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Original article

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# Phagocyte Extracellular Traps in Children with Neutrophilic Airway Inflammation

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**Take home message:** We observed prominent extracellular trap formation in young children with airway inflammation, with and without cystic fibrosis. This innate inflammatory response was down-regulated by a combination of currently available therapeutics.

# ABSTRACT

Childhood lung infection is often associated with prominent neutrophilic airway inflammation and excess production of proteases such as neutrophil elastase (NE). The mechanisms responsible for this inflammation are not well understood. One potentially relevant pathway is the production of extracellular traps by neutrophils (NETs) and macrophages (METs). The aim of this study was to measure NET and MET expression in children and the effect of deoxyribonculease (DNase) 1 and alpha-1 antitrypsin (AAT) on this process.

We studied 76 children (median age of 4.0 years) with cystic fibrosis (CF) or chronic cough who underwent investigational bronchoscopy. NETs, METs and NE activity in bronchoalveolar lavage (BAL) samples were measured using confocal microscopy and functional assays. The effects of DNase 1 and AAT on NET/MET expression and NE activity were examined *in vitro*.

Both subject groups had airway neutrophilia with prominent BAL production of NETs with NE co-expression; the mean % ± standard error of the mean of neutrophils expressing NETs in the CF group was 23.3±2.8% and in the non-CF group was 28.4±3.9%. NET expression was higher in subjects who had detectable NE activity ($p \leq 0.0074$). The percentage of macrophages expressing METs in the CF group was 10.7±1.2% and in the non-CF group was 13.2±1.9%. DNase 1 decreased NET/MET expression ($p < 0.0001$), but increased NE activity ($p \leq 0.0137$). The combination of AAT and DNase 1 reduced NE activity ($p \leq 0.0049$).

We observed prominent extracellular trap formation in symptomatic children with and without CF. This innate inflammatory response was down-regulated by a combination of currently available therapeutics.

# INTRODUCTION

An effective immune response to infection is characterised by an initiation phase with activation of immune effector cells/mediators, then a resolution phase. An inadaquete initiation may result in progressive infection; whilst an ineffective resolution may result in excessive inflammation and tissue damage.

Early childhood is a crucial period for lung development. This period is also characterised by a high frequency of respiratory infection, which may potentially have an adverse effect on this development. Young children have an immune response to infection that is less effective than adults. There are major logistical issues in the study of lung infection in children particularly in terms of obtaining relevant samples and imaging. Consequently, the pathogenesis of respiratory infection in childhood remains relatively poorly understood, as does optimal treatment pathways.

Cystic fibrosis (CF) lung disease is characterised by recurrent lower airway infection and prominent neutrophilic inflammation that begins in the first year of life [1-3]. The persistent airway infection and inflammation causes bronchiectasis and eventually respiratory failure [4]. The presence of protease neutrophil elastase (NE) activity in bronchoalveolar (BAL) fluid in children with CF has been shown to be a primary risk factor for the development of bronchiectasis in this context [5].

Childhood respiratory infection may also have long-term effects in non-CF subjects with decreased lung function and increased risk of development of chronic airways disease [6-11]. A chronic wet cough may be a risk factor for the development of bronchiectasis [12].

One potentially relevant inflammatory pathway is phagocytic extracellular trap formation, an innate immune response. Extracellular traps are produced by neutrophils (NETs) and are composed of extracellular DNA with co-localised mediators such as NE [13,

14]. These NETs help protect against infection, but may also damage host tissue [14-17]. More recently it has been recognised that macrophages also express extracellular traps (METs) and we have demonstrated this in the lung [18, 19]. NETs have been described in the CF lung but this process is not well studied in young children [20]. NETs and METs are dismantled by deoxyribonuclease (DNase) 1 [14]. DNase 1 is available in the form of inhaled dornase alfa, which is used to break down extracellular DNA and improve sputum clearance in subjects with CF [21]. Dornase alfa is currently recommended for children with CF who are six years and older [22]. The activity of neutrophil elastase is reduced by anti-proteases such as alpha-1 antitrypsin (AAT), which is produced by the liver to control protease activity but also may be given exogenously; e.g to subjects with AAT deficiency and emphysema.

Our hypothesis is that phagocytic extracellular traps may be present in early life and contribute to lung inflammation, both in subjects with and without CF. Importantly the process of NET and MET expression in childhood may represent a new therapeutic target.

# MATERIAL and METHODS

## Study populations

Two paediatric groups with prominent airway neutrophilia were studied; 1) subjects with cystic fibrosis and, 2) children with chronic cough.

We recruited children from two tertiary paediatric referral centres in Melbourne, Australia: Monash Children's Hospital (MCH)/Monash Health and the Royal Children's Hospital (RCH). Subjects at MCH had a clinically indicated bronchoscopy as part of standard clinical care. Children with CF and non-CF airways disease were recruited from Monash. Subjects at the RCH were recruited as part of the Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF) protocol [5].

This research project was approved by the Monash Health human research ethics committee and as part of the AREST CF protocol by the ethics committee at RCH. Informed, written consent was obtained from the child's parent/legal guardian prior to enrolment.

It was not feasible to obtain BAL samples in asymptomatic children without airway neutrophilia due to ethical concerns. Therefore we also included as a control group, adults with a chronic cough with normal lung function who had had BAL samples taken previously [18].

## Clinical samples

BAL samples were obtained from anaethetised patients as previously described [5]. Peripheral blood samples were also obtained on the day of the bronchoscopy.

**Assessment of inflammation**

BAL samples were centrifuged to separate cells and supernatant. The BAL supernatants and serum from peripheral blood were frozen at -80$^{o}$C pending further analysis. Cell differential count and viability was performed on the BAL samples as previously described [18, 19].

To assess trap expression, cells were incubated with the prevalent respiratory bacterium nontypeable *Haemophilus influenzae* (NTHi), NTHi and DNase 1 or unstimulated (baseline) for one hour. Cells were then fixed and permeabilised and stained for relevant antibodies to define the expression of NETs and METs. Confocal microscopy was used to take images and results were analysed as previously described [18, 19, 23].

Biomarker/inflammatory mediator expression was assessed in BAL and serum samples. In BAL levels of NE activity, AAT and interleukin-6 (IL-6) were measured. The BAL levels of AAT and IL-6 in both pediatric groups were compared to the adult control group. In the serum samples levels of AAT, then C-reactive protein (CRP) and IL-6 were measured. AAT levels were measured by nephelometric assay in the serum and an enzyme-linked immosorbent assay (ELISA) in the BAL.

Initial samples were used for the analysis of lung extracellular trap expression, whilst subsequent samples were used for the assessment of functional assays of macrophage IL-6 production and effect of AAT on NE activity (there was not enough sample to perform both analyses). Biomarker analysis was done on all samples.

**Statistics**

Statistical analysis using paired/unpaired testing with parametric or non parametric analysis or Chi-squared test was performed as appropriate. Results are expressed graphically as mean ± standard error of the mean (SEM) or median values and interquartile range (IQR) as appropriate.

More details are available in the Supplementary Methods in the Online Supplement.

