Intrinsic alterations in peripheral neutrophils from cystic fibrosis newborn piglets

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Title: Intrinsic alterations in peripheral neutrophils from cystic fibrosis newborn piglets

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Abbreviations:

ICM-MS: intact cell matrix-assisted laser desorption/ionization mass spectrometry.

PWKW: P-value from combined Wilcoxon rank-sum test and Kruskal-Wallis test.

Highlights

- Blood neutrophils from newborn CF-pigs present an altered proteomic profile
- Intact cell MALDI-TOF is able to discriminate between CF and WT blood neutrophils
- Differentially expressed proteins are mainly related to antimicrobial response

Abstract (250 words)

Background

The hallmark of the cystic fibrosis (CF) lung disease is a neutrophil dominated lung environment that is associated to chronic lung tissue destruction and ultimately the patient's death. It is unclear whether the exacerbated neutrophil response is primary related to a defective CFTR or rather secondary to chronic bacterial colonization and inflammation. Here, we hypothesized that CF peripheral blood neutrophils present intrinsic alteration at birth before the start of an inflammatory process.

Methods

Peripheral blood neutrophils were isolated from newborn $CFTR^{+/+}$ and $CFTR^{-/-}$ piglets. Neutrophils immunophenotype was evaluated by flow cytometry. Lipidomic and proteomic profile were characterized by liquid chromatography/tandem mass spectrometry (LC-MS/MS), intact cell matrix-assisted laser desorption/ionization mass spectrometry (ICM-MS) followed by top-down high-resolution mass spectrometry (HRMS), respectively. The ability of CF neutrophils to kill *pseudomonas aeruginosa* was also evaluated.

Results

Polyunsaturated fatty acid metabolites analysis did not show any difference between $CFTR^{+/+}$ and $CFTR^{-/-}$ neutrophils. On the other hand, a predictive mathematical model based on the ICM-MS proteomic profile was able to discriminate between both genotypes. Top-down proteomic analysis identified 19 m/z differentially abundant masses that corresponded mainly to proteins related to the antimicrobial response and the generation of reactive oxygen species (ROS). However, no alteration in the ability of $CFTR^{-/-}$ neutrophils to kill *pseudomonas aeruginosa in vitro* was observed.

Conclusions

ICM-MS demonstrated that $CFTR^{-/-}$ neutrophils present intrinsic alterations already at birth, before the presence of any infection or inflammation.

Keywords:

Cystic fibrosis, CFTR, pig model, neutrophils, ICM-MS

1 Introduction

Cystic fibrosis (CF) is a recessive genetic disease caused by mutations in the cystic fibrosis transmembrane regulator (CFTR) gene, a cAMP-regulated chloride channel (1). CFTR loss of function leads to aberrant ion regulation and disturbed homeostasis in the epithelial surface. Although a multi-systemic disease, lung alterations are the major cause of morbidity and mortality in CF. In the airways, CF is characterised by dehydration of the airway surface liquid (ASL), mucus plugging, and a vicious cycle of recurrent bacterial infections and inflammation (2). CF lung inflammation is characterised by the massive recruitment of neutrophils, release of neutrophil serine proteases, and lung tissue destruction (3). The paradox is that despite the large number of neutrophils presents in the lungs, they fail to kill colonizing bacteria. At the same time, neutrophils are central orchestrators of the inflammatory process as a major source of regulatory lipid mediators, playing an important role in the resolution of the inflammation (4).

Whether neutrophil defective response is due to an innate effect of CFTR alteration or to the lung inflammatory environment is unclear. For instance, chronic infection and inflammation in the lungs of CF patients could lead to altered neutrophil maturation and function (3, 5-7). This is supported by the fact that chronic bacterial colonization in CF is mostly restricted to the lungs. On the other hand, the presence of CFTR in neutrophils and the phagolysosome suggests a direct role in several neutrophil functions, including degranulation bacteria phagocytosis and killing (8, 9).

Studies aiming to clarify the role of CFTR in neutrophils are hampered by several difficulties. First, there is an inherent variability in the degree of inflammation when sampling human patients, due to age and course of disease, and second, sampling newborn babies present several technical and ethical limitations. Animal models can be used to tackle these issues. Mice models present several drawbacks since they fail to recapitulate human CF disease. New animal models have been proposed as an alternative. The $CFTR^{-/-}$ pig model mirrors most features of human CF disease (10, 11). Porcine and human neutrophils present also similar biochemical properties, and the pig is a suitable model for testing new therapies targeting neutrophil inflammation (12, 13). In addition, no signs of lung or systemic inflammation are observed at birth in the CF pig model that could mask or confound the innate differences between CF and non-CF neutrophils.

Here, we aimed to search for potential differences in bioactive inflammatory and pro-resolving lipids as well as peptide/protein content between CF neutrophils and their wild-type counterparts before the appearance of an inflammatory environment that could mask their innate defects.

2 Material and Methods

2.1 Chemicals

Unless otherwise indicated, chemicals were purchased by Sigma-Aldrich (Saint Quentin Fallavier, France). PercollTM was from Fisher (Illkirch, France). Precast gel were from Biorad (Marnes-la-Coquette, France). Antibodies anti-SWC1, SWC3, SWC8, SWC9 for flow cytometry analysis were from Gentaur (Paris, France) and anti-CD11b from Biolegend (Ozyme, Saint Quentin Yvelines, France). Secondary antibody anti-IgM-FITC was from Abcam (Paris, France). Anti-Annexin A1 and anti-H2AFX were from Antibodies online (Paris, France), anti-Pr3 was produced as described in Brea et al (13).

