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Original article

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Reduced neutrophil elastase inhibitor elafin and elevated TGFβ_1 are linked to inflammatory response in sputum of CF patients with Pseudomonas aeruginosa

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Abstract:

Research question: Pulmonary disease progression in patients with cystic fibrosis (CF) is characterized by inflammation and fibrosis, and aggravated by *Pseudomonas aeruginosa* (Pa). We investigated the impact of Pa specifically (1) on protease/antiprotease balance and (2) inflammation, as well as (3) the link of both parameters to clinical parameters of CF-patients. **Methods:** TGFβ_1, IL1β, IL8, neutrophil elastase (NE) and elastase inhibitor elafin were measured (ELISA assays), and gene expression of the NF-κB pathway was assessed (RT-PCR) in the sputum of 60 CF-patients with a minimum age of 5 years. Spirometry was assessed according to ATS guidelines.

Results: (1) NE was markedly increased in Pa-positive sputum, whereas elafin was significantly decreased. (2) Increased IL1β/IL8 were associated with both Pa infection and reduced FEV1, as well as sputum TGFβ_1 was elevated in Pa-infected CF-patients and linked to an impaired lung function. (3) Moreover, gene expression of NF-κB signaling components was increased in sputum of Pa-infected patients; these findings were positively correlated with IL8.

Conclusion: Our study links Pa infection to an imbalance of NE and NE-inhibitor elafin and increased inflammatory mediators. Moreover, our data demonstrate an association between high TGFβ_1 sputum levels and a progress in chronic lung inflammation and pulmonary fibrosis in CF. Controlling the excessive airway inflammation by inhibition of NE and TGFβ_1 might be promising therapeutic strategies in future CF therapy and a possible complement to CFTR-modulators.

Introduction:

Cystic Fibrosis (CF) is the most common lethal autosomal recessive disease, caused by mutations and subsequent absence/dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR). While CF affects multiple organs, the majority of life-limiting sequelae are related to progressive lung disease caused by bronchial inflammation, bacterial infection and lung matrix remodelling resulting in continuous decline of lung function (1). Pseudomonas aeruginosa (Pa) is one of the most prevalent microorganisms chronically infecting the lungs of 50-60% of adult CF patients already early in life (2-4). Persistance of Pa over years substantially contributes to rapid progression of lung disease and higher mortality and morbidity in CF-patients (5, 6). While Pa infection has been widely recognized as an adverse pulmonary outcome parameter, the mechanisms and potential biomarkers linking Pa to these devastating lung changes over time remain elusive.

Pa infection aggravates CF-related lung disease by adversely affecting the impaired mucociliary clearance and increasing influx of inflammatory cells. These pathophysiological changes result in a release of cytokines, growth factors and proteases, and lead ultimately to a protease/antiprotease imbalance (7-9). Various clinical and experimental studies have shown that protease activity is mechanistically important in CF-related lung matrix remodelling (10, 11). In particular, neutrophile elastase (NE), released by activated neutrophils -the most prominent inflammatory cell type, is one of the main proteases inducing structural lung damage in CF by impairing mucociliary clearance, mediating proinflammatory activity by degrading elastic fibers (10-15). Recent studies in CF confirmed a strong association between high NE activity in bronchoalveolar lavage (BAL) fluid and the onset and progression of structural abnormalities including early bronchiectasis, future lung function decline (16) as well as treatment response in pulmonary exacerbations (17). There is only one study with a limited number of CF patients, analyzing NE and elafin concentrations in sputum, providing evidence, that elafin is cleaved by its cognate enzyme NE (18).

There is a growing body of evidence that inflammatory pathways in CF are also extensively influenced by genetic modifiers, notable amongst these Transforming Growth Factor beta 1

($TGF\beta_1$). $TGF\beta_1$ is not only a key regulator of bronchial inflammation, pulmonary fibrosis (19), as well as cell proliferation and cell differentiation (20), but has also been shown to inhibit CFTR expression (21). Moreover, matrix remodelling and local hypoxia as a result of increased mucus deposition (22, 23) promote the release of proinflammatory cytokines, such as interleukins IL1β and activation of inflammatory pathways (11).

Reliable sputum biomarkers for therapeutic monitoring and/or predicting the clinical course of CF are gaining importance. While Pa has been shown to be closely related to the clinical outcome and survival of CF patients (24, 25), the variety and coherences of involved inflammatory mediators are poorly understood and most studies have a limited number of patients. Therefore, we investigated the link between Pa infection and the protease/antiprotease imbalance, inflammatory cytokines, $TGF\beta_1$ as a genetic modifier and the NF-κB signaling pathway in sputum inflammatory cells.

Material and Methods:

Study Population

We investigated 60 CF patients with a confirmed diagnosis of CF according to the consensus guidelines of the Cystic Fibrosis Foundation (26). Further inclusion criteria for this study were a minimum age of 5 years and the capability to produce and expectorate sputum. Patients with current pulmonal exacerbation or acute respiratory infection were excluded. Based on the Leeds criteria Pa infection in our cohort is defined by three positive cultures over 12 months with at least 1-month interval between the samples. Patients who underwent successful Pa eradication (3 negative cultures in a role with at least 1-month interval between the samples) were considered negative. All CF patients infected with Pa were being treated with cycled inhaled antibiotics.

Ethics, consent and permissions: Human guidelines of good clinical practice and the declaration of Helsinki (1964) and Edinburgh (2000) were followed in the conduct of the trial. Ethical approval was obtained from the Medical Ethical Committee of the University Hospital

Cologne (Approval-No.: 12-168). All parents and all patients older than 8 years of age provided written informed consent.

Sputum analysis

Sputum was induced by inhalation of hypertonic saline during a routine physiotherapist session at regular outpatient visits. Sputum processing was performed according to the standard operating procedure (SOP) of the TDN (Therapeutic Drug Development Network, USA). Sputum was processed within 1 hour of collection and sputum plugs segregated from possible saliva. The sputum samples were diluted in 9:1 (weight to volume) phosphate-buffered saline (D-PBS), filtered through 100 µm and 40 µm mesh, and centrifuged for 10min at 260x g at 6°C. Supernatants were stored at -80°C for further analysis; cell suspensions were concentrated by cytospin (1 x 10^6 cells/ml) and stored at -20°C.

Elastase and elafin concentrations in sputum were determined by specific ELISA assays (EnzChek® Elastase Assay Kit, - Molecular Probes Europe, Leiden, Netherlands; Elafin/Skalp Human ELISA Kit - abcam, Cambridge, UK). Pro-inflammatory cytokine concentrations in the sputum were assessed by using a human inflammatory cytokine ELISA-kit (BD Cytometric Bead Array Humane Inflammatory Cytokine Kit, San Jose, CA, USA). TGF-β_1 levels in sputum of all patients were determined by using TGF-specific ELISA-kit (Quantiki-ne®ELISA Human TGFβ_1, R&D systems, Minneapolis, MN, USA). All assays and kits were performed according to the manufacturer´s protocol.