# RESULTS

## Characteristics of the study participants

BAL samples were obtained from 76 participants - 38 children with CF and 38 subjects in the non-CF group. The median age in both groups was 4 years and there were no significant differences in baseline clinical characteristics between the two groups. Both groups had prominent airway neutrophilia and a high prevalence (≥66%) of microbial pathogens; approximately half of the subjects had detectable NE activity in the BAL fluid (Table 1). Further details of the subjects (including features of the adult control subjects) are listed in the Supplementary Methods and Supplementary Tables 1 and 2 in the Online Supplement.

## Phagocyte extracellular trap expression

Both the CF and the non-CF groups had prominent expression of NETs and METs at baseline (analysed in the first 46 subjects (24 CF and 22 non-CF subjects)). Levels of NET expression were 2-fold higher than MET expression. The mean % ± SEM of neutrophils expressing NETs in the CF group was 23.3±2.8% and in the non-CF group was 28.4±3.9%. The percentage of macrophages expressing METs in the CF group was 10.7±1.2% and in the non-CF group was 13.2±1.9 The addition of NTHi to unstimulated BAL cells significantly upregulated trap expression in both groups ($p \leq 0.0431$), validating the relevance of infection to the production of NETs and METs. Results are shown in Figures 1a-f and in Supplementary Figures 1-3.

## NET expression and neutrophil elastase activity

An important component of NET formation is the expression of neutrophil elastase; the presence of detectable NE has been shown to be a key risk factor for the development of CF

in bronchiectasis. In the CF group the number of neutrophils expressing NETs per ml was compared in subjects with detectable and non detectable NE activity using an established threshhold (Figure 1g). The numbers of NETs in subjects with detectable NE was almost 10-fold higher compared to those subjects with no NE (p=0.0074).

In the non-CF group similar results were obtained (Figure 1h). The numbers of NETs in subjects with detectable NE was 14-fold higher compared to those subjects with no NE (p=0.0056).

**Measurement of interleukin 6**

Levels of IL-6 an inflammatory cytokine, were measured in the BAL fluid. Nearly all pediatric subjects had detectable IL-6 and levels were comparable between the CF and non-CF groups (Figure 2a). BAL macrophages were infected *in vitro* with NTHi (for 3 hours), which induced significant upregulation of IL-6 production in the CF group with a 5-fold increase (Figure 2b) and the non-CF group with a 3-fold increase (Figure 2c), (p=0.0002). Serum IL-6 was generally detectable in both the CF (82%) and non-CF groups (97%) with similar levels (Figure S5).

**Measurement of C-reactive protein and alpha-1 antitrypsin levels**

AAT and CRP are acute phase proteins primarily produced in the liver in response to inflammatory signals (e.g. IL-6). Levels of AAT were assessed using an automated nephelometric assay that is used in clinical practice and has a well-established normal range (reference interval). Both groups had median levels that were comparable. Despite these subjects having significant neutrophilic airway inflammation the median values for both groups were in the lower normal range. Both the CF and non-CF populations appeared to be mildly shifted downwards (Figure S6).

In the CF-group the serum CRP levels were 3-fold higher in subjects with detectable NE compared to subjects with no detectable NE and in the non-CF group 6-fold higher (Figures S7a and b), ($p \leq 0.041$). In contrast, levels of serum AAT showed no difference (Figures S7c and d).

BAL levels of AAT in both pediatric groups were compared to the adult control group (Table S3). This adult group despite a lack of neutrophilic airway inflammation had levels of AAT that were more than 7-fold higher than the pediatric groups (Figure 2d), ($p<0.0001$).

**Effect of deoxyribonuclease and alpha-1 antitrypsin**

In both the CF and non-CF groups, the administration of DNase 1 *in vitro* to NTHi-stimulated cells caused a 20-fold reduction in NET (Figures 3a-d) and MET expression (Figure S8), ($p<0.0001$).

AAT is the most important anti-protease involved in downregulation of NE activity. The addition of AAT to NTHi-infected blood neutrophils (that expressed NETs) in both groups induced a more than 5-fold reduction in NE activity (Figures 3e and f), ($p=0.0010$).

The effect of DNase 1 on NTHi-infected neutrophils was assessed. In contrast to AAT, the addition of DNase 1 increased NE activity approximately 2-fold ($p \leq 0.0137$).

The combination of AAT and DNase 1 was then assessed. This resulted in a 6-fold decrease in NE activity in the CF group and a 10-fold decrease in the non-CF group when compared to DNase 1 alone ($p \leq 0.0049$), (Figure 3g and h). Further experiments validated this approach (Figure S9).

The effect of adding AAT on the BAL samples with detectable NE activity was assessed. This resulted in the removal of detectable NE activity in most cases (Figure 3i and j).

A summary of the results and their possible relationship to each other is shown in Figure 4.

## DISCUSSION

We have demonstrated prominent neutrophil and macrophage extracellular trap expression in the lungs of young children and have defined pathways involved in their function. Treatment with medications in current clinical use ameliorated trap formation with potential anti-inflammatory effects.

Although NET expression has been described in CF [20], these studies have generally been undertaken in adults and used sputum analysis. There are issues with the use of sputum samples to assess NET expression as there are a variety of causes of cell death that may be responsible for the presence of extracellular DNA in sputum. There is minimal published literature defining the presence of lung NETs in early childhood .

To our knowledge our work is the largest and most comprehensive study of phagocytic traps in young children. In the current study we have demonstrated the presence of BAL NETs in children with CF from the age of one year onwards. We also studied a non-CF group who were composed of young children with a chronic cough. Similar to the CF group, these children had prominent expression of NETs, which was much higher in those with detectable NE activity. Both groups of children had marked airway neutrophilia and this was much more prominent than we typically observe in adult subjects.

Sly *et al* studied the potential role of a number of factors in the development of bronchiectasis in young children [5]. The key finding of this study was that the presence of detectable NE activity was associated with the dcvelopment of bronchiectasis. We used the same methodology and demonstrated the levels of NETs (that expressed NE) were much

higher in subjects with detectable NE. This result implies that a primary mechanism for the development of lung damage in CF is via NET formation.

In addition to NETs other forms of extracellular traps have been described such as METs. These METs produce mediators such as matrix metalloproteinase (MMP) 9 and 12, which have shown to have a role in the development of emphysema [24, 25] and potentially also in CF [26-28]. To our knowledge pulmonary METs have not been described previously in young children, but were present in both groups of subjects and may potentially contribute to host pathology.

Both the CF and non-CF children groups had prominent BAL inflammation with airway neutrophilia. Levels of NETs and to a lesser extent METs were much higher than we have previously observed in adult subjects (for NETs more than 10-fold higher) [18, 19]. Surprisingly the responses were similar between the two groups. This is likely to be explained by the non-CF group having exacerbated airways disease whilst the CF group were most commonly assessed when at baseline. The results are suggestive that young children make a strong innate immune response in the lung to infection.

