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## **Evidence of early increased sialylation of airway mucins and defective mucociliary clearance in CFTR-deficient piglets**

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**Highlights:**

- CFTR-deficient pigs present increased lung mucin sialylation at birth in the absence of inflammation
- Increased mucin sialylation is linked to increased *Pseudomonas aeruginosa* adhesion to mucins
- CF pigs also present a mucociliary clearance defect, in the absence of inflammation, which may contribute bacterial colonization and development of early lung disease. These defects seem to be linked to the absence of CFTR functionality since they are independent of an inflammatory environment in the lungs

## **Abstract**

### **Background**

Bacterial colonization in cystic fibrosis (CF) lungs has been directly associated to the loss of CFTR function, and/or secondarily linked to repetitive cycles of chronic inflammation/infection. We hypothesized that altered molecular properties of mucins could contribute to this process.

### **Methods**

Newborn CFTR<sup>+/+</sup> and CFTR<sup>-/-</sup> were sacrificed before and 6h after inoculation with luminescent *Pseudomonas aeruginosa* into the tracheal carina. Tracheal mucosa and the bronchoalveolar lavage (BAL) fluid were collected to determine the level of mucin O-glycosylation, bacteria binding to mucins and the airways transcriptome. Disturbances in mucociliary transport were determined by *ex-vivo* imaging of luminescent *Pseudomonas aeruginosa*.

### **Results**

We provide evidence of an increased sialylation of CF airway mucins and impaired mucociliary transport that occur before the onset of inflammation. Hypersialylation of mucins was reproduced on tracheal explants from non CF animals treated with GlyH101, an inhibitor of CFTR channel activity, indicating a causal relationship between the absence of CFTR expression and the sialylation of mucins. This increased sialylation was correlated to an increased adherence of *P. aeruginosa* to mucins. *In vivo* infection of newborn CF piglets by live luminescent *P. aeruginosa* demonstrated an impairment of mucociliary transport of this bacterium, with no evidence of pre-existing inflammation.

### **Conclusions**

Our results document for the first time in a well-defined CF animal model modifications that affect the O-glycan chains of mucins. These alterations precede infection and inflammation

of airway tissues, and provide a favorable context for microbial development in CF lung that hallmarks this disease.

**Keywords:** cystic fibrosis; mucociliary transport; mucin glycosylation; CFTR; *Sus scrofa*

## Introduction

Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene affect the rheology of CF mucus, which becomes thick and difficult to clear from the airways [1]. The combination of bronchial obstruction by mucus, bacterial infection and persistent inflammation eventually leads to lung tissue destruction [2, 3]. There is a long-standing debate regarding the links between CFTR mutations and the development of persistent infection and inflammation [4]. While some suggest that CFTR dysfunction leads to intrinsic inflammation, others hypothesize that excessive inflammation is rather the result of a lung environment that favours bacterial colonization, being indirectly linked to specificities of the CF mucus [5]. Contradictory reports have shown increased or decreased sialylation and sulfation of O-glycan content in CF patients [6-8]. These discrepancies are probably explained by altered expression of sialyltransferases under inflammatory conditions, such as those observed in CF patients [6, 9].

Understanding the precise mechanisms that link the genetic defect to the hyper-inflammatory CF phenotype has been hampered by the paucity of animal models that faithfully reproduce human CF lung disease and on which the study of the early abnormalities of the lung disease would be possible [10]. The CF pig model demonstrates similar airway physiology as humans [11] and, unlike mice, it spontaneously develops CF lung disease [12]. Moreover, CF pig lungs do not show signs of lung inflammation at birth, allowing the evaluation of mucin structure and function before the onset of infection and inflammation [12].

Here, we used newborn CF pigs to determine whether there are alterations in the biochemical composition of airway mucins, mucociliary transport (MCT), as well as the lung inflammatory status before and after *P. aeruginosa* infections, a major pathogen in CF patients. Our results strongly suggest that altered mucin sialylation and MCT predispose to bacterial colonization at birth, in the absence of inflammation.

## **Materials and methods**

### **Animals**

All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee at INRA. The protocol was approved by the French “Ministère de l'éducation nationale, de l'enseignement supérieur et de la recherche” (n° 1166-2015071615392426). Further details are given in the Supplementary material.

### **Newborn cystic fibrosis pig sample collection**

Broncho-alveolar lavage (BAL) from CFTR<sup>+/+</sup> and CFTR<sup>-/-</sup> newborn piglets was collected by instilling and re-aspirating two 15-mL aliquots of sterile PBS in the excised lungs.

Tracheal mucosa was collected at the level of the carina and frozen in liquid nitrogen for mucin isolation, protein extraction and gene expression studies. In addition, tracheal and lung tissues were collected and fixed in either 4% formalin or carnoy's fixative for histological analysis.