The different measurements of our sputum analysis have been done in succession with priorities given to the measurement of TGF-β_1, IL1β and IL8. For some patients the amount of sputum sample was inadequate to assess the levels of all the inflammatory mediators explored in this study, hence the number of investigated samples varied between different measurements.

RNA isolation and quantitative Reverse Transcriptase PCR (qRT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen, Paisley, Scotland, UK), and qRT-PCR was performed using using the 7500 Real-time PCR system (Applied Biosystem, Foster City, CA) (27). The relative amount of the specific mRNA was normalized to β-actin. Primers were designed using Primer Express Software v3.0.1 (ThermoFisher scientific, Waltham. Massachusetts, USA); primer pairs are listed in supplementary table 2.

Spirometry

Spirometric measurements were assessed prior to any study intervention according the ATS guidelines (28) by the use of Master Screen Body (Jaeger, Heidelberg, Germany) and SentrySuite™ version 2.19 software (Carefusion, Becton Dickinson, Franklin Lakes, New Jersey, USA). For all spirometric measurements the Global Lung Function initiative's reference equations were used (GLI-2012). Maximum values of forced expiratory volume in 1 second predicted (FEV_1 in % predicted) was used for analysis, which is defined as FEV_1% of the patient divided by the average FEV_1% in the population for any person of similar age, sex and body composition.

Statistical analysis:

Collected data in the text are reported as means with standard deviation (SD). To compare datasets from two subgroups, we used student´s unpaired t-test for independent samples when the frequency distribution was normal, or Mann-Whitney U test when the distribution was not normal. Cytokine levels were correlated by Pearson or Spearman correlation depending on their distribution. The strength of correlation is definated depending on the correlation coefficient r ($r = 0.3\text{-}0.5$ = weak, $r = 0.5\text{-}0.7$ = moderate, $r > 0.7$ = strong). A p-value <0.05 was considered as statistically significant, statistical analysis was performed using Prism 7 software package (GraphPad 7, San Diego, California, USA). All results were

correlated to age, body mass index (BMI), FEV_1-values and status of Pa infection by using Pearson or Spearman correlation depending on their distribution.

Results:

Clinical data of study population

A cohort of 60 CF patients was recruited based on our inclusion and exclusion criteria. As demonstrated in table 1, the average age was 21.2 years (± 12.2 years); 51.9 % were male; 41 patients were Pa negative (68.3 %) whereas 19 patients were infected with Pa (31.7 %). The FEV_1-values were reduced in the subgroup of Pa positive (59.5 % predicted; ±25.0) in comparison to Pa negative patients (79.8 % predicted; ±22.7), as well as in the subgroup of patients of the age of 18 and older (63.8 % predicted; ±23.8). The average BMI of our cohort was 19.2. Regarding age, BMI, gender and CFTR-mutation we detected no significant relation to the measured inflammatory mediators.

Clinical parameters	**Mean (SD) or n (%)**
Age	21.2 years (±12.2) <18y: 25 (41.7 %) >18y: 35 (58.3 %)
Gender	Female: 29 (48.3 %) Male: 31 (51.7 %)
Pseudomonas aeruginosa infection	19 (31.6 %)
FEV1	73.4 % (±25.1) Pa infected: 59.5 % (±25.0)

	Pa negative: 79.8 % (±22.7) <18y: 86.8 % (±20.7) ≥18y: 63.8 % (±23.8)
CFTR-Mutations	F508del homozygous: 32 (53.3 %) F508del heterozygous: 21 (35.0 %) Other mutations: 7 (11.7 %)

Table 1: Demographics of study population

Increased NE and reduced elafin concentration in soluble CF sputum in P. aeruginosa-positive CF-patients

To determine if poor clinical outcome of Pa-colonized CF-patients is linked to an imbalance of NE and its inhibitor elafin, we assessed both markers in soluble CF sputum. Concentrations of NE were significantly higher in Pa-positive CF sputa when compared with Pa-negative sputa (211.2 ± 31.9 ng/ml vs. 359.1 ± 65.8 ng/ml, $p<0.05$) (Figure 1A). Elafin is primarily expressed by bronchial epithelial cells and inhibits NE. We found that elafin concentration was significantly lower in sputa of Pa-positive CF-patients in comparison to sputa of Pa-negative patients (16311 ± 2184 pg/ml vs. 6975 ± 943 pg/ml, $p<0.001$) (Figure 1B), suggesting a NE/elafin imbalance in Pa-positive CF lungs, favouring elastic fibres degradation and fibrotic matrix remodelling.

Sputum IL1β and IL8 are linked to decline of lung function in in P. aeruginosa-positive CF-patients

To link Pa colonization to the release of proinflammatory cytokines, IL1β and IL8 were determined in CF sputum samples. We detected a significant increase of IL1β (+249.4 %, $p<0.001$) and IL8 concentrations (+218.4 %, $p<0.0001$) in sputum of Pa -positive CF patients

(IL1β: 1278 ± 314 vs. 3187 ± 407 pg/ml; IL8: 2804 ± 465 vs. 6124 ± 483 pg/ml) (figure 2). Sputum IL1β and IL8 concentrations were more than 3-fold and 1.8-fold, respectively, higher in patients with FEV_1 less than 80% when compared to patients with FEV_1 higher or equal than 80% (IL1β: FEV_1≥80% vs. FEV_1 <80%: 902 ± 226 pg/ml vs. 2899 ± 416 pg/ml, p<0.0001; IL8: FEV_1 ≥80% vs. FEV_1 <80%: 2811 ± 553 pg/ml, vs. 5021 ± 548 pg/ml, p<0.01) (figure 3). Finally, we determined a strong positive correlation between IL1β and IL8 levels in our cohort of Pa-positive and Pa-negative CF-patients (r= 0.7645; p<0.0001) (figure 4).

Linking concentrations of TGFβ_1 to clinical parameters and inflammatory cytokines in sputum of CF patients

Since TGFβ_1 has been identified as a genetic modifier for CF lungs disease, we assessed TGFβ_1 concentrations in sputa of CF-patients with or without Pa colonization. High sputum TGFβ_1 was intimately linked to both P. aeruginosa colonization and to lower FEV_1 values at the timepoint of sample collection. Specifically, sputum TGFβ_1 was significantly higher in P. aeruginosa-positive CF patients compared to P. aeruginosa negative CF patients (P. aeruginosa negative: 84.5 ± 11.7 pg/ml, P. aeruginosa positive: 173.8 ± 24.0 pg/ml, p<0.001) (figure 5A). Sputum TGFβ_1 levels were significantly higher in CF-patients with reduced FEV_1 values below 80% predicted than in patients with FEV_1 values above or equal 80% predicted (FEV_1 ≥ 80 %: 85.7 ± 14.2 pg/ml, FEV_1 < 80%: 139.7 ± 18.9 pg/ml, p<0.05) (figure 5B). Moreover, we tested the correlation between TGFβ_1 and the proinflammatory cytokines. Indeed, both IL1β and IL8 showed a significant positive correlation to TGFβ_1 levels in sputum (IL1β: r= 0.707; p<0.0001; IL8: r= 0.670; p<0.0001) (figure 6A, B).

