated the MUC5B bundles and connected the two. The distance between the bundles and the epithelium was measured, revealing a tendency for a shorter distance in CF compared with WT (figure 8a). Interestingly, the number of contact

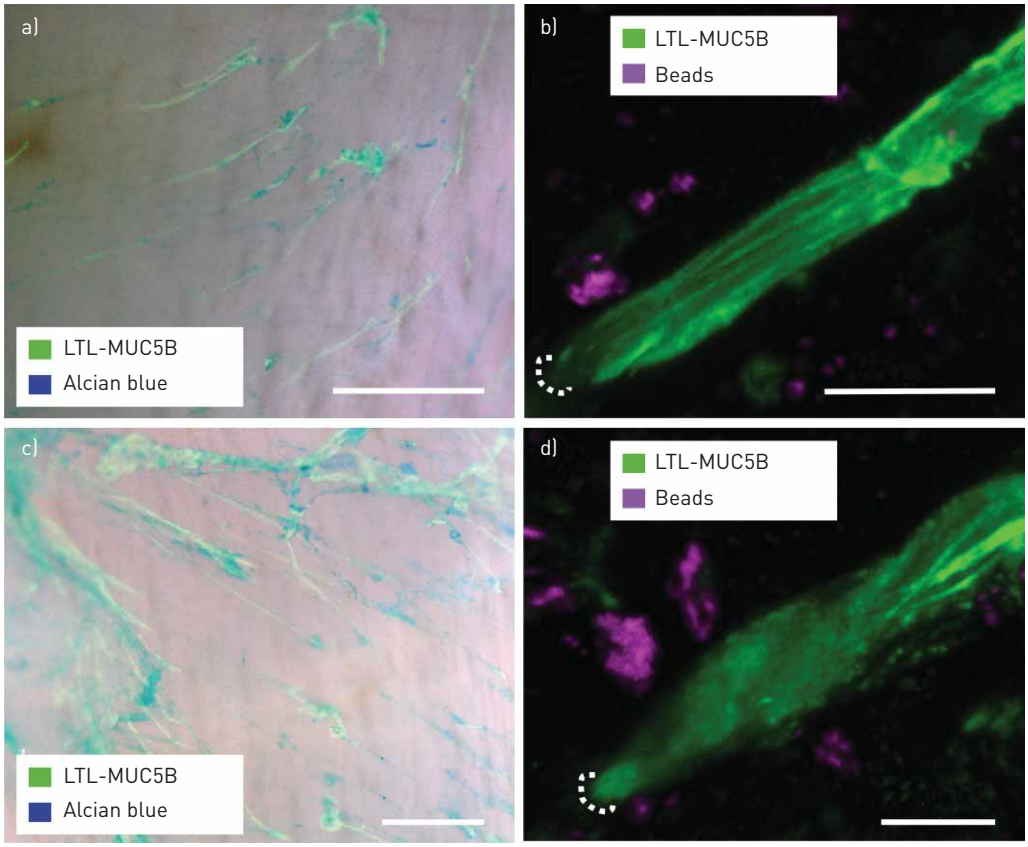

FIGURE 5 Mucus bundles stained with Alcian blue and *Lotus tetragonolobus* lectin (LTL), but not beads. WT: wild-type; CF: cystic fibrosis. a) Mucus bundles stained with Alcian blue and LTL in WT piglet trachea. Scale bar: 1 mm. b) Confocal image of a mucus bundle from a WT piglet trachea stained with LTL and with fluorescent beads. Scale bar: 20 µm. c) Mucus bundles stained with Alcian blue and LTL in CF piglet trachea. Scale bar: 1 mm. d) Confocal image of a mucus bundle from a CF piglet trachea stained with LTL and with fluorescent beads. Scale bar: 20 µm. Note the large overlap in staining with Alcian blue and the lectin in (a) and (c), whereas the beads do not collect on LTL-stained bundles in (b) and (d). Gland opening indicated by dashed line in (b) and (d). Experiments repeated in at least n=3 animals.

points between goblet cells and bundles assessed as MUC5AC-stained connections was significantly higher in CF (figure 8b).