### **Analysis of mucin O-glycosylation**

Using BAL fluid, mucins were purified by isopycnic density-gradient centrifugation (Beckman Coulter LE80K ultracentrifuge; 70.1 Ti rotor, 417 600 g at 15°C for 72 h). Mucins were then submitted to  $\beta$ -elimination under reductive conditions (0.1 M KOH, 1 M KBH<sub>4</sub> for 24 h at 45°C) and the oligosaccharide fractions were isolated by size exclusion chromatography. Permethylation of the mixture of oligosaccharide alditols was carried out with the sodium hydroxide procedure. After derivatization, the reaction products were dissolved in 200  $\mu$ L of methanol and further purified on a C<sub>18</sub> Sep-Pak column. Permethyated oligosaccharides were analysed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) in positive ion reflective mode as

[M+Na]<sup>+</sup>. Quantification through the relative percentage of each oligosaccharide was calculated based on integration of peaks on MS spectra.

### **Measurement of total sialic acids (Neu5Ac and Neu5Gc)**

Proteins were extracted from tracheal tissues of CFTR<sup>-/-</sup> and CFTR<sup>+/+</sup> piglets. Each frozen sample was resuspended in lysis buffer (100 mM Tris HCl, pH 7.4 ; 150 mM NaCl, 1 mM EDTA and 1% Triton X-100), centrifuged at 12 000 g for 10 minutes at 4°C and the supernatant was collected. The concentration of proteins in each sample was determined using a BCA protein assay kit.

Sialic acids were labelled using 1,2-diamino-4,5-methylene dioxybenzene (DMB) and quantified by RP-HPLC. Briefly, total sialic acids were analysed in the protein extracts from tracheal tissues from piglets, following acid hydrolysis release using 2N TFA at 80°C for 3 hours. After vacuum evaporation, sialic acids were derivatized with DMB (7 mM DMB, 18 mM sodium hydrosulfite, 1.4 mM acetic acid and 0.7 mM beta-mercaptoethanol) for 2 hours at 50°C in the dark and separated by HPLC using a C18 column and a mixture of MilliQ water, methanol and acetonitrile (84/7/9) as elution buffer, at a flow rate of 0,9 mL/min. Detection of fluorescently labelled sialic acids was achieved at excitation and emission wavelengths of 373 nm and 448 nm respectively.

### **Incubation of ex-vivo wild-type tracheal explants with CFTR inhibitor**

In order to verify that any possible changes in mucin structure were due to CFTR dysfunction, we inhibited CFTR function in wild type piglet tracheas *in vitro* and examined mucin structure. Wild-type newborn piglets were sacrificed at birth to collect the tracheal explants. Airways were explanted and transported to the laboratory immersed in chilled Krebs-glucose buffer (116 mM NaCl, 1.3 mM CaCl<sub>2</sub>, 3.6 mM KCl, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, 23 mM



NaHCO<sub>3</sub>, and 1.2 mM MgSO<sub>4</sub>, 10 mM d-glucose, 5.7 mM pyruvate, 5.1 mM glutamate, pH 7.4) in the first 2 h after sacrifice. All connective and pulmonary tissue was removed. Tissues were gradually heated and incubated for 24 h in oxygenated Krebs-glucose buffer supplemented with 100 μM GlyH101 or vehicle (control) at 37°C in 5% CO<sub>2</sub> atmosphere. Mucosal tissue was separated and frozen in liquid nitrogen for analysis of mucin O-glycosylation.

### **Experimental infections with *P. aeruginosa* and bioluminescent imaging**

Piglets were anesthetized with Vetflurane®, intubated with cuffless tracheostomy tubes and ventilated with a Fabius® Tiro® Ventilator (Dräger, Telford, USA). Ventilator settings were: volume controlled mode, tidal volume = 8-10 mL.kg<sup>-1</sup>, positive end-expiratory pressure = 5 cmH<sub>2</sub>O, respiratory rate = 15 breath.min<sup>-1</sup>, inspiratory/expiratory ratio = 0.5, 50% oxygen. Piglets were inoculated with 2 mL of luminescent *P. aeruginosa* strain PAKlux [13] suspension (5 x 10<sup>6</sup> cfu/mL) into the carina using an esophageal probe. Piglets were maintained under mechanical ventilation and sacrificed 6 hours after infection.

Lungs were excised and immediately imaged by IVIS Spectrum (PerkinElmer, Waltham MA). A sagittal section of the head was performed to expose the two halves. Regions of interest were placed on a 2D bioluminescent imager to integrate the luminescent signals and analysis was performed using Living Image 4.3.1. Lungs samples for mucin-O-glycosylation and gene expression analysis were collected as above described.