Expression of the NF-kB signalling cascade in sputum cells of CF-patients is regulated by P. aeruginosa.

Our preceding results linking Pa to increased inflammatory cytokines and higher concentrations of TGFβ_1 in lungs of CF-patients, led us to the question if the expression of inflammatory signaling pathways in sputum cells is differentially regulated by P. aeruginosa

colonization in CF lungs. To this end, we measured gene expression of mediators of the NF-kB signalling cascade and detected a significant increase in mRNA of IKK alpha, IL6, p50 and p65 in sputum samples of patients with P. aeruginosa infection (figure 7). Furthermore, we found a significant positive weak correlation between high IL8 levels in sputum and the gene expression of p50 ($r= 0.402$; $p<0.01$) and p65 ($r= 0.356$; $p<0.05$) as markers of the NF-κB signalling cascade (figure 8).

Discussion:

The present study shows that the reduction of lung function in Pa-positive CF lungs is intimately linked to an imbalance of proteases (sputum NE) and anti-proteases (sputum elafin), increased concentrations of sputum $TGF\beta_1$ and proinflammatory cytokines (IL1β, IL8), which might adversely affect the inflammation and remodelling of CF lungs. An activation of NF-κB signalling in sputum cells, presumably neutrophils, might be triggering these processes.

Infections with Pa are associated with significantly poorer outcomes (29). While improvement of life expectancy has been mainly attributed to an early and aggressive treatment of P. aeruginosa infections (30), the initial underlying processes triggering a persistent inflammation and leading to lung injury and destruction remain elusive. Several studies investigated proinflammatory markers in sputum of CF patients (16, 31), but only few reports addressed the impact of Pa colonization on disease progression in CF (24, 25). In our present study, correlation of inflammatory sputum markers with clinical parameters showed that colonization with P. aeruginosa was not only significantly related to higher inflammatory sputum markers, but also to reduced lung function. Specifically, we found that Pa is linked to higher concentrations of IL1β and IL8, which in turn were strongly correlated with increased sputum $TGF\beta_1$. Both inflammatory cytokines IL1β and IL8 as well as $TGF\beta_1$ induce inflammation and lung matrix remodeling favoring fibrosis, thereby contributing to irreversible

structural lung changes and reduced lung function (20). Moreover, inflammatory markers have been identified as risk factors for lung function decline in CF or other chronic lung diseases independent of Pa (32).

Pa elicit massive neutrophile influx in part by release of pyocyanin (33) and modulates neutrophilic myeloid-derived suppressor cells (MDSCs) in CF lungs (34). Here, we show a marked activation of gene expression of the NF-κB signaling in inflammatory sputum cells of Pa colonized CF-patients, suggesting an activation of inflammatory cells, presumably neutrophils, promoting thereby the release of inflammatory cytokines and matrix-remodeling proteases. In parallel, increased sputum NE, a biomarker for monitoring CF lung disease, was significantly related to Pa. Elevated activity of NE is associated with bronchiectasis in CF (35), is predictive of future lung function decline (16) and is related to treatment response in pulmonary exacerbations (19). Furthermore, recent studies in CF demonstrated a strong association between high NE activity in BAL fluid and the onset and progression of structural abnormalities including early bronchiectasis (36). Previous in-vitro experiments confirm this notion by demonstrating an inhibitory effect of inhaled anti-Pseudomonas antibiotic treatment on the activity of NE (37), indicating thereby an activating effect on NE by Pa. This enzyme is pivotal to lung damage because it releases growth factors, e.g. $TGF\beta_1$ and degrades elastic fibers. Elastin fiber breakdown products are highly proinflammatory and promoting the recruitment of activated inflammatory cells (38). Increased release of NE by recruited lung neutrophils and elastin peptide fragments are related to chronic lung diseases, such as pulmonary arterial hypertension (39) or pulmonary fibrosis (40).

Inhibition of elastase by lung endogenous elafin, which is primarily produced in bronchial epithelial cells, mitigates lung destructive processes (41, 42). Measurement of elafin in our cohort showed a marked decrease of elafin in CF lungs colonized with Pa, suggesting a suppressive effect of Pa on elafin expression *in vivo*. Interestingly, Guyot et al. demonstrated, that elafin is proteolytically cleaved by its cognate enzyme NE in BAL fluid of

CF patients infected with Pa (18). The confirmed elafin deficiency as seen in our cohort might be the result of an impaired bronchial epithelial cell homeostasis in Pa positive lungs. Our present findings indicate a relevant imbalance of proteases and antiproteases in CF lungs. While elafin deficiency in CF lungs with Pa may relate to increased NE, other functions of elafin need to be considered. For example, prior studies demonstrated a marked inhibitory effect of elafin on NF-κB and $TGF\beta_1$ activation in the lung (16, 42). Thus, deficient release of elafin may promote activation of NF-κB and TGFβ1 signalling and aggravate lung injury by triggering inflammatory response and lung matrix remodelling, respectively.

Pa and lung inflammation are important in the clinical course of CF (24, 25). The present study shows a marked increased expression of components of the NF-κB pathway in sputum cells and elevated concentrations of sputum IL1β and IL8. These findings were supported by *in vitro* experiments showing that exposure of CF-bronchial epithelial IB3-1 cells or CF-nasal epithelial cells to Pa upregulates the gene expression of IL1β, IL8 or NF-κB activity (43). Alternatively, the lack of inhibitory effect of elafin on NF-κB signaling could in part underly the activation of inflammatory NF-κB signaling and thereby enhance the expression of IL1β and IL8 (16). Additionally, excessive NE as a result of elafin deficiency could promote IL8 expression, neutrophil recruitment, and a self-perpetuating cycle of neutrophil-mediated inflammation (11). Interestingly, Carrabino et al. demonstrated that stimulation of Pa-exposed CF-nasal epithelial cells with IL1β increased IL8 expression (44). This strong link between IL1β and IL8 may explain the correlation of both cytokines in our cohort.

The intimate link between IL1β/IL8 and reduced lung function (lower FEV1 values) does not only emphasize the important functional role of Pa in the clinical course of CF, but also the additional need for pharmacological approaches targeting specific inflammatory mediators. Initial investigations using IL1β receptor inhibitor (Anakinra) demonstrated an amelioration of the inflammasome-dependent inflammation in human CF-mutated bronchial epithelial cells (45). Previous studies demonstrated, that IL8 serves as first line of host defense against

invading microorganisms (46) and as a potent chemoattractant for neutrophils. Moreover, NF-κB-mediated IL8 and IL1β chemokine secretion and neutrophil influx are prominent early in CF disease progression (47). Our study identifies P. aeruginosa colonization as a possible aggravator for both, activation of NF-κB-signalling and related increase of IL1β and IL8.