Lung inflammation may induce pathways to resolve this response. Interleukin 6 is a key inflammatory cytokine. Both groups had detectable IL-6 in BAL fluid and lung macrophages demonstrated enhanced production with NTHi infection; most subjects also had detectable blood levels of IL-6. Blood levels of IL-6 in young children have not been well-established, but the levels were not obviously elevated (as may be expected in inflammatory states). In addition to its inflammatory effects, IL-6 is the primary cytokine that induces the production of acute phase reactants in the lung such as AAT. AAT is predominantly produced in the liver and has a primary role in inactivating proteases, particularly NE. The production of DNase 1 dismantles extracellular trap formation and has an anti-inflammatory effect [19, 29, 30].

We found that both CF and non-CF children had median serum levels of AAT that were in the lower part of the normal range with both populations being skewed downwards (rather than having outlying subjects). This result is surprising as these children generally had significant airway inflammation (and AAT levels might be expected to be increased). Studies have assessed the role of AAT deficiency alleles in CF; these deficiency alleles have not been found been to be significantly different from normal populations nor were they associated with severity of lung disease [31-33].

Most subjects had detectable AAT in the BAL fluid, but there is very limited literature available regarding what are the normal levels of AAT in the lung. One study described subjects who were healthy smokers as having a median BAL AAT level of 795 ng/ml and in COPD smokers levels were 512 ng/ml (as measured by ELISA) [34]. In the current study we also compared BAL AAT levels in adults with chronic cough with the pediatric groups; levels in children were much lower than adults (in contrast to the IL-6 levels). The results from the current study imply that these children may have a relative deficiency of AAT production in response to inflammation and protease production [35]. The mehanisms responsible for this effect are not clear but could include deficient IL-6 signalling or the inability of young children to upregulate liver AAT production in response to inflammation.

There are several limitations of this study. The first relates to the ethical issue of subjecting asymptomatic children to general anaesthesia to obtain a BAL sample, which required us to use adult controls to compare AAT levels. At our institutions we do not perform bronchoscopy and BAL in asymptomatic children. Hence we used an adult control group who had chronic cough but did not have airway infection, neutrophilia or NET formation. In our laboratory the normal serum levels of AAT have a range of 1.0 to 2.4 g/L for the population aged from 1.0 to 70.0 years. Thus serum levels of AAT are roughly

comparable between adult and paediatric groups. The number of subjects was moderate in size compared to adult studies, however the results were significant with large differences. The non-CF group was hetereogeneous and whether chronic cough or infection predisposes to the development of lung disease has not been definitively confirmed. The adult controls did not have serum samples available to assess AAT levels.

The results clearly demonstrate that DNase 1 (dornase alfa) reduces NET/MET expression. We have previously demonstrated *in vivo* in a smoke exposure model that DNase 1 reduces lung proteolysis and similar findings have been described in the liver [19, 36]. In addition we have demonstrated that this therapy markedly reduces lung leukocyte numbers. In other models, DNase 1 has been shown to have a beneficial anti-inflammatory effect [29, 30]. However DNase 1 was used as a long-term maintenance therapy in a cohort of adults with non-CF bronchiectasis with worse outcomes [37].

Exogenenous AAT has been administered for the treatment of emphysema for a number of years as well as in trials of other lung diseases including CF with inconclusive results [35] DNase 1 has previously been shown to faciliate NE release [38, 39]. We found similiar results in our *in vitro* NET model. The findings suggest that NE in a trap may be protected from the action of AAT. Our results suggest that before administering DNase 1, assessment of AAT levels is important as this could potentially indicate subjects who are more at risk from tissue damage from released proteases.

The first five years of life are critical for normal lung growth. Our work suggests that both DNase 1 and AAT may have a role in reducing pathogenic lung inflammation in children. Furthermore, our results imply a possible role for the use of combined DNase 1 and AAT, particularly in subjects who may have a relative deficiency of AAT. The potential benefits of this novel approach has to be weighed up against the risks of inhibiting a host immune response and perhaps may be safest to be used for the treatment of exacerbations

with co-administered antibiotics. DNase 1 has a short half life so this medication may need to be given twice rather than once daily. AAT as it is principally derived from human serum, is not widely available in many countries and also is relatively unstable with variable effectiveness as a consequence. This regimen may reduce excessive inflammation not only in CF but also in the much larger non-CF population such as in children with pneumonia.

In conclusion, phagocyte extracellular trap expression is prominent in children, both with CF and non-CF airways disease. This robust innate inflammatory response may potentially contribute to lung damage and represent a new therapeutic target in early life with the possible combination of DNase 1 and AAT.

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## Table 1: Baseline characteristics of groups

| Characteristic | CF-group | Non-CF group | p |
|---|---|---|---|
| Number of children | 38 | 38 | |
| Age (yrs) | 4.1 (1.8-6.0) | 3.7 (1.8-6.7) | 0.87 |
| Sex – female/male | 15/23 | 13/25 | 0.81 |
| Detectable neutrophil elastase activity-no./total no. (%) | 23/38 (61) | 17/38 (45) | 0.25 |
| Infection-no./total no. (%) | 30/38 (79) | 25/38 (66) | 0.30 |
| Percentage of neutrophils in BAL | 22 (11-52) | 23 (11-39) | 0.81 |
| Subjects with elevated BAL neutrophil %-no./total no. (%) | 29/38 (76%) | 29/38 (76%) | |

Results expressed as median and interquartile range, as appropriate. BAL = bronchoalvearlar lavage. An elavated percentage of neutrophils in BAL is defined as > than 10%.