### **1-D bacterial overlay**

The 1-D bacterial overlay procedure was used to evaluate the binding of *P. aeruginosa* to mucins purified from BAL of CFTR<sup>-/-</sup> and CFTR<sup>+/+</sup> piglets. Briefly, mucins (10 μg) were spotted on dry nitrocellulose membranes which were saturated by PFBB (Protein Free Blocking Buffer) (Thermo-Scientific) for 1 h. Bacteria (10<sup>9</sup> CFU/mL in phosphate-buffered

saline) were labeled with DAPI for 15 min at room temperature in the dark. Labeled bacteria were collected by centrifugation at 3000xg for 5 min, washed three times in PBS, suspended in 1 mL of blocking buffer and added to the membrane in blocking buffer. After incubation for 1 h at room temperature in the dark, followed by three washes of membranes in PBS containing 0.5% Tween 20, the fluorescence of adherent bacteria was detected by a ChemiGenius 2 imaging system (Syngene). Mucins were chemically desialylated for 1h at 80°C in a 0.05M TFA solution.

### **Gene expression profiling**

Total RNA was extracted from 9 CFTR<sup>+/+</sup> and 10 CFTR<sup>-/-</sup> piglet tracheal carinas, using RNA columns (miRNeasy, Qiagen, Les Ulis, France). The experiment was balanced for sex, infection and genotype. There were two females and three males in each experimental group (i.e. “-/- infected”, “-/- not infected”, “+/+ infected”, “+/+ not infected”), except in the “+/+ infected” group where there were only 2 males. All sequencing results were submitted in the GEO database under the accession number GSE99862. Real-time quantitative PCR (RT-qPCR) was used to validate the results for a selected subset of genes involved in the inflammatory response (Table S1). Detailed RNA-seq, bioinformatics and RT-qPCR analysis are described in the online supplement.

### **Evaluation of cytokines and chemokines in BAL**

Tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-6, IL-8 and Il-10 were measured in the BAL supernatant using specific Pig ELISA kits (Abcam®, Paris, France) following manufacturer’s instructions.

### **Histological and immunohistochemical analysis**

Formalin-fixed, paraffin-embedded tracheal and lung sections (5  $\mu$ m) from CFTR<sup>-/-</sup> and CFTR<sup>+/+</sup> piglets were either stained with haematoxylin and eosin (H&E), with PAS/alcian

blue at pH 2,5 or incubated with anti MUC5AC (clone 45-M1; 1:150) or rabbit polyclonal anti-MUC5B (H-300; sc-20119, Santa Cruz Biotechnology, 1:150) antibodies. For further details see Supplementary material.

### **Determination of *P. aeruginosa* growth on CFTR<sup>+/+</sup> and CFTR<sup>-/-</sup> airway epithelia**

Bronchial epithelial cells from 3 CFTR<sup>-/-</sup> and 3 CFTR<sup>+/+</sup> were grown in air-liquid interface (ALI) conditions, as described in the online supplementary material, and incubated with *P. aeruginosa* PAKlux (MOI= 0.1) during 6h at 37°C in the IVIS Spectrum (PerkinElmer, Waltham MA, USA). The growth rate of *P. aeruginosa* PAKlux on either CFTR<sup>+/+</sup> or CFTR<sup>-/-</sup> differentiated bronchial epithelial cells was calculated by measuring PAKlux bioluminescent signal. Bioluminescence was captured every fifteen minutes with integration times ranging from few seconds to five minutes depending of the intensity of the signals. Regions of interest (ROIs) were placed on the 2D bioluminescent image to encompass the luminescent signals and analysis was done using Living Image 4.3.1.

### **Statistical analysis**

Results are expressed as mean±S.E.M. Statistical analyses were performed using GraphPad Prism versions 5.0 (GraphPad Software, La Jolla, CA) or the statistical package R version 3.3.3. Differences in mucin O-glycosylation, *P. aeruginosa* adherence to mucins, gene expression and cytokine levels between CFTR<sup>-/-</sup> and CFTR<sup>+/+</sup> piglets were analysed using a Mann-Whitney-Wilcoxon test. *P. aeruginosa* growth rate in CFTR<sup>-/-</sup> or CFTR<sup>+/+</sup> airway epithelium was fitted using a non-linear exponential growth model (Graphpad Prism 5.0) and best-fits compared using the extra-sum-of-squares F test. A p-value <0.05 was considered statistically significant.