2.2 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).

Male and female $CFTR^{+/-}$ transgenic pigs were provided by the LMU Munich, Germany (10), transferred to INRA, Nouzilly (France) and mated to generate $CFTR^{+/+}$, $CFTR^{+/-}$ and $CFTR^{-/-}$ piglets. Piglets were allowed to suckle colostrum and genotype was confirmed by multiplex PCR as described below. All samples were collected within 6 hours of birth and an approximate equal distribution of male and female newborn piglets was used. For sacrifice, pigs were sedated with intramuscular Ketamine (20 mg/kg; Imalgene®, Mérial, France) and Xylazine (2 mg/kg; Rompun®, Bayer, Germany), and exsanguinated. Blood was collected over 0.2% EDTA (pH 8.0) and processed within 1 h of collection.

2.3 Genotyping of newborn piglets

The genotype of the $CFTR^{+/+}$, $CFTR^{+/-}$ and $CFTR^{-/-}$ piglets was determined by multiplex PCR using a combination of 3 different primers as described in Guillon et al (14).

2.4 White blood cell (WBC) count

Total and differential WBC were counted with a MS9-5 Hematology Counter® (digital automatic hematology analyzer, Melet Schloesing Laboratories, France) following manufacturer's recommendations.

2.5 Isolation of porcine blood neutrophils

An aliquot of blood (15 ml) was incubated with 35 ml of red blood cells lysis buffer [0.1 mM EDTA, 10 mM potassium bicarbonate and 150 mM ammonium chloride (pH 7.4)] for 15 min at room temperature and then centrifuged at 500 g for 5 min. The pellet was suspended with 15 ml of PBS and incubated as previously with lysis buffer. The resulting pellet was suspended with 12 mL of PBS and a sample was removed for flow cytometric analysis; the remaining cells were gently layered on a discontinuous Percoll™ density gradient prepared with 1.2 ml layers of the following densities: 1.105, 1.100, 1.093, 1.087 and 1.081 g/ml. After centrifugation at 800 g for 30 min, the neutrophil band sedimented at the interface of the 1.1 and 1.093 g/ml layer, whereas monocytes were found between 1.087 and 1.081 g/ml density. The PMNs were gently recovered and washed with PBS, suspended in PBS, counted and their viability was determined by Trypan Blue exclusion.

2.6 Polyunsaturated fatty acid metabolites analysis

Peripheral neutrophils extracted from $CFTR^{+/+}$ and $CFTR^{-/-}$ newborn piglets were prepared for lipidomic analysis. Briefly, 6-keto-prostaglandin 1α (6k$PGF_{1\alpha}$), resolvin E1 (RvE1), Tromboxan B2 (TxB_2), PGE_3, 11B-$PGF_{2\alpha}$, $PGF_{2\alpha}$, PGE_2, RvD3, lipoxin B4 (LxB4), PGD2, RvD2, LxA4, RvD1, 8isoPGA_2, leukotriene B5 (LTB_5), 7-maresin 1 (7MaR1), LTB_4, protectin Dx (PDx), RvD5, 18-hydroxyeicosapentaenoic acid (18-HEPE), 15dPGJ_2, 13-hydroxyoctadecadienoic acid (13-HODE), 9-HODE, 15-hydroxyeicosatetraenoic acid (15-HETE), 17-hydroxydocosahexaenoic acid (17-HDoHE), 14-HDoHE, 8-HETE, 12-HETE, 5-HETE, 14,15-epoxyeicosatrienoic acid (14,15-EET), 5-oxo-eicosatetraenoic acid (5oxoETE), 11,12-EET, 8,9-EET and 5,6-EET (all from Cayman Cheamicals, Interchim, Montluçon, France) were quantified in neutrophil lipid extracts (n= 8 $CFTR^{+/+}$ and 7 $CFTR^{-/-}$ piglets) by liquid chromatography/tandem mass spectrometry (LC-MS/MS) as previously described (15).

Data analysis were performed using Mass Hunter Quantitative analysis software (Agilent Technologies).

2.7 Molecular profiling through ICM-MS and top-down HRMS.

The intact cell matrix-assisted laser desorption/ionization mass spectrometry (ICM-MS) approach is a well-established molecular phenotyping method, allowing the direct detection of endogenous peptides and proteins in whole cells without sample pre-treatment. This technique allows fast, routine high-throughput biotyping and discrimination of different cell populations (16). Neutrophils from 7 $CFTR^{+/+}$ and 7 $CFTR^{-/-}$ piglets were analysed by ICM-MS using a Bruker UltrafleXtreme MALDI-TOF instrument (Bruker Daltonics, Germany) operating in positive linear mode in the mass range of 2-20 kD. Spectral processing and analysis were performed with ClinProTools v3.0 software (Bruker Daltonics, Germany). Protein/peptide identification was performed by top-down high-resolution mass spectrometry (HRMS) on a dual linear ion trap Fourier Transform Mass Spectrometer (FT-MS) LTQ Orbitrap Velos (Thermo Fisher Scientific, Germany) coupled to an Ultimate® 3000 RSLC Ultra High Pressure Liquid Chromatographer (Dionex, The Netherlands) controlled by Chromeleon Software (version 6.8 SR11; Dionex) and the data analysed using ProSight PC software v 3.0 SP1 (Thermo Fisher, San Jose, CA). Proteins showing differential abundance were confirmed by western blot. Functional annotation of differentially abundant proteins was performed manually using the UniProtKB database. Detailed methods are provided in supplementary information