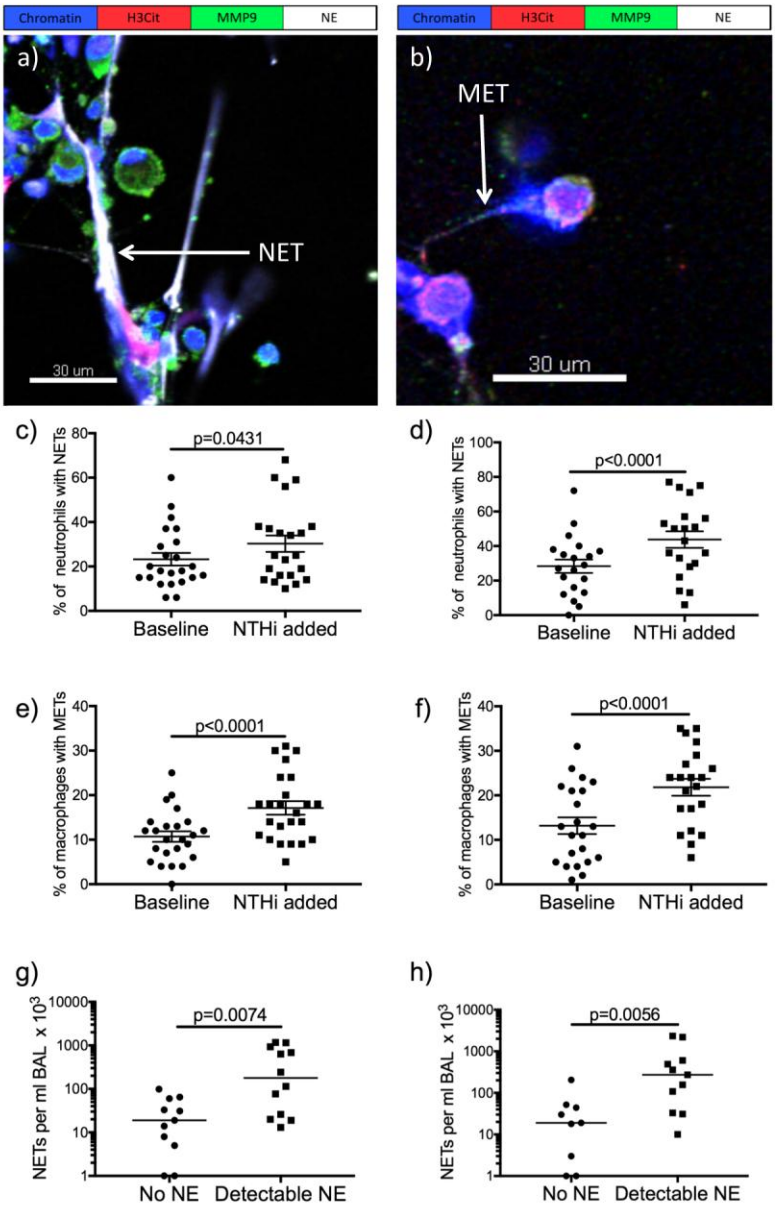


Figure 1: Phagocyte extracellular trap expression. Neutrophil (NET) and macrophage (MET) extracellular trap expression was assessed in both the CF and non-CF groups using confocal microscopy; at baseline and with the addition of the bacterium nontypeable Haemophilus influenzae (NTHi). a) Demonstrates NET expression and b), MET expression. Images shown are merged with chromatin (blue), citrullinated histone (H3Cit) (red), matrix metalloproteinase (MMP) 9 (green) and neutrophil elastase (NE) (white). c) NET expression in CF group and d) NET expression in non-CF group. e) MET expression in CF group and f) MET expression in non-CF group. BAL NE activity was assessed in both CF g) and non-CF groups h). The number of neutrophils expressing NETs was compared between subjects without detectable NE and with detectable NE. Results shown as mean±SEM or median.

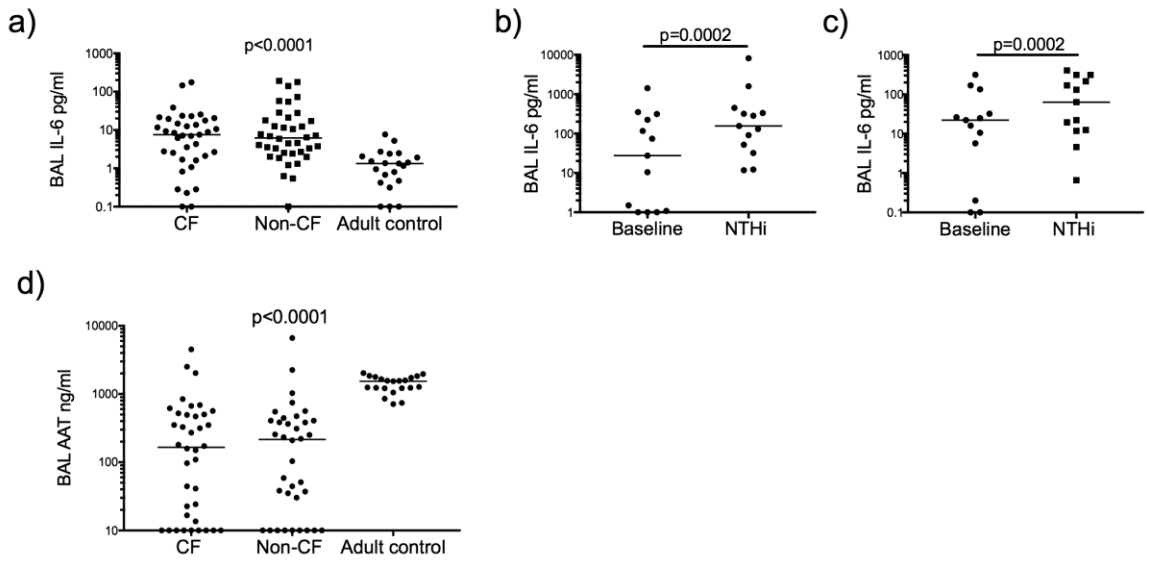


Figure 2: Measurement of interleukin (IL) 6 and alpha-1 antitrypsin (AAT) levels. Potential biomarkers/inflammatory mediators were assessed in the BAL fluid. Both childhood groups had detectable BAL IL-6 that was higher than the adult control group a). BAL macrophage production of IL-6 with nontypeable Haemophilus influenzae (NTHi) infection in CF b) and non-CF c) groups. BAL levels of AAT (measured with ELISA) were compared between CF, non-CF and adult control groups d). Results expressed as medians.

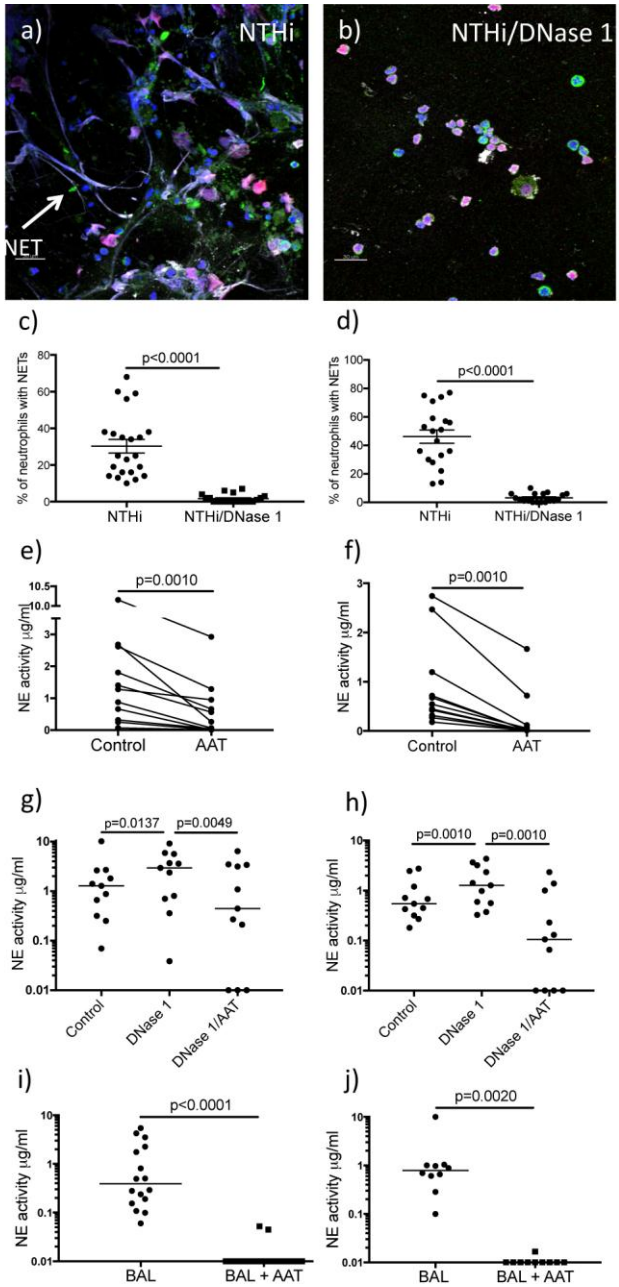


Figure 3: Effect of deoxyribonuclease (DNase) 1 and alpha-1 antitrypsin (AAT). The effect of DNase 1 on NET expression was assessed in both the CF and non-CF groups. Panel a) demonstrates extracellular trap expression in NTHi stimulated cells and b), demonstrates the effect of the addition of DNase 1. Panel c) shows NET expression in CF group and d) shows NET expression in non-CF group. The addition of AAT decreased NE activity compared to control in CF subjects e) and similar results were noted in non-CF subjects f). DNase 1 increases NE activity which is reduced by the addition of AAT in CF subjects g) and non- CF subjects h). The addition of AAT to BAL reduces NE activity in CF i) and non-CF j) populations. Results shown as mean±SEM or median.