$TGF\beta_1$ is a pleiotropic growth factor, involved in the regulation of cell differentiation and survival, inflammatory response, and fibrotic processes of chronic lung diseases (18). Furthermore, recent studies identified $TGF\beta_1$ as an important genetic modifier in the lung pathobiology of CF. For example, inhibition of CFTR expression has been shown to be one mechanism by which $TGF\beta_1$ modulates pathomechanisms in CF. However, it remains unclear if the changes in proteases/antiproteases and the increase of inflammatory cytokines in Pa positive sputum samples are linked to impaired mucociliary clearance; or if an elevation in active $TGF\beta_1$ aggravates these processes and therefore serve as a potential biomarker. Our results demonstrate a correlation between high sputum $TGF\beta_1$ in CF-patients and the degree of pulmonary inflammation, as well as an association to P. aeruginosa colonization and lower FEV_1-values. These findings indicate the possible role of $TGF\beta_1$ as a sputum biomarker for disease progression in CF.

This study has some limitations. It is well known that longer duration of Pa colonization is associated with CF lung disease progression (48), our study did not evaluate the duration of Pa colonization regarding measured mediators. Moreover, for some patients the amount of sputum sample was inadequate to assess the levels of all the inflammatory mediators explored in this study, lack of associations between potential confounders may be related to the smaller sample size for some of the inflammatory mediators. In addition, investigating the influence of an inhibition of elafin and $TGF\beta_1$ on the inflammatory response in the CF lung would be of interest. Therefore, further cell culture studies are needed and planned by our working group in future.

In conclusion, our results demonstrate a significant association between high inflammatory sputum mediators and Pa infection and confirms the importance of an early eradication

therapy for newly colonized patients as well as an aggressive chronic treatment of P. aeruginosa in already chronically infected CF patients. Our findings also demonstrate the important impact of Pa infection on NE/elafin imbalance and hyperinflammation by the release of TGF-β_1, increase of IL1beta /IL8 as well as NF-κB- activity, all ultimately resulting in a progress of chronic inflammatory lung disease and pulmonary fibrosis (figure 9). Reducing the excessive airway inflammation by inhibition of NE and TGFβ_1 might be a promising therapeutic strategy in future CF therapy and a promising complement to CFTR-modulators.

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Figures and legends:

Figure 1: (A) Assessment of NE concentrations (ng/ml) and (B) elafin concentrations (pg/ml) in sputum of CF patients related to Pseudomonas infections: P. aeruginosa-negative CF-patients (gray bar, n=32) and P. aeruginosa-positive CF patients (white bar, n=16). Median ± interquartile range, Mann-Whitney U test, **p<0.01, ***p<0.001.

Figure 2: (A) Interleukin 1β (IL1β) and (B) Interleukin 8 (IL8) concentrations (pg/ml) in sputum of CF patients related to Pseudomonas infection: P. aeruginosa-negative CF-patients (black bar, n=34) and P. aeruginosa-positive CF-patients (white bar, n=18). Median ± interquartile range, Mann-Whitney U test, ****p<0.0001.

Figure 3: (A) IL1β and (B) IL8 concentrations (pg/ml) related to FEV_1 values: Elevated IL1β and IL8 levels were detected in CF patients with FEV_1 < 80% (white bar, n=27) compared to CF patients with FEV_1 values ≥ 80% (black bar, n=25); Median ± interquartile range, Mann-Whitney U test, **p<0.01, ****p<0.0001.

Figure 4: Pearson correlation between IL1β and (B) IL8 concentrations (pg/ml) in sputum of CF patients (n=52): A positive correlation between IL1β and IL8 levels was detected (r= 0.763; ****p<0.0001).

Figure 5: Assessment of sputum TGF-β_1 concentrations (pg/ml) in CF patients by specific TGFβ1-ELISA: Correlation to **(A)** P. aeruginosa colonization. P. aeruginosa-negative (black bar, n=33); P. aeruginosa-positive (white bar, n=17) and **(B)** FEV_1 values predicted in %, (FEV1 ≥ 80 % = black bar, n=23; FEV< 80 % = white bar, n=27). Median ± interquartile range, Mann-Whitney U test *p<0.05, ****p<0.0001.

Figure 6: Spearman correlation between concentrations of sputum TGF-β_1 (pg/ml) and proinflammatory cytokines IL1β and IL8 in CF patients (n=50): (A) Positive correlation between IL1β and TGF-β_1 levels (r= 0.707; **** p<0.0001) and **(B)** IL8 and TGF-β_1 levels (r= 0.670; ****p<0.0001).

Figure 7: Gene expression of NF-*k*B signalling cascade mediators in CF sputum cells of 43 patients: p50, p65, IKK alpha and IL6 levels were determined by quantitative RT-PCR. Significantly elevated mRNA expression of mediators of the NF-kB signalling cascade: **(A)** IKK alpha (**p<0.01) and **(B)** IL6 (*p<0.05), as well as **(C)** p50 (**p<0.01) and **(D)** p65 (*p<0.05). Median ± interquartal range, Mann-Whitney U test.

Figure 8: Spearman correlations between level of IL8 and quantitative mRNA expression of p50 and p65: Significant positive correlation between IL8 levels and quantity of mRNA expression of the NF-kB signalling proteins **(A)** p50 (r= 0.402; ** p<0.01) and **(B)** p65 (r= 0.356; * p<0.05).

Figure 9: Simplified presentation of the vicious circle of chronic inflammation in CF as a result of high neutrophilic activity, disruption of the homeostatic protease/antiprotease balance and microbial infection with P. aeruginosa in the CF lung causing chronic inflammatory lung disease and pulmonary fibrosis.