2.8 Flow cytometry analysis of purified neutrophils.

Total white blood cells and purified neutrophils were characterized by flow cytometry using specific cell surface markers. Briefly, 10^5 purified cells in 96-well plates were incubated for 1h in blocking buffer (2 mM EDTA, 5% SVF in PBS) and then incubated with primary antibody (anti-SWC1, anti-SWC3, anti-SWC8, anti-SWC9, anti-CD11b or IgM control) for 1h at 4°C. Cells were washed twice, suspended in 100 µl of blocking buffer and analysed with a MACSQuant® Analyser flow cytometer (Miltenyl Biotec); data for at least 10,000 events were recorded.

2. 9 *Pseudomonas aeruginosa* killing assay

P. aeruginosa strain PAK was grown in Luria broth (LB) at 37°C. After approximatively 8h, bacteria were washed twice with RPMI. Bacterial suspension was diluted to have an OD of 0.4. Freshly isolated neutrophils (5 x 10^5 cells in 100 µl of RPMI medium supplemented with 10 mM hepes) were placed in low-binding polypropylene 96-wells plates (Corning; Avon, France) and *Pseudomonas aeruginosa* (25 µl corresponding to MOI of 10) was added. Plates were centrifuged at 800 g for 5 min and incubated without agitation for 2h at 37°C. Total bacterial numbers was determined by the addition of 0.1% Triton X-100 to the co-culture and performing three passages through a 25-gauge needle. Following serial dilution, bacteria were plated on LB plates for numeration.

2.10 Statistical analysis

Results are expressed as mean ± S.E.M. of at least three replicates. Statistical analysis were performed using GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA). Total and differential WBC count and bacteriological measurements were analysed using non-parametric Mann-Whitney U tests. Differences between groups were considered significant when $p<0.05$.

3 Results

3.1 Total and differential WBC count and phenotypic characterization of $CFTR^{+/+}$ and $CFTR^{-/-}$ peripheral neutrophils from newborn piglets

The total leukocyte population in peripheral blood did not show significant differences between $CFTR^{+/+}$, and $CFTR^{-/-}$ newborn piglets (figure 1A). Differential cell counts of leukocyte subsets did not show significant differences between genotypes, either (figure 1B). Isolated neutrophils did not show any difference neither in the population purity (>88%) nor their morphology (supplementary figure S1). Phenotypical characterization of purified peripheral neutrophils by flow cytometry showed no difference between genotypes. Furthermore, no difference was observed in the expression of CD11b and SWC3 (CD172a), two cell surface markers involved in neutrophil migration (17, 18) (figure 1C).

3.2 CF and WT neutrophils present similar ability to kill *pseudomonas aeruginosa in vitro*

We incubated $CFTR^{+/+}$ and $CFTR^{-/-}$ peripheral blood isolated neutrophils with *P. aeruginosa* to assess differences in neutrophil ability to kill *P. aeruginosa* between both genotypes. Both $CFTR^{+/+}$ and $CFTR^{-/-}$ neutrophils were able to significantly decrease *P. aeruginosa* bacterial count after 2 h of incubation *in vitro*. However, no difference was observed between genotypes in their *P. aeruginosa* killing ability (figure 2).

3.3 Bioactive lipid evaluation of $CFTR^{+/+}$ and $CFTR^{-/-}$ peripheral neutrophils from newborn piglets

Bioactive lipids derived from polyunsaturated fatty acids (PUFA) are critical mediators of the inflammatory response. The $6kPGF_{1\alpha}$, RvE1, TxB_2, PGE_3, 11B-$PGF_{2\alpha}$, $PGF_{2\alpha}$, PGE_2, RvD3, LxB4, RvD2, LxA4, RvD1, $8isoPGA_2$, LTB_5, 7MaR1, RvD5, 18-HEPE, $15dPGJ_2$, 17-HDoHE, 5oxoETE and 11,12-EET were not detectable in neutrophils. Their concentrations did not reach the limit of detection corresponding to the lowest concentration leading to a signal to noise over 3. The concentration of 12-HETE, 8-HETE, 8,9-EET, 5,6-EET, LTB4, 9-HODE, 13-HODE, PGD_2, 14-HDoHE, PDx, 14,15-EET, $PGD_2$15-HETE and 5-HETE reached the limit of quantification corresponding to the lowest concentration leading to a signal to noise over 10 in neutrophils from both genotypes. No significant differences were observed between genotypes for any of the evaluated lipids, and, as expected, principal component analysis did not show a clear separation between genotypes (figure 3 and supplementary figure S2).