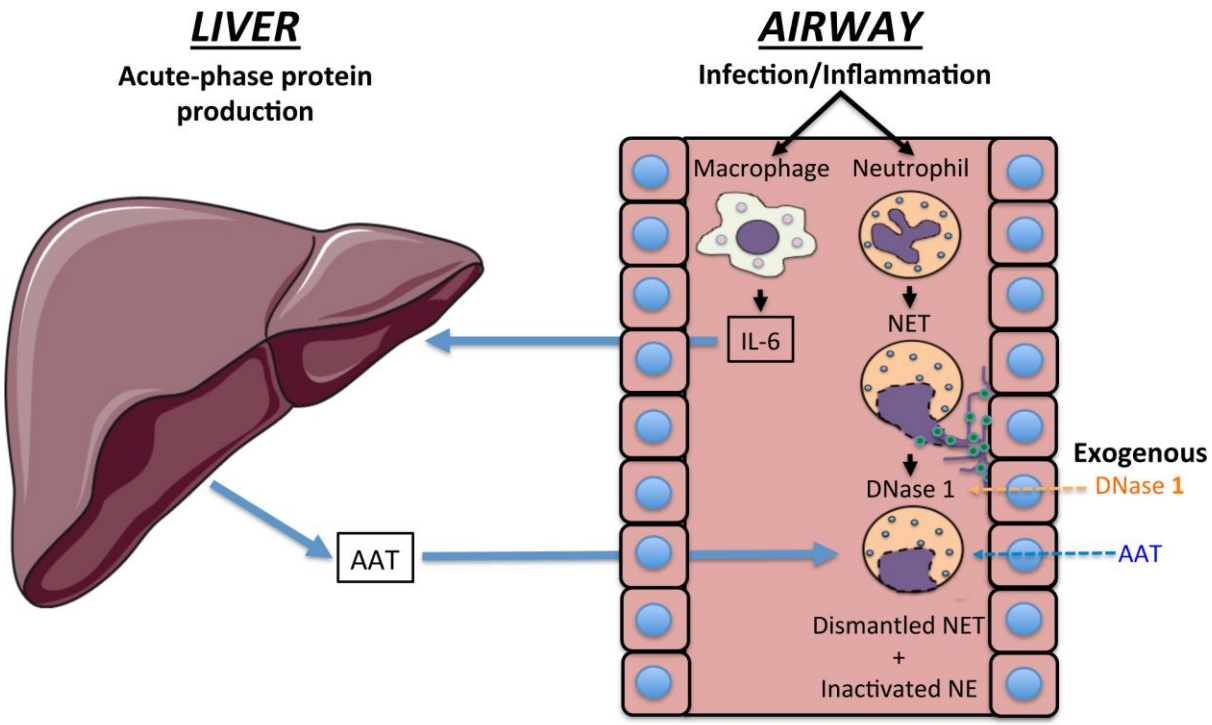


Figure 4: Potential relation between key results. Figure 4 summarises the possible relation between the airway and systemic immune responses. Infection/inflammation in the airway causes the expression of NETs with NE; as well as activating macrophages to produce inflammatory mediators such as IL-6. One of the potential effects of inflammatory mediators is to act on the liver to induce acute phase reactants such as CRP and AAT. Circulating AAT may then come back to the airway. DNase 1 breaks down NETs and AAT inactivates NE. Both DNase 1 and AAT may be given exogeneously.

# SUPPLEMENT

**To**

# Phagocytic extracellular traps in children with neutrophilic airway inflammation

**Table of Contents**



**Authors**

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## SUPPLEMENTARY METHODS

### Patient groups

Subjects were recruited from both Monash Children's Hospital (MCH) and Royal Children's Hospital (RCH); both institutions are tertiary referral pediatric centres in Melbourne. All children at both centres were assessed by a respiratory pediatrician prior to the performance of bronchoscopy. Bronchoscopy and BAL at MCH and RCH was performed under general anaesthesia as previously described [1]. A paired blood sample was taken at the time of bronchoscopy.

At MCH bronchoscopies were performed as part of clinical care and part of the BAL sample was taken for research along with a paired blood sample. Generally bronchoscopies were done in the context of exacerbated airways disease, particularly to optimize management in children who had not responded to initial therapy. As bronchoscopy and BAL requires a general anaesthetic this procedure is only performed in our institutions in children who have severe symptoms that have not responded to aggressive medical therapy. Children from RCH were recuited as part of the AREST CF project as previously described [1]. Bronchoscopy was done on annual basis on the child's birthday.

Eleven children with CF were recuited from MCH and 27 from RCH. Their characteristics are listed in Table S1. Twenty three subjects had detectable neutrophil elastase (NE) activity. There were a variety of isolates from the bronchoalveolar lavage (BAL), some of these such as *Haemophilus parainfluenzae* may have relatively low pathogenicity. Upper respiratory tract flora (URTF) was a common finding and was classified as being non-pathogenic. Eleven children with CF at MMC had a bronchoscopy; seven of these were part of an inpatient tune-up with the use of intravenous antibiotics,

three of these were done to optimise therapy after courses of oral antibiotics and one was done as a combined procedure with a change of a percutaneous endoscopic gastrostomy (PEG) tube.

Thirty eight children in the non-CF group had a bronchoscopy performed. Thirty five children were recruited from MCH and three from RCH as a non-CF group. Their characteristics are listed in Table S2. These subjects had a history of chronic cough (> 4 weeks) that was mostly wet (35/38). These subjects generally had prominent neutrophilic airway inflammation as measured by the high neutrophil differential count (which was similar to the CF group). Two subjects had significant underlying neurological disorders (Myasthenia Gravis and Ohdo syndrome). One child in the CF group had confirmed bronchiectasis. However we rarely perform CT scans in young non-CF children due to the risk of radiation and need for sedation/anaesthesia.

Twelve of the 38 CF subjects had abnormalities of their liver function tests; 11 had mild elevation of alanine aminotransferase levels and one had a markedly elevated alkaline phosphatase level.

No child in the non-CF group was on dornase alfa (Pulmozyme) treatment. In the CF group 17 children were on dornase alfa (2.5 gm daily). In all subjects this dornase alfa was given in the evening as a standard regimen. All bronchoscopies were done in the afternoon and no subject received dornase alfa on the day of the bronchoscopy.