Figure 1

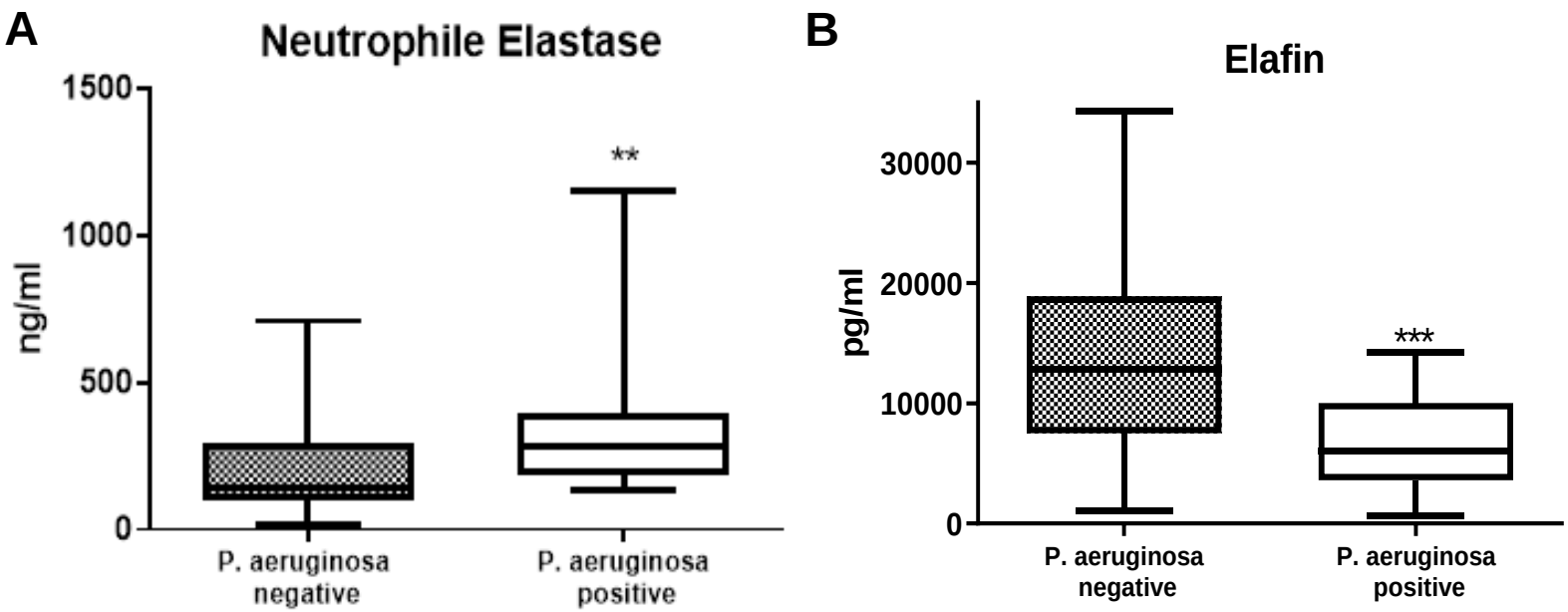

A
Neutrophile Elastase
1500
1000
500
0
ng/ml
**
P. aeruginosa negative
P. aeruginosa positive
B
Elafin
30000
20000
10000
0
pg/ml
***
P. aeruginosa negative
P. aeruginosa positive

Figure 2

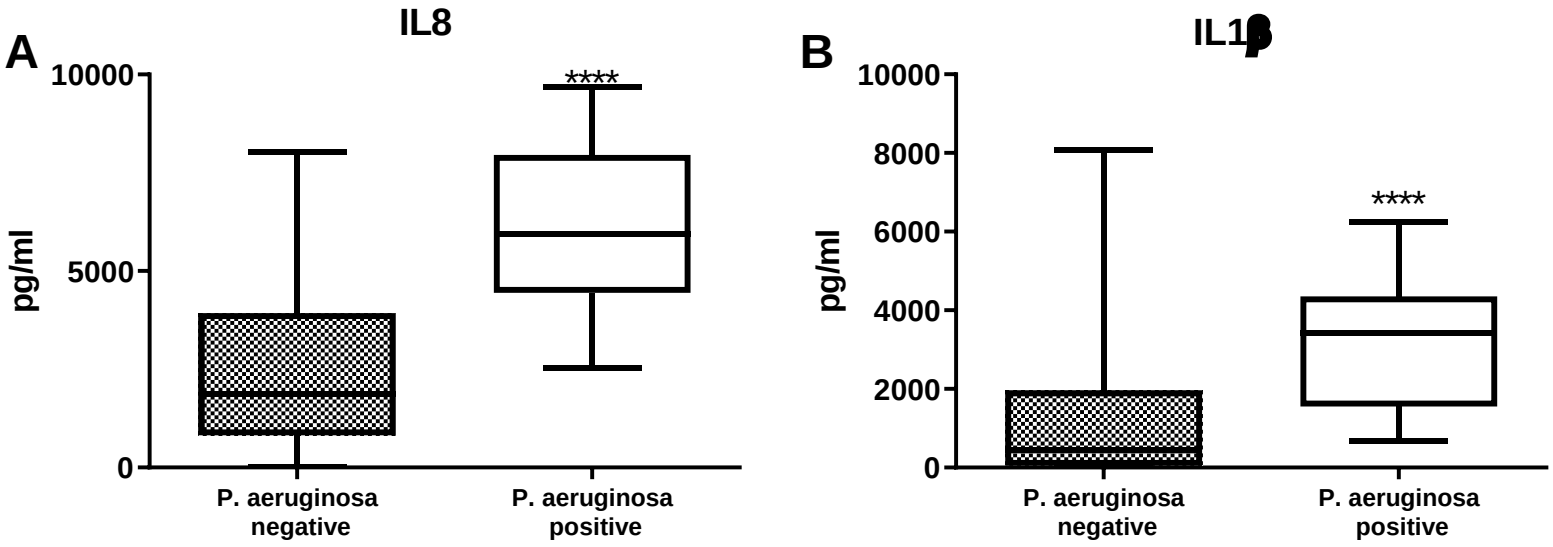

A
IL8
10000
5000
0
pg/ml
****
P. aeruginosa negative
P. aeruginosa positive
B
IL1β
10000
8000
6000
4000
2000
0
pg/ml
****
P. aeruginosa negative
P. aeruginosa positive