3.4 Identification of molecular signatures of $CFTR^{-/-}$ peripheral neutrophils through ICM-MS and top-down HRMS.

$CFTR^{+/+}$ and $CFTR^{-/-}$ neutrophils showed significant global phenotypic differences as evidenced after supervised machine-learning analysis with spectra from neutrophils of both genotypes. More specifically, the prediction model based in the use of the genetic algorithm was able to distinguish between the ICM-MS spectra generated from $CFTR^{+/+}$ and $CFTR^{-/-}$ neutrophils with a recognition capability of 100% independently of the variability among spectra (indicated by a cross-validation value of 92.73%). The analysis of ICM-MS spectra from $CFTR^{+/+}$ and $CFTR^{-/-}$ neutrophils indicated that 89 out of the 214 m/z masses detected in the range of 2-20 kDa showed significant signal intensity differences (PWKW $p<0.01$), and from these, 49 showed very significant signal intensity differences (PWKW $p<0.001$). Eleven of these differentially abundant masses showed a good diagnostic performance, indicated by values of area under receiver operating characteristic curves higher than 0.75. Two-dimensional peak distribution of peaks 6 at *m/z* 2136 and peak 36 at *m/z* 3247 allowed for a good discrimination of $CFTR^{+/+}$ and $CFTR^{-/-}$ neutrophils (supplementary figure 3). The top-down proteomic analysis was able to identify 57 *m/z* masses from the total peak list retrieved

from the $CFTR^{+/+}$ and $CFTR^{-/-}$ neutrophils spectra. Among these, 19 corresponded to masses showing significant changes (PWKW $p<0.01$), and corresponded mainly with proteins related with the antimicrobial response. Four of the masses showing good diagnostic performance (AUC> 0.75) were identified (Table 1). Western blotting analysis confirmed the differential expression of Annexin A1, Histone H2A and Pr3 observed by the ICM-MS and top-down analysis (table 2).

4 Discussion

Here, we present novel data showing that CF pig peripheral blood neutrophils are intrinsically different to their WT counterparts at birth. For this study, we used a CF pig model, which present several advantages to study neutrophil physiology. First, newborn CF piglets do not present lung infection or inflammation at early stages after birth (11), which could alter maturation of the neutrophils in the bone marrow, and therefore their functionality (19). In accordance to this lack of lung or systemic inflammation, we did not observe signs of systemic or lung inflammation, such as differences in the total and differential WBC between CF and WT piglets. Second, porcine and human neutrophils are suggested to present similar response *in vitro* to *pseudomonas aeruginosa*, as well as biochemical properties (12, 13).

Despite reports pointing to alterations in CF patients ability to biosynthesize pro-resolving lipid mediators. No difference was observed in our study where we considered only one cell type. Metabolism of PUFA into specialized pro-resolving mediators such as resolvins implicate two different enzymes usually expressed by two different cell types. For instance, RvD1 synthesis is mediated by the 15-lipoxigenase expressed by resident cells and 5-lipoxigenase expressed by infiltrating cells (4). This data led us to focus on the use of ICM-MS, a proteomics approach that allows cell profiling by identifying specific peptide/proteins MS fingerprints (20). We identified for the first time differences in the phenotype of peripheral blood neutrophils from CF newborn pigs (6-12h after birth). ICM-MS spectra was used to build mathematical models that accurately differentiated neutrophils from either $CFTR^{+/+}$ or $CFTR^{-/-}$ origin. These results strongly pointed to intrinsic differences in the peptide/protein composition of $CFTR^{-/-}$ neutrophils already at birth, in the absence of an inflammatory environment, which could have a potential impact in their functionality. Similarly, a recent report showed an increase neutrophil survival as well as neutrophil extracellular trap formation in CF neutrophils that was associated to a primary defect of CFTR rather than a secondary effect of inflammation (21).

Once determined that $CFTR^{+/+}$ and $CFTR^{-/-}$ peripheral neutrophils are intrinsically different, we aimed to identify those proteins that are differentially abundant and whether they are related with defects in neutrophil function. Further analysis by flow cytometry did not show any difference in the expression of CD11b, a β2 integrin subunit that is involved in the process of neutrophil transmigration, between CF and WT circulating neutrophils, as well. Similar data has been previously reported in unstimulated neutrophils (7), suggesting that altered chemotaxis might be related to different concentration of chemoattractants in the lung environment rather than intrinsic defects in CF neutrophils.

At the site of infection, neutrophils must mount an adequate immune response to clear pathogenic bacteria. Previous studies have reported alterations in CF neutrophil degranulation, phagolysosomal chlorination and bacterial killing (9). In this regard, the list of differentially abundant proteins between $CFTR^{+/+}$ and $CFTR^{-/-}$ neutrophils showed that $CFTR^{-/-}$ neutrophils were defective in several proteins implicated in neutrophil maturation and response to pathogens. Antimicrobial peptides, such as PR-39 (22) and Prophenin-2 (23), as well as SRGN, PTPRN2 and CANX, which are implicated in phagosome and granule