We were not able to obtain sufficient BAL samples from children without airway inflammation. Therefore an adult control group that we had previously studied was used (Table S3) [2]. These subjects had a bronchoscopy for the investigation of a cough. They had normal lung function and no definable lung disease. None of these adult subjects had detectable NE activity, NETs or isolated pathogens. These subjects also had BAL IL-6

levels that were 6 fold lower than both the CF and non-CF groups (demonstrating a lower inflammatory state).

**General Methods**

BAL

BAL samples were obtained as previously described [1]. The right middle lobe (RML) was lavaged with three aliquots of warmed normal saline (1 ml per kilogram of body weight), with one additional aliquot lavaged into the lingua or the most affected lobe identified on X-ray. The first aliquot from each lobe was processed for microbial analysis. The second and third aliquots retrieved from the right middle lobe were pooled and used for analyses of inflammation, as described below.

BAL fluid was processed at either RCH or MCH in the microbiology laboratory for the presence of bacteria, fungi or viruses. BAL and blood samples from the RCH were transported to Monash Health on the day of the bronchoscopy for the analysis of inflammation.

In the adult control group BAL samples had been obtained from the RML as previously described [2]. Three to four 25ml aliquots of warmed saline were lavaged into the RML then aspirated. The BAL was centrifuged to separate cells and supernatant and stored at -$80^0$C.

Blood samples

Immediately prior to the bronchoscopy, blood was obtained from an arm venepuncture. In six patients it was not possible to obtain blood (3 CF subjects and 3 non-CF subjects).

Sample Processing

BAL samples were spun at 1500rpm for 10 minutes, and supernatant was collected into smaller aliquots and stored at -80C. Cells were washed with phosphate buffered saline (PBS), and resuspended in RF10 media (RPMI (Sigma-Aldrich, USA), supplemented with 10% fetal calf serum and 0.1%L-glutamine (Sigma-Aldrich)). Total cell count and viability as performed manually using a hemocytometer (cells were stained with 0.4% trypan blue). Although total cell numbers varied, cell viability was above 90%. Approximately 5 x $10^4$ BAL cells were prepared via cytocentrifugation for later histological analysis and remaining BAL cells were utilised for confocal microscopy.

Serum was obtained from clotted peripheral blood samples by centrifugation and aliquoted and stored at -80$^o$C until use. Neutrophils were isolated from peripheral blood and aliquoted onto cover slips as previously described [2].

NTHi

NTHi was prepared in-house from a previously-collected clinical sample [2-4] and grown on pre-prepared agar plates (Mueller Hinton +5% Horse Blood +20mg/L NAD, Thermo Fisher, USA) kept at 37$^o$C.

Cell histology

Following cytocentrifugation, cells were fixed and stained via a commercial Romanowsky stain (Sigma-Aldrich). Percentages of immune cells were calculated after manually counting at least 500 cells using an Olympus Stereologer on a 40x magnification (with oil) and FIJI image software.

Neutrophil Elastase Assay

An established method was used to determine levels of NE activity in both BAL and blood assay. A specified threshhold that has been shown to be associated with the development of bronchiectasis in children with CF (0.2μg/ml) was used to denote absent or detectable NE activity in the BAL as shown in Figure 1 and Figure S6 [1]. Undiluted, thawed BAL fluid supernatant (or serially diluted human Neutrophil Elastase (Sigma-Aldrich, E7885)) was incubated with an equal volume of substrate N-methoxysuccinyl-ala-ala-pro-val p-nitroanilide (Sigma-Aldrich, M4765) at 37$^{o}$C for 60 minutes, and read at 405nm. Substrate was reconstituted in Methyl-2-pyrolidone (Sigma-Aldrich) then diluted in 110mM Tris HCL (pH 8.0) for a final volume of 0.5mM per well.

**Confocal microscopy.**

Previously described methods were used [2, 5, 6]. A single coverslip (#1.5, 13mm, Thermo Scientific, USA) was placed into each well of a 24 well plate, and incubated in 0.01% of Poly-L-Lysine (Sigma-Aldrich) at 37$^{o}$C for 20 minutes. Poly-L-Lysine was removed and coverslips were dried.

Wells were loaded with 3-7 x $10^5$ BAL cells suspended in 400μl RF10 media (as above) and incubated at 37$^{o}$C for 20 minutes to adhere. Samples were then incubated for 60 minutes: 1) untreated, 2) NTHi (multiplicity of infection (MOI) of 100:1) or 3) NTHi

(MOI 100:1) and 5 international units DNase 1 (Pulmozyme, Genentech, USA). Samples were then fixed with 2% periodate-lysine-paraformaldehyde (PLP) and permeabilized with 0.2% Tween 20. Following PBS wash, samples were blocked with 10% chicken sera in 5% bovine serum albumin (BSA) in OBS for 30 minutes at room temperature. Primary and isotype antibodies (Table E4) were incubated for 16hrs at 4$^{o}$C in 1% BSA in PBS. Following by PBS wash, samples were incubated in secondary antibody (see below) for one hour at room temperature, then mounted with DAPI prolong gold (Thermo Fisher). Samples were also prepared using only secondary antibody for use as background control.

Images were obtained using Olympus FV1200, excited with 405, 488, 597 and 647 nm lasers. Twenty fields of view per coverslip were obtained for analysis. To obtain representative images a grid pattern was used [5].

Rabbit IgG and Sheep IgG isotype control antibodies were prepared in-house and purified using Protein G columns from sera.

For the first three subjects only METs were measured (1 CF and 2 non-CF) and in the next 43 subjects both METs and NETs were measured. For the samples only a limited number of cells was available. Analyses were prioritised for 1) baseline trap expression, then 2) NTHi-stimulated trap expression, then 3) combined NTHi/DNase 1. Therefore samples analysed for; 1) CF NET expression numbers were 23:22:20, 2) Non-CF NET expression numbers were 20:20:18, 3) CF MET expression numbers were 24:24:22, and 4) Non-CF NET expression numbers were 22:21:17.

Image analysis

Imaris software was used to analyse trap expression uisng previously described methods [2]. NETs were defined by the presence of co-localisation of extracellular chromatin,

H3Cit and NE, whilst METs were defined by co-localisation of extracellular chromatin, H3Cit and MMP9 (and absence of NE staining). Controls for both background and isotype staining were used.

**Biomarker Analysis**

Biomarker analysis was done both on blood and BAL samples. There were 70 blood samples and 76 BAL samples available for analysis. An initial analysis was done on the first 39 blood specimens for biomarkers with bioplex assay, DNA complexes and CRP. Subsequent analyses were could be done on the 68 available serum samples so there were 68/70 measurements of AAT/CRP/IL-6.