Figure 3

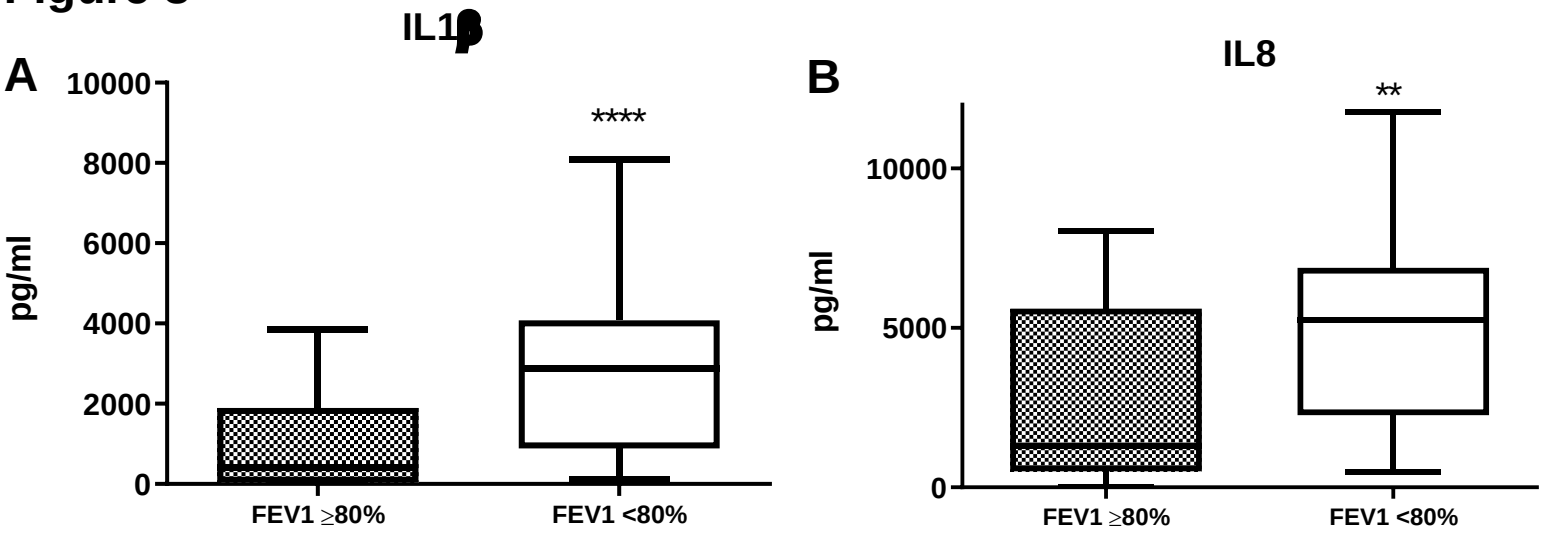

A
IL1β
pg/ml
10000
8000
6000
4000
2000
0
****
FEV1 ≥80%
FEV1 <80%
B
IL8
pg/ml
10000
5000
0
**
FEV1 ≥80%
FEV1 <80%

Figure 4

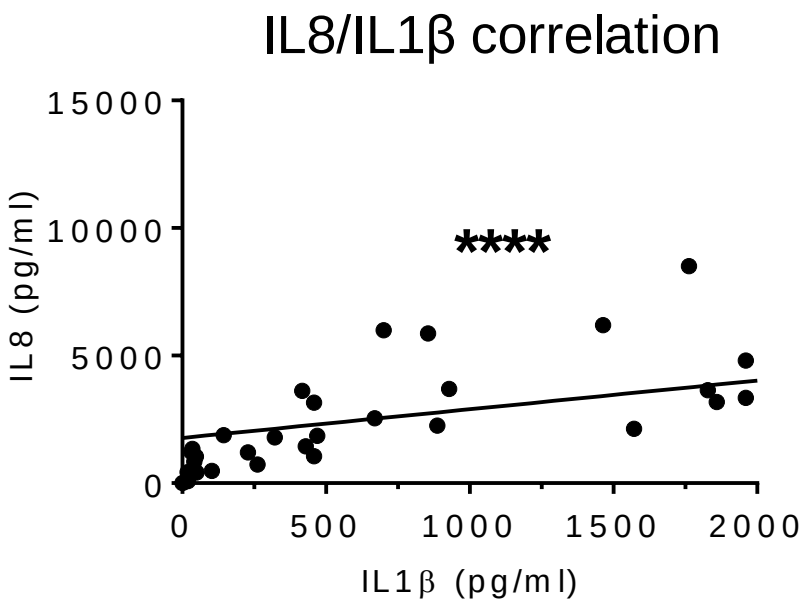

IL8/IL1β correlation
15000
10000
5000
0
IL8 (pg/ml)
****
0
500
1000
1500
2000
IL1β (pg/ml)

Figure 5

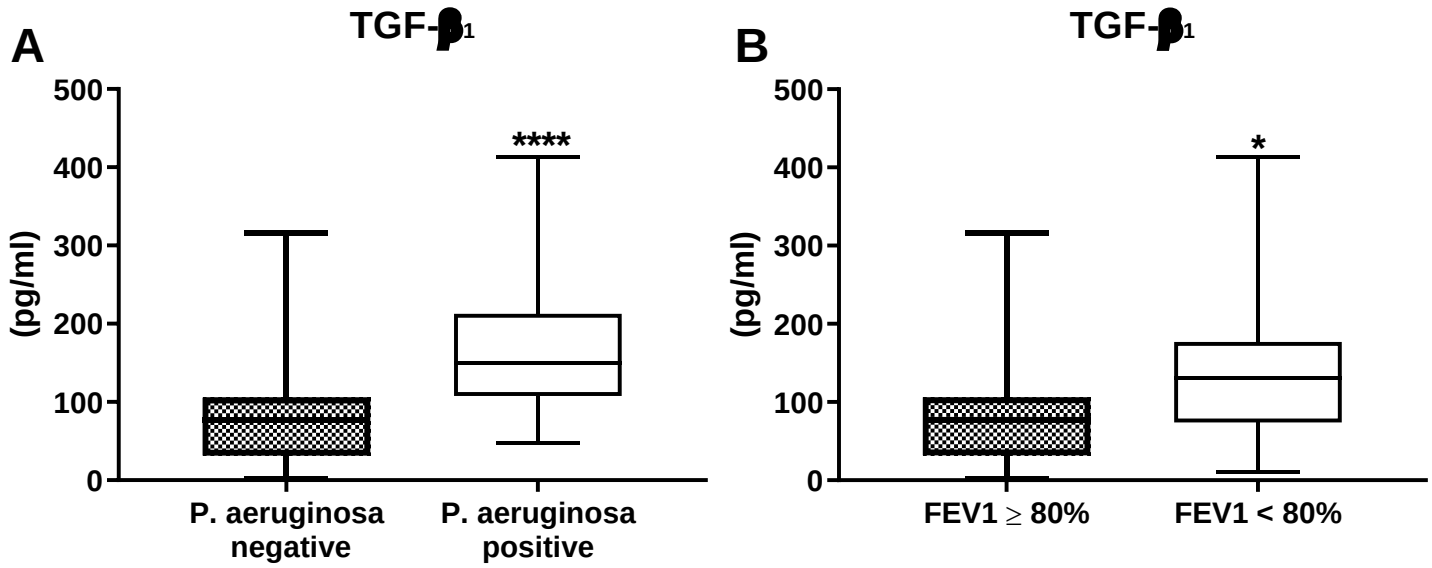

A
TGF-β1
500
400
300
200
100
0
(pg/ml)
****
P. aeruginosa negative
P. aeruginosa positive
B
TGF-β1
500
400
300
200
100
0
(pg/ml)
*
FEV1 ≥ 80%
FEV1 < 80%