To illustrate the consequences of the observed differences in mucus bundle movement for cleaning the tracheal surface of bacteria, we added TAMRA-stained *P. aeruginosa* bacteria (red) to LTL-stained bundles (green) on piglet trachea (figure 8c and d). In WT, the bacteria were quickly collected on the moving bundles, leaving the surface essentially free of bacteria (figure 8c and supplementary movie S6). In contrast, the bacteria covered the surface of the epithelium in CF and the bacteria on the bundles were essentially stagnant (figure 8d and supplementary movie S7). The mucus bundles sweeping over the tracheal surface are thus essential for cleaning the tracheobronchial surface and the stagnant bundles in CF result in inefficient clearing of bacteria from the epithelial surface.

Discussion

Analysing mucus *in vivo* or *ex vivo* in a physiological way is challenging. A major problem is that mucus is completely transparent and thus invisible. The cationic Alcian blue dye binds to the multiple negative charges found on the glycans of the mucin domains and has been used for decades to stain mucus on tissue sections. We have now taken advantage of this and used Alcian blue to stain live tracheobronchial explants. To our initial surprise, we observed thick Alcian blue-stained mucus bundles appearing from submucosal gland openings [6]. Other researchers have used 40 nm fluorescent beads to stain "mucus" [14]. These beads are carboxylate-modified and thus negatively charged. These beads did not bind the mucus bundles, but gathered on other structures of unknown identity very weakly stained by Alcian blue or LTL. Alcian blue bundle and bead transport were affected in opposite directions by Cch stimulation in both WT and CF; this further supports that Alcian blue and fluorescent beads label distinct materials. The

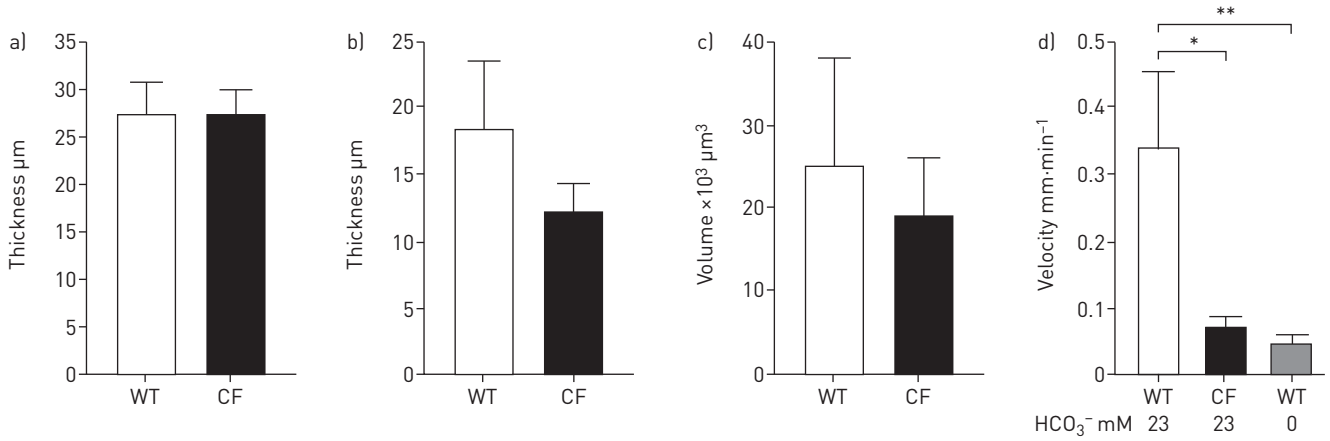

FIGURE 6 Mucus bundle thickness and amount are similar in wild-type (WT) and cystic fibrosis (CF), whereas the transport velocity is lower in CF than WT. LTL: *Lotus tetragonolobus* lectin; HCO_3^-: bicarbonate. a) Thickness of Alcian blue-stained bundles. WT: n=8 bundles, n=3 animals; CF: n=11 bundles, n=4 animals. b) Thickness of LTL-stained bundles. Mean±SEM bundle thickness in WT 18±4.9 µm (n=11 bundles) and in CF 12±2 µm (n=9 bundles); at least five measurements per bundle, n=3 animals per genotype. c) Mean volume of LTL-stained mucus bundles on an identical surface area of WT and CF trachea (WT: 31 data points; CF: 16 data points; n=3 animals each). Data analysed by the two-tailed Mann–Whitney U-test. d) Alcian blue-stained bundles moved with higher velocity on WT than CF trachea and with higher velocity than WT when HCO_3^- was omitted from the buffer; *: p=0.02; **: p=0.007. n=39 WT, n=32 CF and n=36 WT (no HCO_3^-) time-lapse recordings. Data analysed by the Kruskal–Wallis multiple comparisons test with Dunn's multiple comparisons test.