Bioplex assay

Serum samples (diluted 1:4) were examined using Bio-Rad's Bio-Plex Pro human chemokine assays using a MagPix Luminex system, following the manufacturer's instructions. Briefly, standards and samples were incubated overnight with specialised magnetic beads containing antibodies to 40 proteins of interest (ENA-78, CTACK, Eoxtaxin-2, IL-16, SCYB16, SDF-1a+b, 6Ckine, MDC, MIF, MIP-1d, Gro-1a, Gro-1b, IL-8, IP-10, MCP-1, MCP-2, MCP-4, MIG, MIP=3b, Fractalkine, MPIF-1, TARC, TECK, BCA-1, Eotaxin, Eotaxin-3, I-309, I-TAC, MCP-3, GCP-2, GM-CSF, IFN-g, IL-1b, MIP3a, TNF-a, IL-1b, IL-2, IL-6 IL-10, MIP-1a). Following wash (using a handheld magnetic plate washer), standards and samples were incubated for 30 minutes on a shaker with detection antibody. Each detection antibody created an individual fluorescence following incubation with the reporter molecule Streptavidin Phycoerythrin. The MagPix

Luminex system was then used to identify and calculate the quantity of each antibody in the sample, and thus, the concentration its correlating protein of interest.

DNA complexes

DNA complexes were measured using the sandwich ELISA method developed by Kano et al [7]. Wells of a 96-well plate were coated with anti-MPO antibody (Merck Millipore, USA) was diluted to 5ug/ml in M Carbonate-bicarbonate buffer (pH 9.6), incubated overnight at 4C The following morning, wells were washed 3x with PBS, then blocked for 120 minutes with 1% BSA with 0.05% sodium azide in PBS. Following another PBS wash, serum (diluted 1:3 with PBS) and the PBS only control was incubated overnight at $4^{o}$C. On the third day, wells were washed with PBS and incubated for 90 minutes at room temperature in peroxidase-conjugated anti-DNA antibody, diluted in the incubation buffer provided (Cell Death Detection Assay, Roche, Switzerland). Wells were washed 3 times with 0.5% Triton X. Photometric colour was provided by ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), prepared according to manufacturer's instructions (Roche, Cell Death Detection Assay), and samples were incubated, with shaking, for 30 minutes for colour development. The optical density (OD) of each well was measured at 405nm, with a reference wavelength of 490nm and calculated as a percentage of the PBS control.

CRP analysis

CRP analysis was performed on serum by the Monash Pathology. Highly sensitive CRP was measured from serum using the routine automated Beckman Coulter AU5812. The quantitative determination of CRP is measured by immuno-turbidimetric method

(Beckman Coulter). CRP reacts specifically with anti-human CRP antibodies coated on latex particles to yield insoluble aggregates and the absorbance of the aforementioned aggregates is directly proportional to the CRP concentration in the sample.

AAT analysis

AAT levels were assessed two methods.

Nephelometry was used to measure AAT levels in the serum by Monash Pathology using the routine Beckman Coulter IMMAGE 800 Protein Chemistry Analyser. The quantitative determination of AAT is measured by rate nephelometry method (Beckman Coulter, USA). The test measures the rate of increase in light scattered from particles suspended in solution as a result of complexes formed during an antigen-antibody reaction. Results were expressed in normal range (or reference interval).

A standardised ELISA (R&D systems, USA) was used to measure AAT levels in the BAL fluid. This method is able to detect the lower levels of AAT present in the BAL fluid (in addition the nephelometry method has only been established for the analysis of blood samples in Monash Pathology). ELISA was performed according to manufacturer's instructions and results were normalised using a positive control.

**Functional Assays**

BAL macrophage IL-6 production

From BAL fluid 2 to 4 x $10^5$ cells were incubated in 24 well plates with 500µl of culture medium and left overnight at 37$^o$C. The next day the media was removed, washed once with PBS and replace media with RPMI (+L-glutamine, no serum). Based on previous

method nearly all the adherant residual cells were then macrophages [2]. Live NTHi was then added to each well at a MOI of 100:1 or as uninfected (control). Samples were collected at 1 and 3 hour timepoints after infection (and matched control samples), spun down (to removed any cells) and were then frozen at -80$^{o}$C. For final analysis samples were thawed and IL-6 levels measured using ELISA as described above.

Effect of AAT on NE activity

Human serum was obtained and NE activity was measured as described above. Approximately 4ml of serum from a healthy adult donor was mixed with 1ml of CaptureSelect Protein Affinity Resin selective for AAT (Thermo Fisher) and incubated overnight at 4$^{o}$C on a roller. A column was eqilibrated with PBS, then the resin/serum mixture was added. AAT-free serum was immediately collected, then the column was washed with 10x 10ml of PBS. Concentrated AAT was then collected using approximately 3ml of elution buffer (0.1M glycine, PH3.0). Depleted serum (DS) was then assessed for AAT via the previously described AAT ELISA and found to be undetectable. Likewise, the eluted AAT was assessed to determine the concentration of AAT.

Three preparations of the human serum were used for subsequent experiments 1), whole original serum (WS), 2) eluted AAT and, 3) depleted AAT (DS). Preliminary experiments were done in adults to ascertain doses that significantly reduced/oblitered NE activity that were used in subsequent experiments in children.

A 96 well plate was prepared by adding 50-100µl/well of poly-l-lysine (for neutrophil adherance) and left at 37$^{o}$C for 20 minutes, then PLL was removed and plates left at 37$^{o}$C to dry.

Whole blood was lysed with Ammonium Chloride as previously described.[2] Cells were resuspended in culture medium (phenol-free RPMI, Thermo Fisher) and cells counts performed. Neutrophils (5 to 10 x $10^4$) were then added to each well (Poly-L-Lysine coated) with NTHi at an MOI of 100:1 to induce NET formation and incubated at 37$^o$C for one hour as previously described (E2,5).

Culture media was removed and fresh culture medium was added for 1) Control (serum-free, phenol-free RPMI), 2) eAAT (approximately 20pg/ml AAT in serum-free, phenol-free RPMI), 3) DNase 1 and 4) DNase 1 and eAAT.

An equal volume of NE substrate was then added to each well. A positive control was also included (0.1mg/ml porcine elastase, as previously described). Plates were read immediately at 405nm and following 1 hour incubation at 37$^o$C. Results were expressed as OD absorbance at the one hour time point.

Further validation experiments were also peformed in which control was compared with 1) whole serum (10% human serum in phenol-free RPMI) and 2) depleted serum (10% AAT-depleted serum in phenol-free RPMI).

**Statistics**

Data was analysed using paired and unpaired testing with parametric or non parametric methods as appropriate. For contingency analysis, Chi square testing was used. Analysis of three independent groups was performed using the non-parametric Friedman test. Data was analysed using Prism 7 software (Graphpad, USA). After the first 46 samples for NET/MET assessment, an interim analysis was performed; this demonstrated significant differences with large between group changes. Therefore it was decided that further assessment of this parameter was not required and subsequent samples were used to

further define functional assay studies of the extracellular trap pathway (given the small amount of tissue available it was not possible to do both).

## SUPPLEMENTARY RESULTS

Staining for NET formation is demonstrated in Figure S1. Panel A shows staining for chromatin (DAPI), B staining for citrullinated histone (H3Cit), C staining for MMP9, D staining for NE and E shows the merged image. Insert is isotype control.

Staining for MET formation is shown in Figure S2. Panel A shows staining for chromatin (DAPI), B staining for H3Cit, C staining for MMP9, D staining for NE and E shows the merged image. Insert is isotype control.