## Results

### **Altered glycosylation of airway mucins in CFTR<sup>-/-</sup> piglets is present at birth.**

In order to identify possible alterations in the level of sialylation of airway mucins of CFTR<sup>-/-</sup> pigs, we used a modified AB/PAS (alcian blue/Periodic-Acid-Schiff) histochemical staining of airway tissues [14]. This technique stains sialylated mucins in blue, neutral mucins stain in magenta and mixtures stain in various shades of purple. As shown in Figure 1A, staining of the airways of CFTR<sup>+/+</sup> pigs showed a majority of goblet cells of the cell surface stained in purple with a very small number of goblet cells stained in blue, indicating that CFTR<sup>+/+</sup> mucins are mainly composed of a mix of neutral and sialylated mucins. On the other hand, CFTR<sup>-/-</sup> pigs trachea showed a majority of goblet cells stained in blue, indicative of a high density of sialylated mucins (Fig. 1B). Mucins from the submucosal glands of both CFTR<sup>-/-</sup> and CFTR<sup>+/+</sup> piglets stained magenta or in various shades of purple.

These results were confirmed by mass spectrometry analysis of mucins from bronchoalveolar lavages (BAL) samples, which showed a 2.4 fold increase ( $p < 0,0001$ ) in the percentage of sialylated oligosaccharides from newborn CF piglets compared to non-CF (Fig. 1C and Supplementary Fig. 1). Differences are statistically significant ( $p < 0.05$ ) for most sialylated structures when considered individually. We also collected cell surface mucins by scraping the mucosal layer from the trachea of CFTR<sup>-/-</sup> and CFTR<sup>+/+</sup> piglets. In agreement with the results from BAL mucins, a significant increase of sialylation by a factor 1.8 was found for surface mucins from CFTR<sup>-/-</sup> piglets compared to CFTR<sup>+/+</sup> ones ( $p < 0.001$ , Fig. 1C).

### **Impairment of the CFTR channel activity by the CFTR inhibitor GlyH101 enhances mucin sialylation in wild-type (WT) newborn piglets.**

A third piece of evidence was provided with *ex vivo* tracheal explants of WT piglets which were treated for 24h with the CFTR inhibitor GlyH101 followed by separation and

purification of mucosal mucins. We observed a significant increase in mucin sialylation in tracheas treated with GlyH101 ( $p < 0,05$ , Fig. 1D).

### **Hypersialylation of cell surface mucins does not affect other glycoproteins.**

In order to determine whether alterations in sialylation could be due to a general effect that would affect other glycoconjugates, we evaluated the total sialic acids (N-acetylneuraminic acid, Neu5Ac and N-glycolylneuraminic acid, Neu5Gc) from proteins extracted from newborn pigs tracheas. As shown in Figure 2, there were no significant differences in protein sialylation between the two genotypes.

### **Altered glycosylation of mucins in newborn CFTR<sup>-/-</sup> animals is not a consequence of inflammation or bacterial infection**

The airway transcriptome of newborn CFTR<sup>-/-</sup> animals was characterized after RNA-seq analysis, under basal conditions or after a 6 hour-infection with *P. aeruginosa*. Fig 3B compares the log<sub>2</sub>ratio between CF and non CF animals for the infected (x-axis) and non-infected (y-axis) conditions. It shows that very few modifications of gene expression were detectable between the two genotypes, except for CFTR which was strongly downregulated (Fig 3A,B).

Pre-existing inflammation in CFTR<sup>-/-</sup> airways was ruled out since no difference in the expression of inflammatory genes was noticed between the two genotypes under basal conditions (Fig. 3B). Table 1 summarizes gene expression for 12 sialyltransferases that were identified in our pig transcriptomes. No difference in their expression was observed at birth before infection with *P. aeruginosa* (Table 1). RNA-seq data is described in more details in the online supplement.

These data, which support the absence of inflammation in non-infected newborn piglet airways, were confirmed: (i) by real-time quantitative PCR on a selection of 7 markers (Supplementary Fig. 2); (ii) by the absence of neutrophils in the lumen of CFTR<sup>-/-</sup> and

CFTR<sup>+/+</sup> piglets (Supplementary Fig.3A); (iii) by low levels of IL-8 cytokine in BAL (Supplementary Fig. 3B).

### **Expression and localization of airway mucins are similar between CFTR<sup>-/-</sup> and CFTR<sup>+/+</sup> pigs at birth**

MUC5AC was exclusively found in goblet cells bordering the tracheal lumen (Fig. 4A,D). MUC5B was detected in goblet cells of the surface epithelium (Fig. 4B,E), but it did not appear to be co-expressed in the same granules. MUC5B was also detected in submucosal glands, regardless the genotype. There was no difference in the expression of both mucins between genotypes. These results were in agreement with those published previously by Ostedgaard et al. [15].