increased transport velocity of the beads after Cch is similar to what has been observed for mucociliary clearance using, for example, micro-optical coherence tomography [2, 15]. This surface liquid has often been called "mucus", but in the normal lung this is the same as ASL. To avoid confusion, we use the term "ASL" for bead transport. This normal ASL should be kept separate from the mucus that accumulates at the epithelial surface during disease observed on human bronchial epithelial cultures [16].

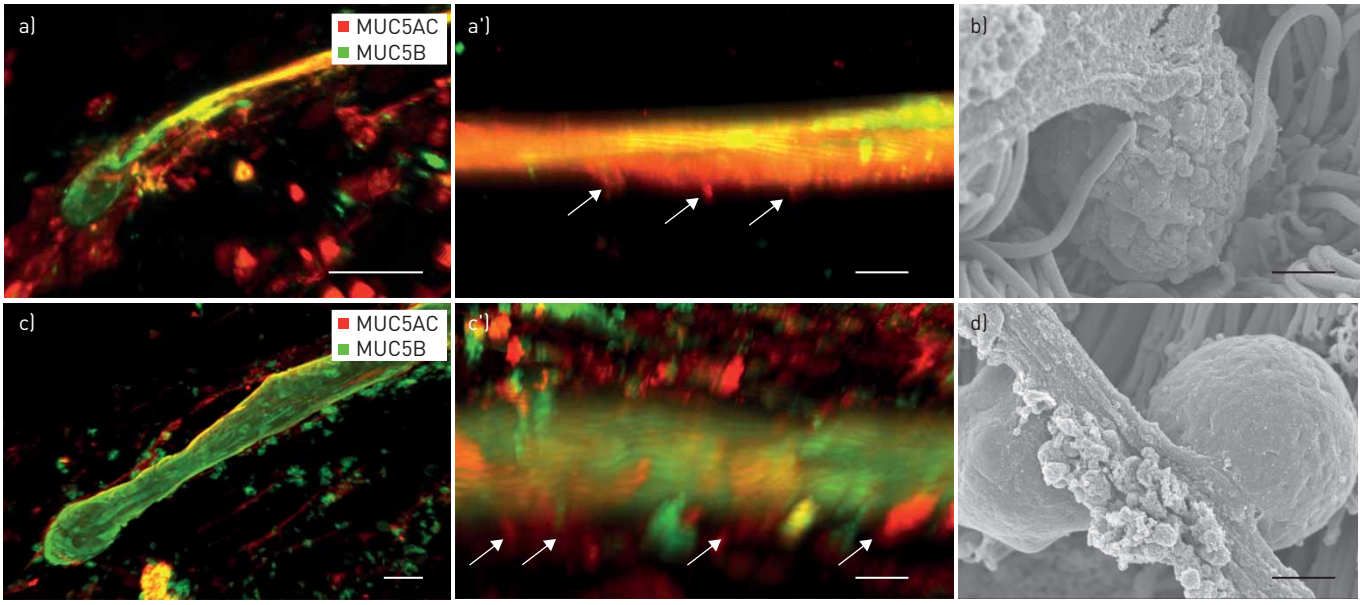

FIGURE 7 Mucus bundles are retained by mucus from surface goblet cells in cystic fibrosis (CF). WT: wild-type; SEM: scanning electron microscopy. Intensity for the lectins does not reflect relative amounts in confocal images. a) Bundles exiting submucosal glands consist of a core of MUC5B and a coating of MUC5AC. The coating of MUC5AC comes from surface goblet cells. Scale bar: 20 µm. a′) Detail of a mucus bundle at higher magnification shows how goblet cell mucus reaches out to coat the bundle. Scale bar: 10 µm. b) The contact between a mucus bundle and a goblet cell was also observed with SEM. Scale bar: 1 µm. c) Similar bundles were observed in CF piglet trachea as in WT. Scale bar: 20 µm. c′) Larger magnification showed that the CF bundles had a higher number of contact points than WT bundles. Scale bar: 10 µm. White arrows in (a′) and (c′) point to goblet cell–bundle attachment points. d) The contact between bundles and goblet cells in CF was observed with SEM. Scale bar: 1 µm.