maturation and degranulation processes were downregulated in CFTR$^{-/-}$ neutrophils. In addition, we identified several proteins that play an important role in reactive oxygen species (ROS) generation to be differentially abundant in the CFTR$^{-/-}$ neutrophils. Generation of ROS is an important factor in the neutrophil ability to kill pathogens, but it is also responsible for major tissue damage. Thus, uncontrolled release of ROS substances into the extracellular milieu must be kept under control. Annexin A1 (AnxA1) is a potent anti-inflammatory protein that is implicated in neutrophil maturation, and induction of neutrophil apoptosis (24). In agreement with our observations, AnxA1 has been reported to be downregulated in CF mice and CF human patients (25), as well as mice treated with a specific CFTR inhibitor (24). AnxA1 downregulation could be related to the reported delayed apoptosis in CF neutrophils from human patients and newborn CF pigs (21). Moreover, exogenous supplementation of AnxA1is able to inhibit LPS-induced ROS production in a dose-dependent manner in RAW 264.7 macrophages (26). These data suggests that AnxA1 downregulation could lead to an exacerbated oxidative response. ATP6AP2 (also known as (pro)renin receptor) activates the renin-angiotensin system and intracellular signal transduction enhancing inflammation. ATP6AP2 also plays an important role in energy metabolism by interacting with the pyruvate dehydrogenase (PDH) complex. ATP6AP2 knockdown resulted in reduced PDH activity and, a reduction in glucose-induced ROS generation in retina cells (27).

Despite the observed differences, in the abundance of antimicrobial proteins, no alteration was observed in neutrophil killing ability from CFTR$^{-/-}$ pigs *in vitro*. In this regard, the effect of a CFTR deficiency in neutrophils ability to kill bacteria has not been clarified (28). Upon bacterial phagocytosis, NADPH oxidase subunits assemble at the phagolysosome and the cell membrane to direct the production of hypoclorous acid (HOCl), an oxygen reactant with high antimicrobial activity. This procedure seems to be defective in CF neutrophils phagolysosome, but not extracellularly, and lead to a decrease ability of peripheral blood CF neutrophils to kill opsonized *P. aeruginosa* (8). Other reports have not found differences in the ability to kill *P. aeruginosa*, which could vary depending on the bacterial isolate (29) or the proteolytic environment (28). In addition to their ability to kill bacteria, other important features of neutrophil functionality remain to be evaluated. Generation of ROS, apoptosis rates and chemotaxis can greatly influence neutrophil response to a pathogen. Further studies would be needed to determine the specific role of the observed alterations in CFTR$^{-/-}$ neutrophils in their different functional aspects and how they can impact their ability to kill bacteria.

In conclusion, we have demonstrated for the first time that CFTR$^{-/-}$ and CFTR$^{+/+}$ neutrophils present intrinsic differences in their peptide/protein content already at birth, where no influence of a pro-inflammatory environment could alter neutrophil maturation and/or functionality. We suggest that those differences may place CF neutrophils in an intrinsic "altered state".

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Conflict of interest statement

The authors declare no conflict of interest.

Table 1. Characteristics of the intact cell MALDI-TOF Mass Spectrometry m/z masses found to be differential between CFTR+/+ and CFTR -/- pig neutrophils ($p<0.05$) that were confidently identified after top-down High Resolution Mass Spectrometry. Gene symbols are indicated according with the Hugo Gene Nomenclature Committee-approved gene nomenclature. AUC: area under the receiving-operating curve.

Mass	Intensity Average Difference	p-value	Fold-change CFTR+/CFTR-	AUC	Symbol	Protein name	Main function
8634.95	0.51	0.0002	1.9	0.74	NPG2	Prophenin-2	Antibacterial protein
8678.73	0.24	0.0053	1.5	0.75	HIST2H2AA4	Histone H2A type 2-A	Nucleosome; Immunostimulation
2100.11	0.63	0.0097	1.3	0.68	ANXA1	Annexin A1	Regulator of the inflammatory process
8338.1	0.12	0.0001	1.3	0.74	PMAP37	Antibacterial peptide PMAMP-37	Antibacterial protein
3247.48	0.14	0.0000	1.2	0.82	CANX	Calnexin	Chaperone; Maturation of phagosomes
2980.76	0.13	0.0000	1.2	0.84	PR39	Antibacterial protein PR-39	Antibacterial protein
9368.06	0.09	0.0016	1.2	0.68	MLLT10	Protein AF-10	Transcriptional regulation
2300.84	0.11	0.0034	1.2	0.77	LOC100736951	Cathelicidin	Antibacterial protein
8396.36	0.09	0.0008	1.2	0.68	SUPT5H	Transcription elongation factor SPT5	Regulates mRNA processing and transcription elongation
3377.72	0.09	0.0071	1.2	0.65	HNRNP	Heterogeneous nuclear ribonucleoprotein	RNA binding protein
9313.07	0.07	0.0080	1.1	0.65	HIST2H2BE	Histone H2B type 2-E	Nucleosome; Immunostimulation
6382.27	0.08	0.0067	1.1	0.63	CAP2	Adenylyl cyclase-associated protein	Regulates filament dynamics
4599.95	0.08	0.0013	1.1	0.66	PR39	Antibacterial protein PR-39	Antibacterial protein
6787.34	0.07	0.0032	1.1	0.64	LOC100154508	Histone H2A type 1-F like	Nucleosome; Immunostimulation
5146.05	0.08	0.0067	1.1	0.61	LOC100512420	Histone H2B type 1-K	Nucleosome; Immunostimulation
7388.33	0.06	0.0093	1.1	0.62	NPG2	Prophenin-2	Antibacterial protein
6715.07	0.16	0.0087	0.8	0.68	H2AFX	Histone H2A	Nucleosome; Immunostimulation
4090,36	1.96	0.0048	0.7	0.68	SERPINB1	Leukocyte elastase inhibitor	Regulates the activity of the neutrophil proteases
7260.62	4.2	0.0001	0.5	0.75	ATP6AP2	Renin receptor	Neutrophil degranulation

Table 2. Fold-changes (CF/WT) observed in peptide/protein abundance of the Annexin A1, Pr3 and Histone 2A between peripheral neutrophils from $CFTR^{+/+}$ and $CFTR^{-/-}$ newborn piglets calculated by ICM-MS or western blot analysis.