### **Altered glycosylation of CFTR<sup>-/-</sup> airway mucins promotes *P. aeruginosa* adhesion at birth.**

To determine a possible impact of altered O-glycan sialylation, we evaluated the capacity of *P. aeruginosa* to bind to immobilized mucins. *P. aeruginosa* adhesion to immobilized CF mucins was significantly increased compared to those from CFTR<sup>+/+</sup> (p<0.01). This binding was lost after chemical desialylation of mucins, supporting a role of sialylated O-glycans as a ligand for *P. aeruginosa* (Fig. 5A).

### ***Pseudomonas aeruginosa* growth is increased in CFTR<sup>-/-</sup> bronchial epithelial cells**

In order to test any impairment of anti-bacterial host defence in CFTR<sup>-/-</sup> pigs, live *P. aeruginosa* were inoculated at the apical surface of a 3D-reconstituted bronchial epithelium, obtained after differentiation under air liquid interface conditions. We noticed a significant increase of bacterial growth when inoculation was performed on the CFTR<sup>-/-</sup> bronchial epithelium (p<0.0001) (Fig. 5B).

### **Impairment of *Pseudomonas aeruginosa* clearance in lungs from CFTR<sup>-/-</sup> piglets**

*In vivo* evaluation of mucociliary clearance of *P. aeruginosa* differed between CFTR<sup>+/+</sup> and CFTR<sup>-/-</sup> piglets. Six hours after bacterial inoculation into the tracheal carina, *P. aeruginosa* was mainly found in the larynx, pharynx and nose of CFTR<sup>+/+</sup> piglets. On the contrary, *P. aeruginosa* remained in the tracheal carina of CFTR<sup>-/-</sup> piglets, near the site of inoculation, suggesting a defective MCT (Fig. 5C).

### **The inflammatory response to infection by *Pseudomonas aeruginosa* is not affected by the CFTR genotype**

The inflammatory response after infection was compared between the 2 genotypes (Fig. 6A-B). A highlight of the transcriptional response of newborn CFTR<sup>-/-</sup> and CFTR<sup>+/+</sup> lungs to infection by *P. aeruginosa* is shown on Fig. 6C. Full data are available in Supplementary Excel file. No major modification of the inflammatory response was noticed between the two genotypes, except for a slight increase of IL-8 in the BAL of CFTR<sup>-/-</sup> piglets (Supplementary Fig. 4).

### **Discussion**

Here, we show for the first time that lung mucins from newborn CF pigs demonstrate increased sialylation, and enhanced *P. aeruginosa* adhesion to these mucins. This experimental model, which closely reproduces the human disease, demonstrates the presence of these alterations early after birth, i.e. before any infection or inflammation had occurred. We also demonstrate by using two different modes of *in vivo* or *in vitro* infections with live bacteria that mucociliary clearance is defective at birth in CF animals, and that airway cell cultures derived from the same tissues display an increased capacity of bacterial growth. All these properties favour the bacterial colonization and inflammation that are observed in CF lungs.

Contrasted differences in the degree of mucin sulfation and sialylation of CF mucins have been reported in the literature [6, 8, 16-18]. These differences have been explained by: (i) differences in the lung inflammatory status of the CF patients, (ii) use of non-CF pulmonary diseases as control patients, who often have difficulties in expectorating mucus [17, 19-21], (iii) regional differences along the vertical axis, with higher levels of sialylation in the distal than in the proximal lung. Our studies on newborn CF pigs were designed in order to control these different parameters. They were performed immediately after birth, i.e. before any cycle of infection and/or inflammation, and in the absence of metacholine stimulation, so that mucins purified from the BAL were representative of the airway surface, including in CFTR<sup>-/-</sup> piglets, where mucus is more difficult to detach [22]. All mucus samples were systematically purified after BAL collection or scraping of the mucosal surface, so that our measurements were specific to mucins. We believe that these points probably explain the differences between our study and a report by Tang *et al.*, who reported no difference of mucin sialylation between newborn CF and WT piglets [23]. Those authors performed mass spectrometry analysis from total airway surface liquid (ASL) after methacholine stimulation. This treatment enriches ASL with submucosal gland secretions [23], which do not present glycosylation alterations in CF patients [7], and could mask the differences in glycosylation present in the surface mucins. Even if their conclusion was that no differences in the glycosylation of mucins between genotypes were found, it should be noticed that the measurement of the monosaccharide composition of ASL revealed an increased ratio of sialic acids (Neu5Ac+Neu5Gc) normalized to galactosamine level in CFTR<sup>-/-</sup> piglets, from a ratio of 1.25 in CFTR<sup>+/+</sup> piglets to a ratio of 2.13 in CFTR<sup>-/-</sup> piglets, which is fully consistent with our own measurement. It should also be noticed that the CF pigs in the two studies originate from two different genetic background. This could result in differences in the glycosylation of mucins, as observed in the glycosylation levels of MUC2 mucins from the small intestine