Figure 6

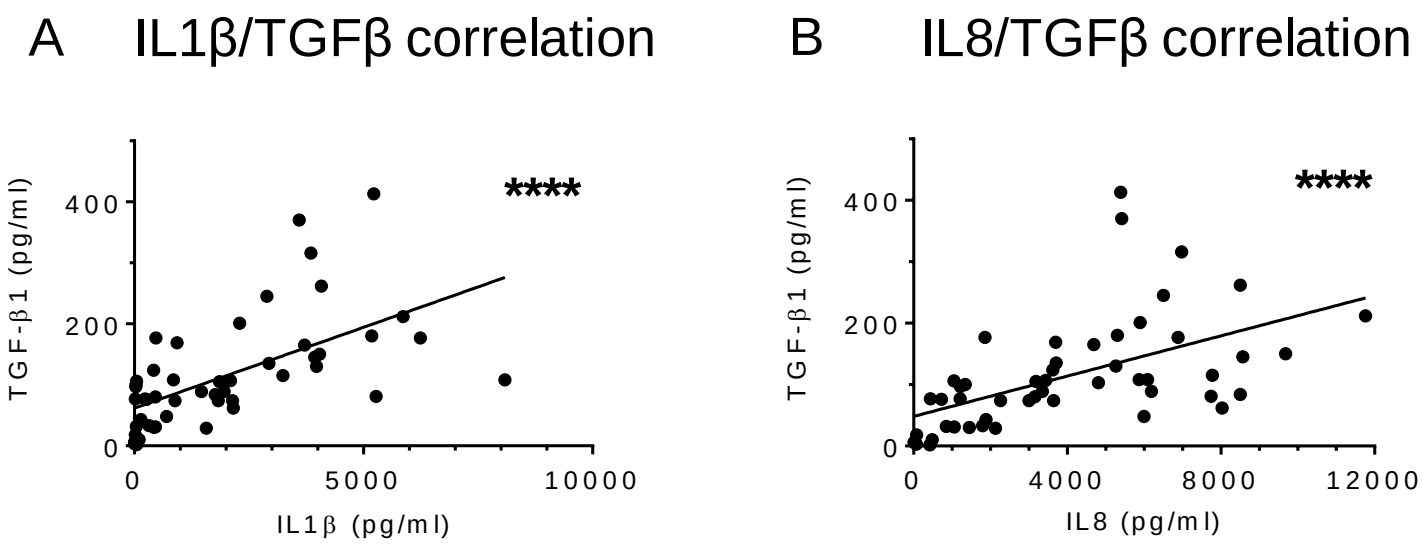

A IL1β/TGFβ correlation
B IL8/TGFβ correlation
TGF-β1 (pg/ml)
400
200
0
0
5000
10000
IL1β (pg/ml)
****
TGF-β1 (pg/ml)
400
200
0
0
4000
8000
12000
IL8 (pg/ml)
****

Figure 7

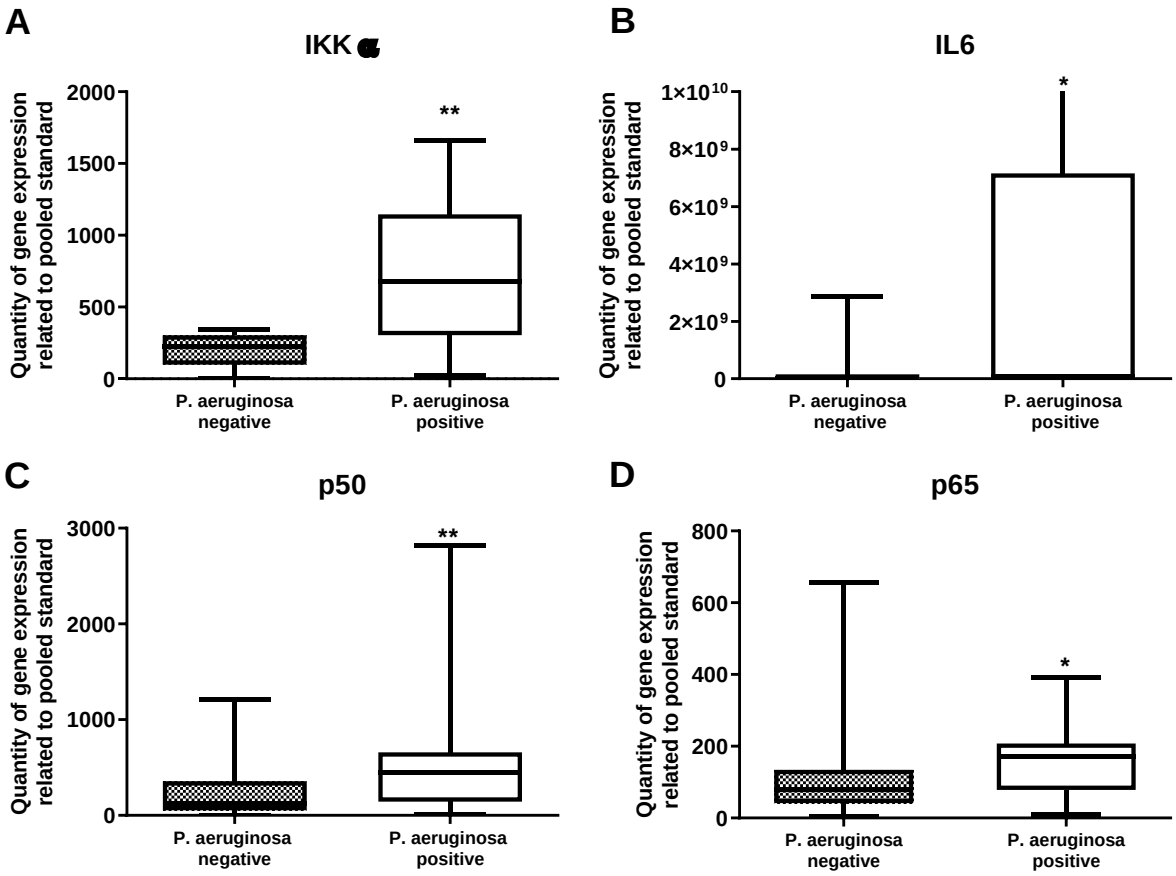

A
IKK
Quantity of gene expression related to pooled standard
2000
1500
1000
500
0
**
P. aeruginosa negative
P. aeruginosa positive
B
IL6
1×10^10
8×10^9
6×10^9
4×10^9
2×10^9
0
*
C
p50
3000
2000
1000
0
**
D
p65
800
600
400
200
0
*