Protein name	Fold-change ($CFTR^{-/-}/CFTR^{+/+}$)	
	ICM-MS	Western blot
Annexin A1	0.75	0.667
Pr3	1.32	1.47
Histone 2A	0.66	0.725

Figure 1. Total white blood cell counts and cell profiles in the peripheral blood and phenotypic characterization of peripheral neutrophils from CFTR$^{+/+}$ (WT), and CFTR$^{-/-}$ (KO) newborn piglets. (A)Total white blood cells (WBC) in peripheral blood. (B) Profile of the WBC populations in peripheral blood. (C) Flow cytometry analysis of purified neutrophils using antibodies raised against the surface marker of leucocytes (SWC1), myeloid cells (SWC3), neutrophils (SC8), macrophages (SWC9) and CD11b with IgM isotype as control.

Figure 2. Neutrophil-mediated killing of *Pseudomonas aeruginosa in vitro* is similar between CFTR$^{+/+}$ and CFTR$^{-/-}$ piglets. Non-opsonized *P. aeruginosa* strain PAK (MOI of 10) were incubated with neutrophils from CFTR$^{+/+}$ (WT) and CFTR$^{-/-}$ (KO) origin for 2 hours at 37°C. Scatter plot indicates the mean bacterial count. Each dot represents a different replicate. Comparisons between WT and KO neutrophils were non-significant ($p>0.05$) by Mann-Whitney U tests.

Figure 3. Lipidomic analysis of peripheral neutrophils from wild-type (WT) and cystic fibrosis (CF) newborn piglets. Comparison of mean levels of PGD_2, LTB4, PDx, 13-HODE, 9-HODE, 14-HDoHE, 15-HETE, 12-HETE, 8-HETE, 5-HETE, 14,15-EET, 8,9-EET, 5,6-EET. Each dot represent a different animal. Comparisons between WT and KO neutrophils were non-significant ($p>0.05$) by Mann-Whitney U tests.