between different rat strains [24]. There are different mechanisms that could explain the observed increase in mucin sialylation. Mucins could take longer to be released from the cells under CF conditions allowing for greater modification of the O-glycan chains. Cellular endoplasmic reticulum (ER) stress and the unfolded protein response (UPR), which is documented in CF [25-26], could also lead to altered mucin sialylation. In this regard, we have observed that human nasal epithelial cells, cultivated at the air-liquid interface, treated by a CFTR inhibitor or thapsigargin, an inducer of ER stress, display a similar increase in mucin sialylation (our unpublished data). This is in line with our observation of borderline variations affecting molecules such as TRIB3, CHAC1 and ATF3, which are linked to this pathway. A tempting hypothesis would be therefore that a loss of CFTR would directly trigger an endoplasmic reticulum stress, possibly by affecting the internal pH. This could, in turn, promote a weak but significant inflammation, affect the activity of sialyltransferases and increase sialylation of mucins. Interestingly, we didn't observe global differences in sialylation of other glycoproteins in the trachea of CFTR<sup>-/-</sup> piglets. This is in agreement with the study of Tang *et al.* where they analyzed the level of protein sialylation, but not purified mucins, from differentiated primary culture of airway epithelia and found no differences between pig genotypes [23]. Altogether, these results suggest a specific regulation of mucin sialylation expressed at the surface of epithelial cells.

We also noticed that mucins from CF pigs had an increased binding capacity to *P. aeruginosa*, which is probably linked to the ability of *P. aeruginosa* to bind to the sialic acid moiety of mucins [8, 27-29], *via* the bacterial flagellar cap FliD protein [30]. Increased binding should help bacterial clearance from the lungs. In a previous report, Hoegger *et al.* suggested that a defective mucociliary transport occurs in lung from CF pigs due to mucus tethering to gland ducts, which impairs detachment [22]. Hoegger's results were only observed after a cholinergic stimulation of airway newborn CF piglets by methacholine

known to stimulate submucosal gland secretion. Their measurements were done using inert tantalum particles and fluorescent beads to label mucus, and no particles were recovered under basal conditions. In their non CF piglets, mucus tethered to submucosal glands were not observed at all, even after treatment by methacholine, indicating that CF mucus was abnormal before it emerged onto the airway surface. However, this set-up did not address the possible impact of bacteria on MCT and secretion of ASL [31-32]. Because our measurements were directly performed with live *P. aeruginosa*, we believe that our assessment of MCT reproduces more faithfully the physiological situation that occurs during infection. It remains that our data fully confirmed the severe impairment of MCT in CF airways at an early stage of development observed by these authors. Our data did not detect any modification of expression of MUC5AC or MUC5B. However, we noticed a slight increase in Duox2 and DuoxA<sub>2</sub> in the airways of CFTR<sup>-/-</sup> piglets, with borderline statistical scores. Whether a higher expression of Duox2 could drive oxidative and ER stress in cystic fibrosis remains to be investigated. However, oxidative stress and high levels of reactive oxygen species in CF mucus have been shown to positively correlate with high concentrations of the oxidized products of cysteine (disulfide cross-links), leading to an increase in mucin cross-linking and a higher elasticity [33], which may contribute to the observed defective mucociliary transport.

Incidentally, our observation that CF airway epithelium favoured *P. aeruginosa* growth, is probably in line with the reduced pH of the ASL that can inactivate host antimicrobials [34].

Altogether, the CF lung environment promoted bacterial colonization and increased bacterial residency time in the lungs. This can indeed represent a likely explanation for the slight increase in the host defence response observed in RNA-seq experiments. A blunted immune response to heat-killed *Staphylococcus aureus* has been previously reported by Bartlett *et al.*

[12]. This difference could be explained by the use of killed bacteria, unable to colonize the lungs.

Overall, the most notable differences that we measured in gene expression were related to an increased expression of a few transcripts associated with the endoplasmic reticulum stress response. This observation is consistent with the known relationship between endoplasmic reticulum stress and increased activity of the inflammatory pathways [35]. It has however not been reported before in other gene expression studies performed on other CF models. Understanding the underlying mechanisms will thus require future investigations.

In conclusion, we report here for the first time an early increase of respiratory mucin sialylation in the lungs of CF pigs that results in an increased adherence of *P. aeruginosa* to the CF lung, in the absence of any previous inflammation. This increased sialylation, which can also occur as a result of inflammation in non-CF individuals, is potentially beneficial for host defence as it may allow better mucociliary clearance of inhaled pathogens. However, in the CF lung these altered mucin properties may be detrimental as a result of a concomitant decrease in mucociliary clearance and impaired bacterial killing at the epithelial surface. This may result in an increased time of residency of live bacteria, allowing them to escape from the mucus layer and survive long enough in the periciliary liquid to infect neighbouring tissue. We propose that these three properties cooperatively contribute to bacterial colonization and development of CF lung inflammation.