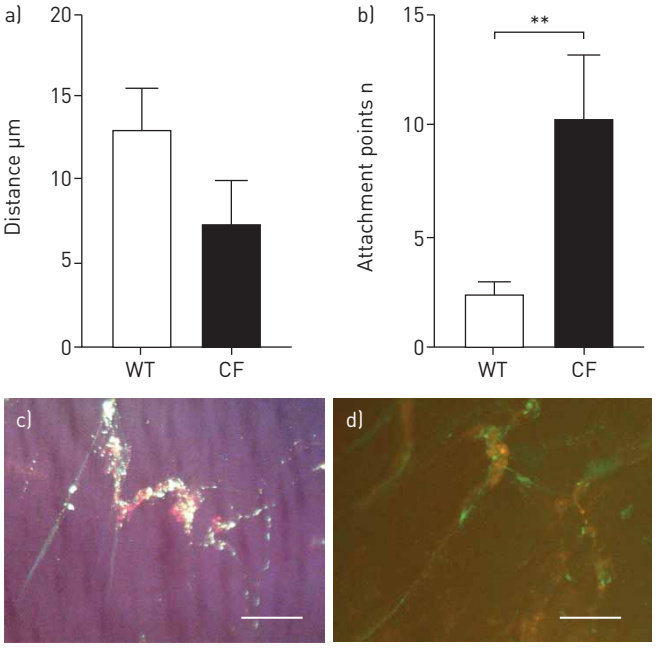

FIGURE 8 Model bacterium *Pseudomonas aeruginosa* PAO1 is cleared in wild-type (WT) but not cystic fibrosis (CF) trachea. LTL: *Lotus tetragonolobus* lectin. a) Measuring the distance between the epithelium and lectin-stained bundles in live tissue revealed a tendency for a shorter distance in CF. WT: n=6 bundles, n=5 animals; CF: n=3 bundles, n=3 animals; not significant (p=0.1). b) The number of attachment points was lower in WT than CF. WT: n=7 bundles, n=5 animals; CF: n=3 bundles, n=3 animals; **: p=0.008. Data analysed with the two-tailed Mann–Whitney U-test. Representative images are presented in figure 7a and c. c) Mucus bundles stained with LTL (green) and bacteria (red) in WT trachea. d) Mucus bundles stained with LTL (green) and bacteria (red) in CF trachea. Note the lack of bacteria on the surface between the bundles in WT. Scale bar: 500 µm.

Lungs in pigs and humans have numerous submucosal glands ideally organised to make mucus bundles from linear MUC5B mucin polymers [5]. In the normal lung, these mucus bundles are transported cephalically and by this sweep the tracheal surface [6]. The normal tracheobronchial surface is covered by ASL of ~10 µm, of which 4–5 µm is occupied by cilia in pigs and humans [2]. The mucus bundles are substantially thicker (average thickness 27 µm) than the ASL depth and thus the bundles are moving on top of and partly dipping into the ASL.

During the transport toward the larynx the mucus bundles from the glands were covered with material from surface goblet cells both in WT and CF. This material contained MUC5AC mucin, was stained by UEA1 lectin and connected the surface goblet cells with the bundles. The distance between the bundles and epithelial surface was similar in WT and CF, but the number of connections was four times higher in CF. As bundles appeared from both WT and CF glands, and were found on the tracheal surface to about the same extent, we find it difficult to envision that the mucus bundles were attach specifically to the gland openings, as has been suggested elsewhere [12, 17]. In contrast, our results suggest that the mucus bundles were anchored to the surface goblet cells in CF. The observations that the MUC5B core of the mucus bundles is coated with MUC5AC mucin and that goblet cells are found also in the gland ducts [2, 6] provide an explanation for how the mucus bundles can be seen to be retained in gland openings in CF. Our results suggest that the surface mucus bundles are anchored to the epithelial surface goblet cells, and that the bundles are kept at the surface and thereby hindered from falling out into the tracheal lumen. As the mucus bundles are transported up to 10 times slower than the ASL, we further hypothesise that bundle transport is controlled by attachment/detachment of goblet cell bundle anchors. This is similar to what we observed in the small intestine, where a specific protease is required for detaching the mucins from the goblet cell anchor [13, 18].