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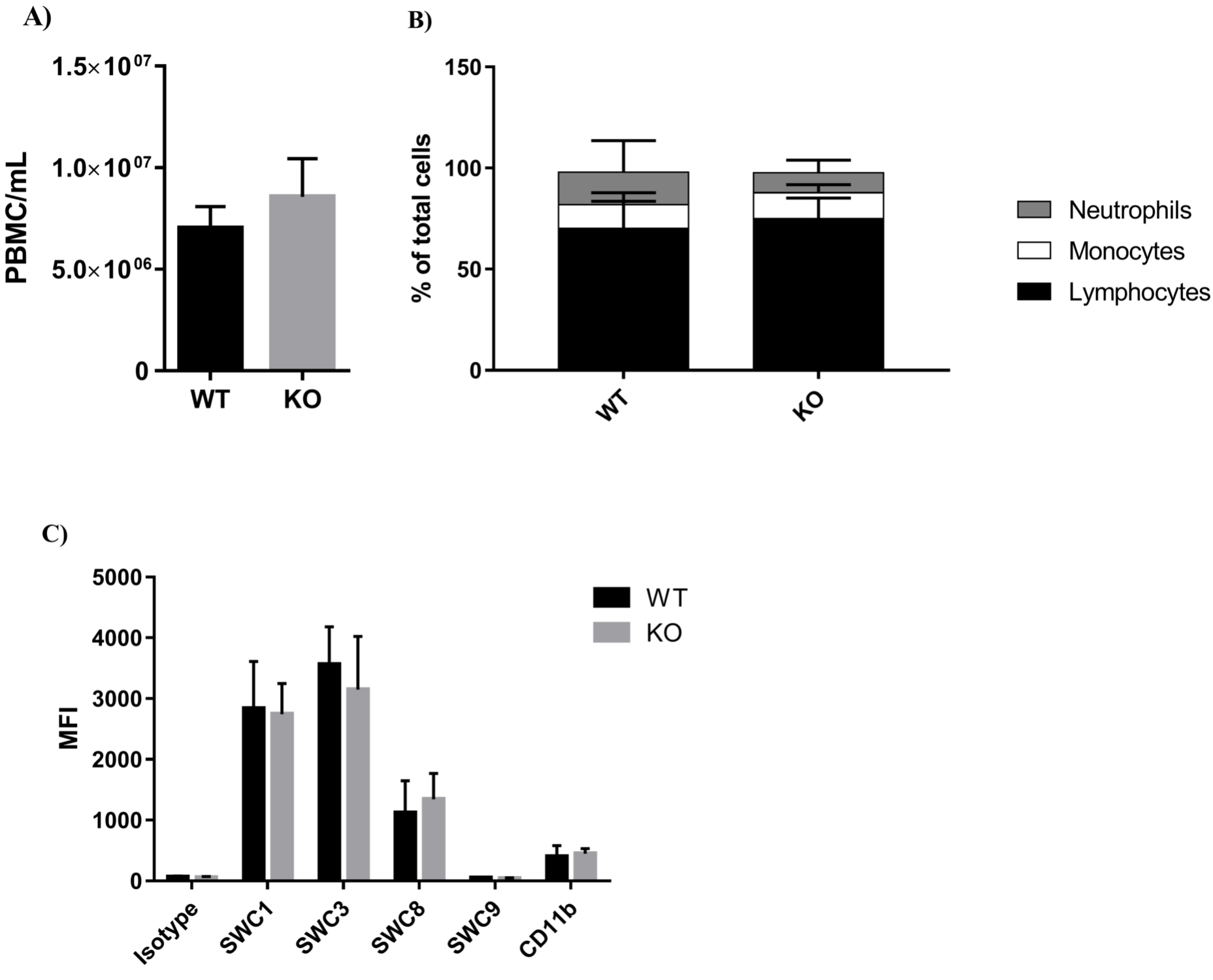
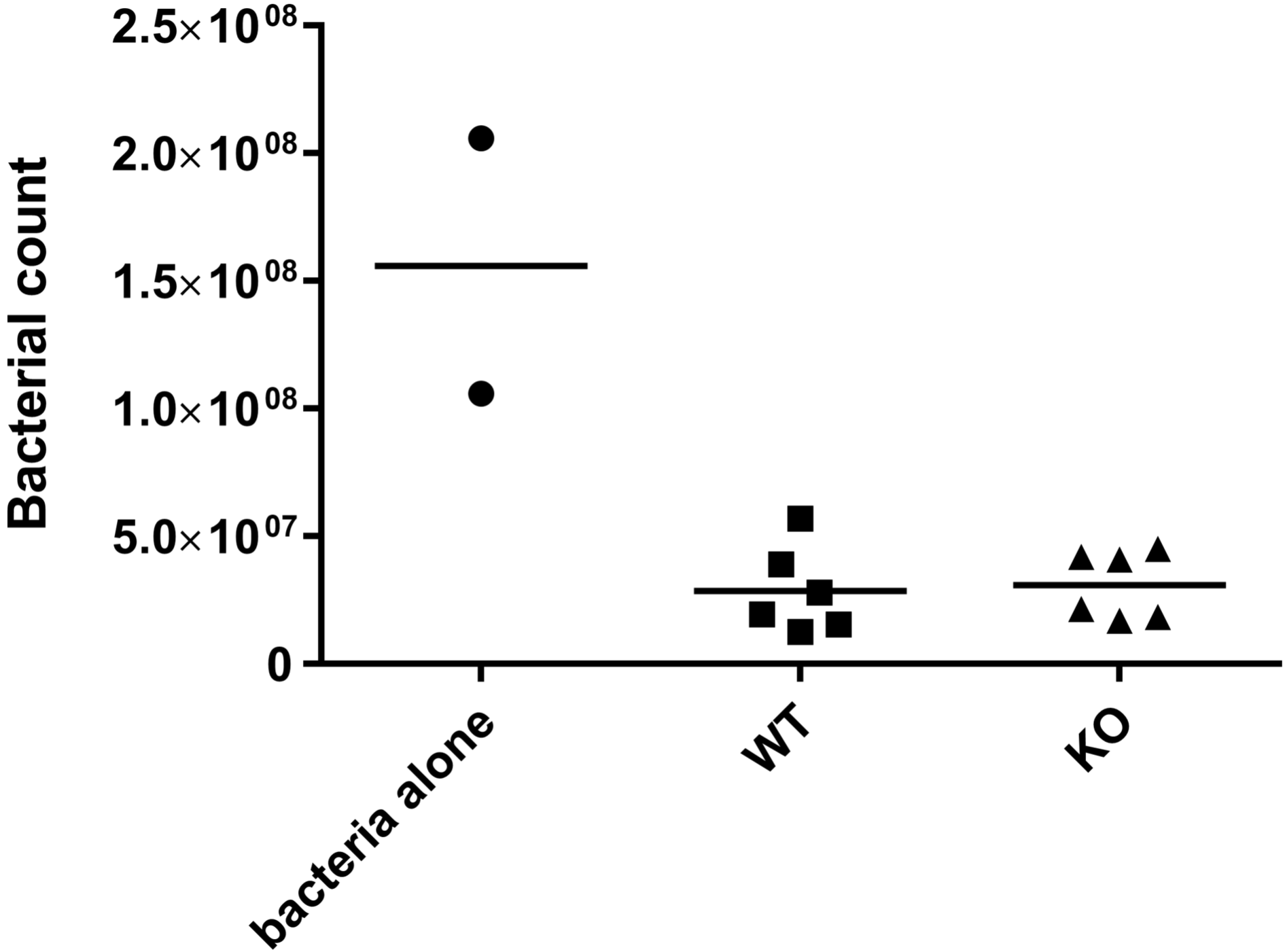

A)
PBMC/mL
1.5×10^07
1.0×10^07
5.0×10^06
0
WT
KO
B)
% of total cells
150
100
50
0
WT
KO
Neutrophils
Monocytes
Lymphocytes
C)
MFI
5000
4000
3000
2000
1000
0
WT
KO
Isotype
SWC1
SWC3
SWC8
SWC9
CD11b