Our results suggest that decreasing the level of sialylated mucin O-glycans in the lungs might be of therapeutic benefit for CF patients by helping fighting bacterial adhesion to the mucus. Thus data gleaned from these study provide new leads for the design of sialylated glycomimetics targeting *Pseudomonas aeruginosa* and the development of new therapeutic strategies for treating bacterial infections.

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**Conflict of interest statement**

The authors declare no conflict of interests.

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**Table 1. Comparison of pig sialyltransferases gene expression in the airways between CFTR<sup>+/+</sup> and CFTR<sup>-/-</sup> piglets at birth either infected or not with *Pseudomonas aeruginosa*.**

ANNOTATIONS			-/- vs +/+		-/- vs +/+, infected		+/, infected vs ctrl		-/-, infected vs ctrl	
Label	Geneid	Reads	log2FC	adjusted p-value	log2FC	adjusted p-value	log2FC	adjusted p-value	log2FC	adjusted p-value
ST3GAL1	ENSSSCG00000005943	1073	0.31	1.00	0.21	0.81	0.68	0.01	0.58	0.02
ST3GAL2	ENSSSCG000000025089	156	0.12	1.00	-0.50	0.45	-0.15	0.71	-0.76	<0.01
ST3GAL3(1)	ENSSSCG000000023597	30	0.15	1.00	0.08	0.95	0.11	0.80	0.03	0.92
ST3GAL3(2)	ENSSSCG000000029461	33	-0.02	1.00	-0.39	0.64	0.38	0.31	0.01	0.98
ST3GAL4	ENSSSCG000000015232	1074	-0.05	1.00	0.03	0.98	-0.33	0.15	-0.25	0.21
ST3GAL5	ENSSSCG000000008227	30	-0.11	1.00	-0.12	0.92	-0.76	0.01	-0.77	0.01
ST6GAL1	ENSSSCG000000025770	1610	-0.25	1.00	-0.11	0.93	0.08	0.88	0.21	0.53
ST6GAL2	ENSSSCG000000008141	31	-0.29	1.00	0.08	0.95	-0.16	0.72	0.22	0.53
ST6GALNAC1	ENSSSCG000000017172	598	-0.21	1.00	0.17	0.85	0.32	0.28	0.69	<0.01
ST6GALNAC2	ENSSSCG000000017177	925	-0.04	1.00	0.26	0.61	-0.65	<0.01	-0.36	0.05
ST6GALNAC4	ENSSSCG000000005628	281	0.18	1.00	-0.44	0.45	0.42	0.13	-0.20	0.46
ST6GALNAC6	ENSSSCG000000005623	309	0.11	1.00	-0.39	0.52	-0.04	0.92	-0.55	0.02
ST8SIA4	ENSSSCG000000014180	759	-0.02	1.00	0.01	0.99	-0.37	0.04	-0.34	0.03

## Figure legends

### Figure 1. CFTR<sup>-/-</sup> pig mucins show altered glycosylation at birth.

A-B). PAS/Alcian blue histochemical staining of CFTR<sup>-/-</sup> and CFTR<sup>+/+</sup> pig tracheas. A) Section (200x) of a CFTR<sup>+/+</sup> proximal trachea showing neutral/acidic mucins in the surface goblet cells (stained in purple). The right figure is a zoom of the surface epithelium goblet cells of CFTR<sup>+/+</sup> pig proximal trachea. B) Section (200x) of a CFTR<sup>-/-</sup> proximal trachea showing acidic mucins in the surface goblet cells (stained in blue). The right figure is a zoom of the surface epithelium goblet cells of CFTR<sup>-/-</sup> pig proximal trachea. Images are representative of 20 CFTR<sup>-/-</sup> and 22 CFTR<sup>+/+</sup> pig tracheas. C) Relative percentage of whole sialylated O-glycans in mucins. Mucin O-glycans were released from BAL (n=20 CFTR<sup>-/-</sup> and n=22 CFTR<sup>+/+</sup> pigs) and from epithelial surface of the tracheas (n=4 CFTR<sup>-/-</sup> and n=7 CFTR<sup>+/+</sup> pigs). O-glycans were permethylated before analysis by MALDI-TOF mass spectrometry in the positive ion mode [M+Na]<sup>+</sup>. D) Increased sialylation of airway mucins after cystic fibrosis transmembrane conductance regulator (CFTR) inhibition. Relative percentage of whole sialylated O-glycans in mucins treated with the CFTR inhibitor GlyH101. Mucin O-glycans were released from tracheas (n=5 for each condition) and O-glycans permethylated before analysis by MALDI-TOF mass spectrometry in the positive ion mode [M+Na]<sup>+</sup>. Data correspond to mean ± S.E.M.