CF is characterised by stagnant mucus that permits bacterial overgrowth. We now show that mucus bundles were essentially stationary in newborn CF piglets as well as in WT when bicarbonate was removed. As the piglets were <24 h old at analysis, our results suggest that human CF lung disease also starts at birth, strongly suggesting that treatment of human CF should start early.

In addition, *P. aeruginosa* bacteria added to the tracheas were rapidly removed from the WT, but not CF tissues. This can be observed as a higher background in the CF images. Whether the cause of this difference is more or less specific binding to the epithelial surface or the absence of bundle sweeping, or a

combination of these, is not known. The important point is that in CF there is already a defect in airway cleaning from birth and the bacterial accumulation can be assumed to cause lung disease.

The cholinergic system is important for respiratory physiology as the vagus nerve uses ACh to increase airway smooth muscle tone, submucosal gland secretion and ciliary beat frequency at the same time as it inhibits surface liquid absorption [2, 3]. Increased cholinergic tonus is common in asthma and COPD [8, 9], and the muscarinic inhibitor ipratropium bromide (Atrovent) is used for pharmacological treatment of COPD. The use of this treatment is rather counterintuitive except for its inhibitory effect on bronchoconstriction. However, we discovered that ACh quickly stopped the transport of mucus bundles and this suggests another novel mechanism that could explain some of the beneficial effect of anticholinergic treatment. Ipratropium, with its antimuscarinic M_1, M_2 and M_3 receptor inhibition, efficiently reversed this effect. Newborn normal piglet tracheas do not represent the lung phenotype of a patient with COPD as these patients have accumulated mucus and plugged airways. However, COPD patients have a clinically documented beneficial effect of ipratropium bromide and we suggest that this is due to its anticholinergic effect counteracting ACh-induced reduced mucus bundle transport. This effect might be localised to less affected parts in COPD patients. Clinical outcomes of ipratropium bromide in COPD are presented elsewhere [19]. Ipratropium bromide had little effect in newborn CF piglets, indicating that initial CF disease acts by a different mechanism than in COPD. However, lack of effect in initial CF disease does not mean lack of effect in advanced CF disease [20].

Increased vagal tone and ACh is the physiological response to dust inhalation, and the effect to block bundle movement while the ASL is simultaneously moving faster is a logical one to more efficiently collect the debris onto the mucus bundles before these are detached and capable of efficiently transporting the debris to the larynx. Our results further suggest that mucus bundle movement is controlled by attachment/detachment of the mucus bundles to the surface goblet cells.

Acknowledgements: We are indebted to Jeffrey Wine (Stanford University, Stanford, CA, USA) and David Thornton (University of Manchester, Manchester, UK) for valuable discussions.

Conflict of interest: G.C. Hansson reports grants from the National Institutes of Health, European Research Council, Wallenberg, Lederhausen and CFF, during the conduct of the study.

Support statement: This work was supported by the European Research Council, Swedish Research Council, The Swedish Cancer Foundation, The Knut and Alice Wallenberg Foundation, IngaBritt and Arne Lundberg Foundation, Sahlgrenska University Hospital (ALF), Wilhelm and Martina Lundgren's Foundation, National Institute of Allergy and Infectious Diseases (U01AI095473), Erica Lederhausen's Foundation, The Swedish Heart–Lung Foundation, the Swedish Cystic Fibrosis Foundation, Magnus Bergvall's Foundation, Lederhausen's Center for CF Research at the University of Gothenburg, and the Cystic Fibrosis Foundation (CFFT, Hansso14X0). Funding information for this article has been deposited with the Crossref Funder Registry.