Bacterial count
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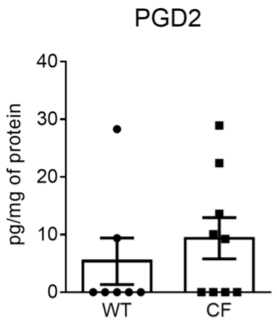
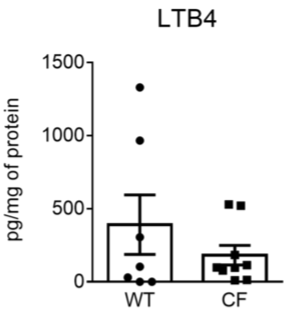
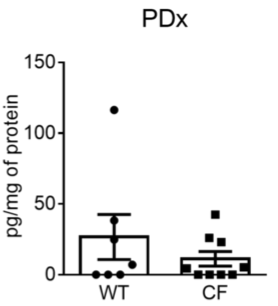
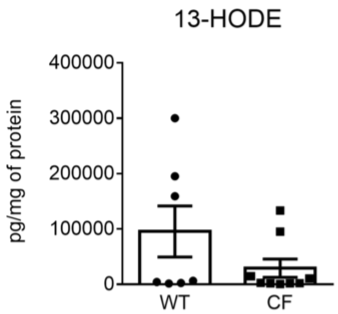
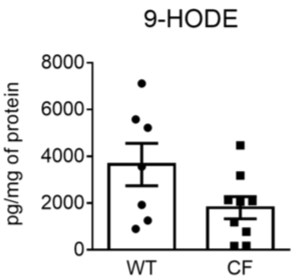
2.0×10 08
1.5×10 08
1.0×10 08
5.0×10 07
0
bacteria alone
WT
KO

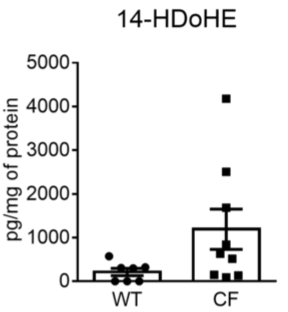

PGD2
pg/mg of protein
40
30
20
10
0
WT
CF


LTB4
pg/mg of protein
1500
1000
500
0
WT
CF


PDx
pg/mg of protein
150
100
50
0
WT
CF


13-HODE
pg/mg of protein
400000
300000
200000
100000
0
WT
CF


9-HODE
pg/mg of protein
8000
6000
4000
2000
0
WT
CF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pg/mg of protein
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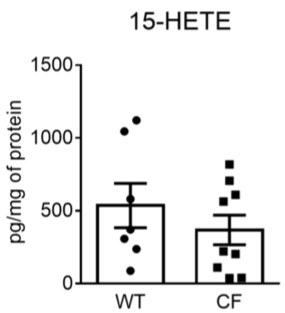
4000
3000
2000
1000
0
WT
CF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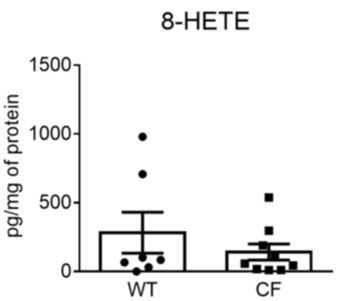
pg/mg of protein
1500
1000
500
0
WT
CF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pg/mg of protein
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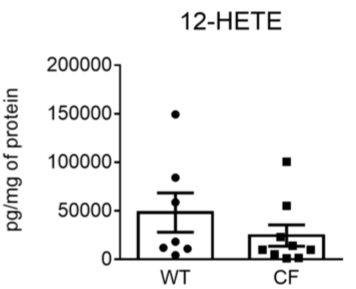
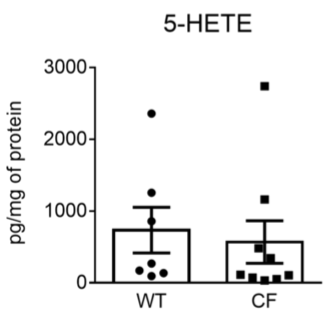
1000
500
0
WT
CF


12-HETE
pg/mg of protein
200000
150000
100000
50000
0
WT
CF


5-HETE
pg/mg of protein
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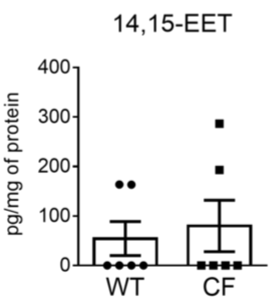
2000
1000
0
WT
CF


14,15-EET
pg/mg of protein
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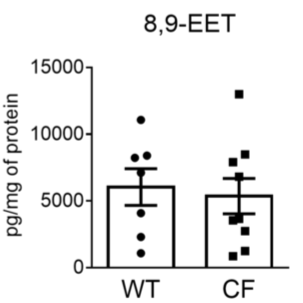
300
200
100
0
WT
CF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pg/mg of protein
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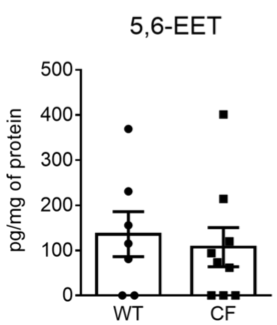
5,6-EET
pg/mg of protein
500
400
300
200
100
0
WT
CF