Figure 8

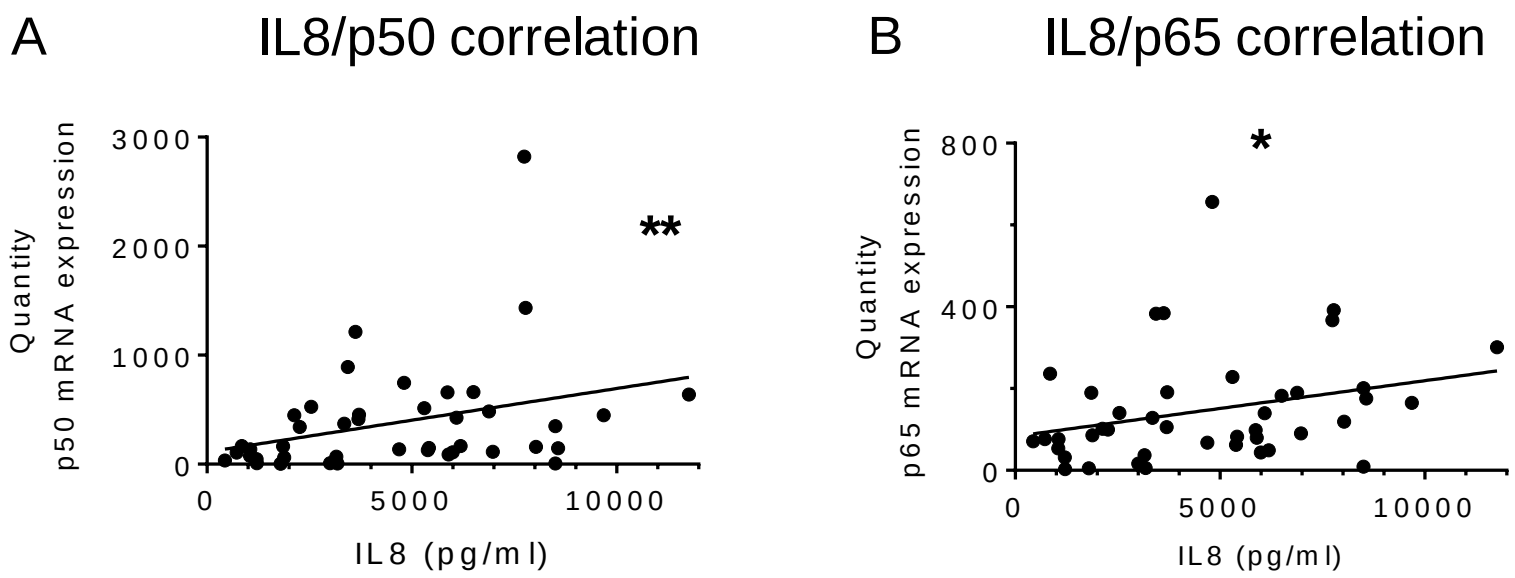
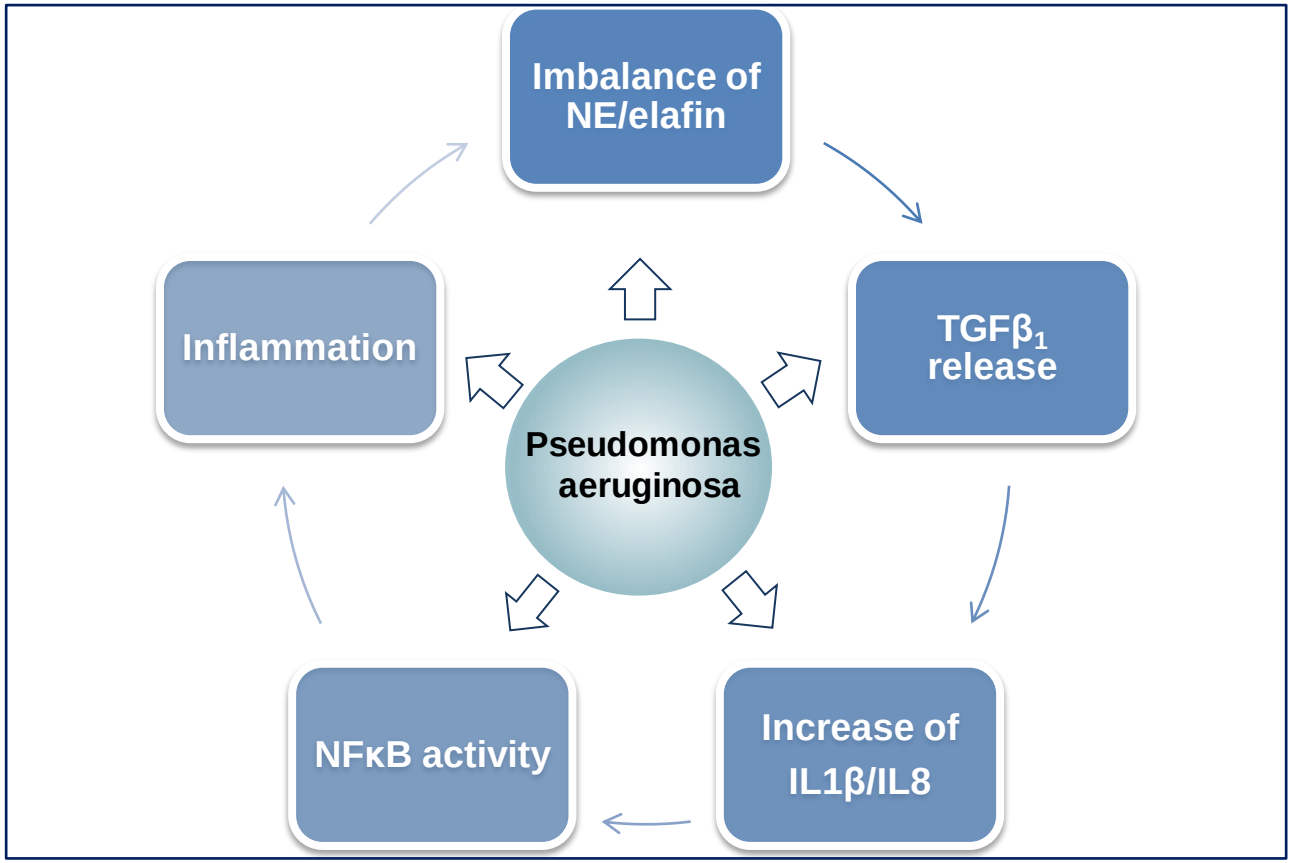

A
IL8/p50 correlation
Quantity
p50 mRNA expression
3000
2000
1000
0
0
5000
10000
IL8 (pg/ml)
**
B
IL8/p65 correlation
Quantity
p65 mRNA expression
800
400
0
0
5000
10000
IL8 (pg/ml)
*

Figure 9


Imbalance of NE/elafin
TGFβ1 release
Increase of IL1β/IL8
NFκB activity
Inflammation
Pseudomonas aeruginosa

Gene	Forward	Reverse
p50	GGCTACTCTGGCGCAGAAAT	GACTGTACCCCCAGAGACCTCATA
p65	GGCTCTGCTTCCAGGTGACA	GGTTCACTCGGCAGATCTTGA
IKK alpha	TGGAGCTACAGAAGAGCCCCTAT	GTGATCTGAAGGTCTGTGTTTTAACTG
IL6	GATGAGTACAAAAGTCCTGATCCA	CTGCAGCCACTGGTTCTGT

Supplementary table 1: Primer sequences used for the detection of the mediators of the NF-kB signalling cascade.