References

1 Gosens R, Zaagsma J, Meurs H, *et al.* Muscarinic receptor signaling in the pathophysiology of asthma and COPD. *Respir Res* 2006; 7: 73.
2 Widdicombe JH, Wine JJ. Airway gland structure and function. *Physiol Rev* 2015; 95: 1241–1319.
3 Buels KS, Fryer AD. Muscarinic receptor antagonists: effects on pulmonary function. *Handb Exp Pharmacol* 2012; 208: 317–341.
4 Ridley C, Kouvatsos N, Raynal BD, *et al.* Assembly of the respiratory mucin MUC5B: a new model for gel-forming mucin. *J Biol Chem* 2014; 289: 16409–16420.
5 Trillo-Muyo S, Nilsson HE, Recktenwald CV, *et al.* Granule-stored MUC5B mucins are packed by the non-covalent formation of N-terminal head-to-head tetramers. *J Biol Chem* 2018; 293: 5746–5754.
6 Ermund A, Meiss LN, Rodriguez-Pineiro AM, *et al.* The normal trachea is cleaned by MUC5B mucin bundles from the submucosal glands coated with the MUC5AC mucin. *Biochem Biophys Res Commun* 2017; 492: 331–337.
7 Klymiuk N, Mundhenk L, Kraehe K, *et al.* Sequential targeting of CFTR by BAC vectors generates a novel pig model of cystic fibrosis. *J Mol Med* 2012; 90: 597–608.
8 Gross NJ, Co E, Skorodin MS. Cholinergic bronchomotor tone in COPD. Estimates of its amount in comparison with that in normal subjects. *Chest* 1989; 96: 984–987.
9 Ayala LE, Ahmed T. Is there loss of protective muscarinic receptor mechanism in asthma? *Chest* 1989; 96: 1285–1291.
10 Larsen GL, Barron RJ, Cotton EK, *et al.* A comparative study of inhaled atropine sulfate and isoproterenol hydrochloride in cystic fibrosis. *Am Rev Respir Dis* 1979; 119: 399–407.
11 Wanner A. Clinical aspects of mucociliary transport. *Am Rev Respir Dis* 1977; 116: 73–125.
12 Hoegger MJ, Fischer AJ, McMenimen JD, *et al.* Impaired mucus detachment disrupts mucociliary transport in a piglet model of cystic fibrosis. *Science* 2014; 345: 818–822.
13 Gustafsson JK, Ermund A, Ambort D, *et al.* Bicarbonate and functional CFTR channel is required for proper mucin secretion and link cystic fibrosis with its mucus phenotype. *J Exp Med* 2012; 209: 1263–1272.
14 Hoegger MJ, Awadalla M, Namati E, *et al.* Assessing mucociliary transport of single particles in vivo shows variable speed and preference for the ventral trachea in newborn pigs. *Proc Natl Acad Sci USA* 2014; 111: 2355–2360.

15 Birket SE, Chu KK, Liu L, *et al.* A functional anatomic defect of the cystic fibrosis airway. *Am J Respir Crit Care Med* 2014; 190: 421–432.
16 Matsui H, Grubb BR, Tarran R, *et al.* Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell* 1998; 95: 1005–1015.
17 Ostedgaard LS, Moninger TO, McMenimen JD, *et al.* Gel-forming mucins form distinct morphologic structures in airways. *Proc Natl Acad Sci USA* 2017; 114: 6842–6847.
18 Schütte A, Ermund A, Becker-Pauly C, *et al.* Microbial induced meprin beta cleavage in MUC2 mucin and functional CFTR channel are required to release anchored small intestinal mucus. *Proc Natl Acad Sci USA* 2014; 111: 12396–12401.
19 Li X, Obeidat M, Zhou G, *et al.* Responsiveness to ipratropium bromide in male and female patients with mild to moderate chronic obstructive pulmonary disease. *EBioMedicine* 2017; 19: 139–145.
20 Bradley JM, Koker P, Deng Q, *et al.* Testing two different doses of tiotropium Respimat in cystic fibrosis: phase 2 randomized trial results. *PLoS One* 2014; 9: e106195.