### Figure 2. Hypersialylation does not affect other glycoproteins.

Total sialic acids (Neu5Ac and Neu5Gc) in proteins extracted from tracheal CFTR<sup>-/-</sup> and CFTR<sup>+/+</sup> pigs, determined by RP-HPLC. N= 20 CFTR<sup>-/-</sup> and 22 CFTR<sup>+/+</sup>. Data correspond to mean ± S.E.M.

**Figure 3. Gene expression modifications in airway mucosa from newborn CFTR<sup>-/-</sup> piglets.**

A) Outline of the study groups. B) RNA-seq read densities (vertical scale of reads) in the CFTR genomic locus in CFTR<sup>-/-</sup> and CFTR<sup>+/+</sup>, showing the absence of CFTR mRNA in CFTR<sup>-/-</sup> piglets. C) Relationship between log<sub>2</sub>(KO/WT) in infected (horizontal) and not infected (vertical) piglets, essentially showing the absence of a transcriptomic signature that would distinguish CFTR<sup>-/-</sup> from <sup>+/+</sup> piglets, in infected (inf) or not infected (ctrl) animals.

**Figure 4. Expression and localization of secreted MUC5AC and MUC5B mucins are similar in CFTR<sup>-/-</sup> and CFTR<sup>+/+</sup> pig airways.**

Double immunostaining for MUC5AC detected by mouse monoclonal antibody 45-M1 in green (A, D) and MUC5B detected by rabbit polyclonal antibody H-300, sc-20119 in red (B, E). Nuclei were stained in blue. C and F are merged images of the corresponding line. MUC5AC was exclusively recovered in the surface epithelium goblet cells whereas MUC5B was localized in the submucosal glands and in the surface goblet cells in the trachea of CFTR<sup>+/+</sup> (A-C) and CFTR<sup>-/-</sup> pigs (D-F). Scale bar = 50 μm

**Figure 5. CFTR<sup>-/-</sup> pigs show impaired bacterial clearance from the lungs.**

A-B) *Pseudomonas aeruginosa* preferentially binds to and grows in CFTR<sup>-/-</sup> airway mucins.

A) Binding of DAPI-labeled *P. aeruginosa* bacteria to purified airway mucins from CFTR<sup>-/-</sup> and CFTR<sup>+/+</sup> pigs was quantified by slot-blot overlay assays. *P. aeruginosa* binds to CFTR<sup>-/-</sup> airway mucins. After chemical desialylation of mucins, no binding of the bacteria was observed. Data shown is a representative experiment +/- S.D of 3 replicates, representative of at least 20 experiments.

B) Time series of luminescent *P. aeruginosa* imaged under the IVIS spectrum system. Bacteria was inoculated to a CFTR<sup>+/+</sup> or a CFTR<sup>-/-</sup> bronchial epithelium (MOI=0.1) and cultured for 6h. Data are mean±S.E.M. Curves were fitted using a non-linear exponential growth model and best-fits compared using the extra-sum-of-squares F test.

C) Defect in mucociliary clearance in CFTR<sup>-/-</sup> pigs. CFTR<sup>+/+</sup> and CFTR<sup>-/-</sup> pigs (n=3) were inoculated with 2 mL of luminescent *P. aeruginosa* (5 x10<sup>6</sup> cfu/mL) into the tracheal carina for 6 h. Lungs and a sagittal section of the head were imaged under the IVIS Spectrum system. *Pseudomonas aeruginosa* can be observed in the larynx and pharynx of CFTR<sup>+/+</sup> pigs, while is mainly restricted to the tracheal carina of CFTR<sup>-/-</sup> pigs.

**Figure 6. CFTR<sup>-/-</sup> piglet transcriptional response following infection by *Pseudomonas aeruginosa*.**

A) RNA-seq read densities (vertical scale of reads) for TNF, IL-6, CXCL2 and IL1B1 genomic loci illustrating the similar inflammatory response observed after infection by *Pseudomonas aeruginosa* of the CFTR<sup>-/-</sup> and CFTR<sup>+/+</sup> piglet airways. B) Log<sub>2</sub>(infected/control) relationship between CFTR<sup>+/+</sup> (horizontal) and CFTR<sup>-/-</sup> (vertical). C) Heatmap of the most differentially expressed transcripts during infection. Corresponding data are available in Supplementary Table 2.