Staining for NET/MET formation is shown using a lower magnification field in Figure S3. Panel A shows staining for chromatin (DAPI), B staining for MMP9, C staining for H3Cit, D staining for NE and E shows the merged image. Insert is isotype control.

A bioplex assay was used to measure 40 potentially relevant biomarkers in serum and assess whether these could be used as a non-invasive way to predict detectable BAL NE activity. Results were compared between subjects who had no measurable NE and those who had detectable NE. Results were analysed from 20 CF subjects and 19 non-CF subjects and both groups were pooled for the analysis. The results showed no significant differencs (data not shown).

DNA complexes were measured in the serum as an indirect measure of circulating extracellular trap activity. This was done in the CF group (20 subjects) and the non-CF group (19 subjects). To increase statistical power the two groups were combined. Results were compared between subjects who had no measurable NE and those who had detectable NE. There were no significant differences (Figure S4).

Serum levels of IL-6 were measured. Most subjects who had measurements performed had detectable IL-6 (82% of CF group and 97% of non-CF group). Levels were similar in both groups (Figure S5).

Serum AAT levels in the CF and non-CF groups were measured using nephelometry with similar results (Figure S6)

Serum CRP and AAT levels (AAT by nephelometry) were measured in subjects with and without detectable neutrophil elastase. CRP levels were higher in subjects who had detectable NE in both CF (Figure S7A) and non-CF groups (Figure S7B). For AAT levels there were no significant differences in the CF group (Figure S7C) or the non-CF group (Figure S7D).

Similar to its effect on NETs, DNase 1 markedly reduced MET expression in the CF group (A) and non-CF group (B). Results are shown in Figure S8.

AAT significantly reduced NE activity in all subjects. It did not obliterate activity in about half of subjects, predominantly in those with higher AAT levels. The dose of AAT used was determined in preliminary experiments in adults. In retrospect a higher dose of AAT would have been required in these children with their active and primed neutrophils to obliterate the NE activity. In addition AAT may be unstable due to a variety of factors and as the assays required a minimum of one hour to do it is possible in some subjects that the function of AAT may have been reduced.

Whole serum (WS) contains multiple anti-proteases of which the most important involved in the inactivation of NE is AAT. To further validate the experiments shown in the main text in Figure 3, the effect of WS on NE was assessed as was the effect of WS that had been depleted of AAT (dAS). The results showed that WS decreased NE activity when compared to control but dAS increased NE when compared to WS. Results assessed

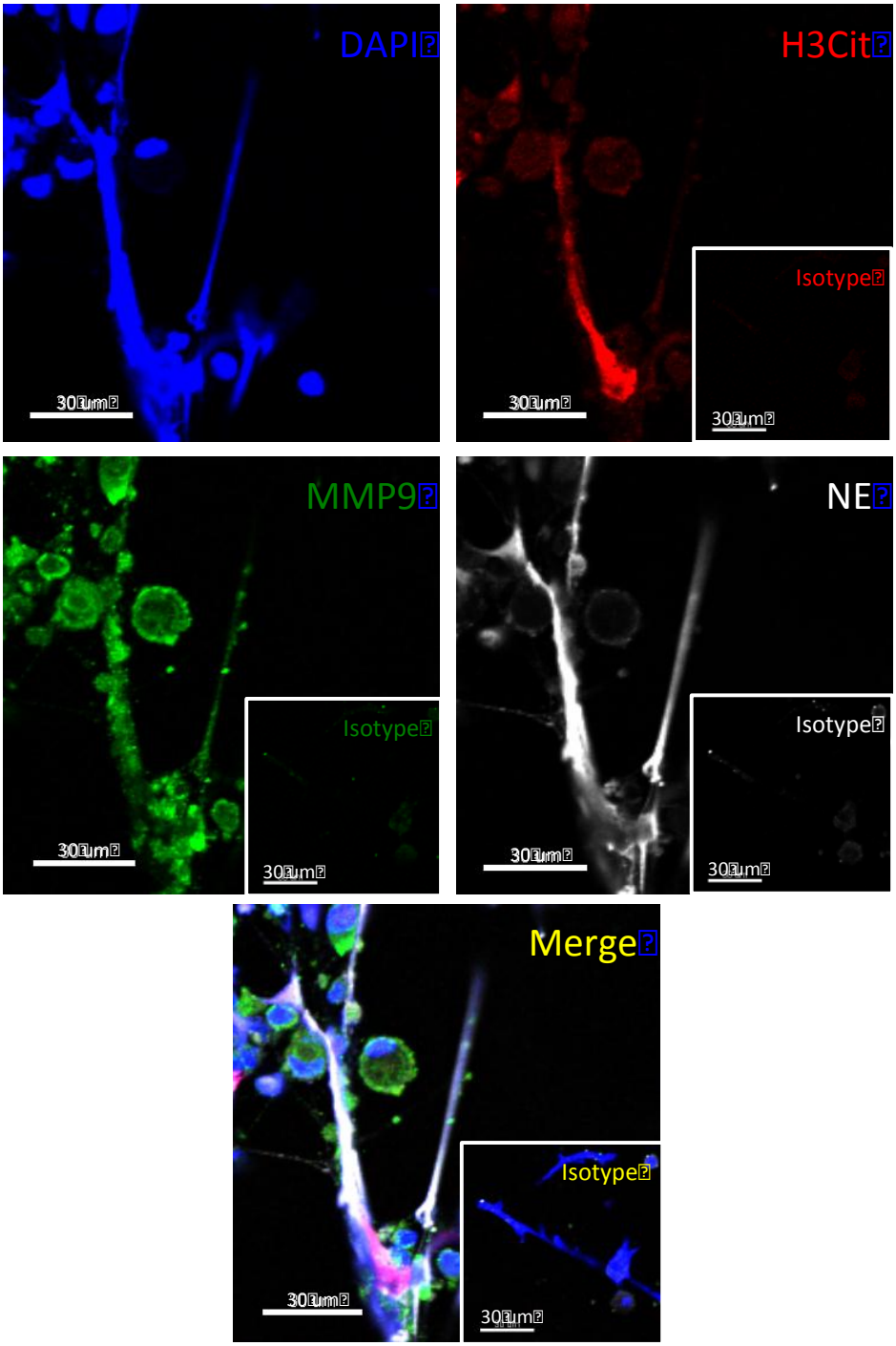
in CF subjects (there were not enough cells to do this in the non-CF subjects). Results are shown in Figure S9.

Of the 23 CF subjects who had NET expression measured by confocal microscopy, 9 subjects were receiving dornase alfa and 14 were not (and this was given the previous day). This dornase alfa group of 9 subjects were older than the other group, 5.1 yrs ± 2.8 versus 3.3 yrs ± 2.3 (p=0.13). The % of neutrophils with NETs was similar in the dornase alfa group 24±16% versus non-Pulmozyme group 21±10% (p=0.53). Dornase alfa is rapidly metabolised in the lung (one study found an 80% reduction in sputum concentration after two hours)[8], and it's half life in serum is reported to be 3-4 hours [9], so it is difficult to draw any conclusions from this data.

We also assessed potentail associations between other NETosis related markers including CFTR status, pathogens and CF medication but were not able to find any definitive associations. This may have been due to the numbers being too small.

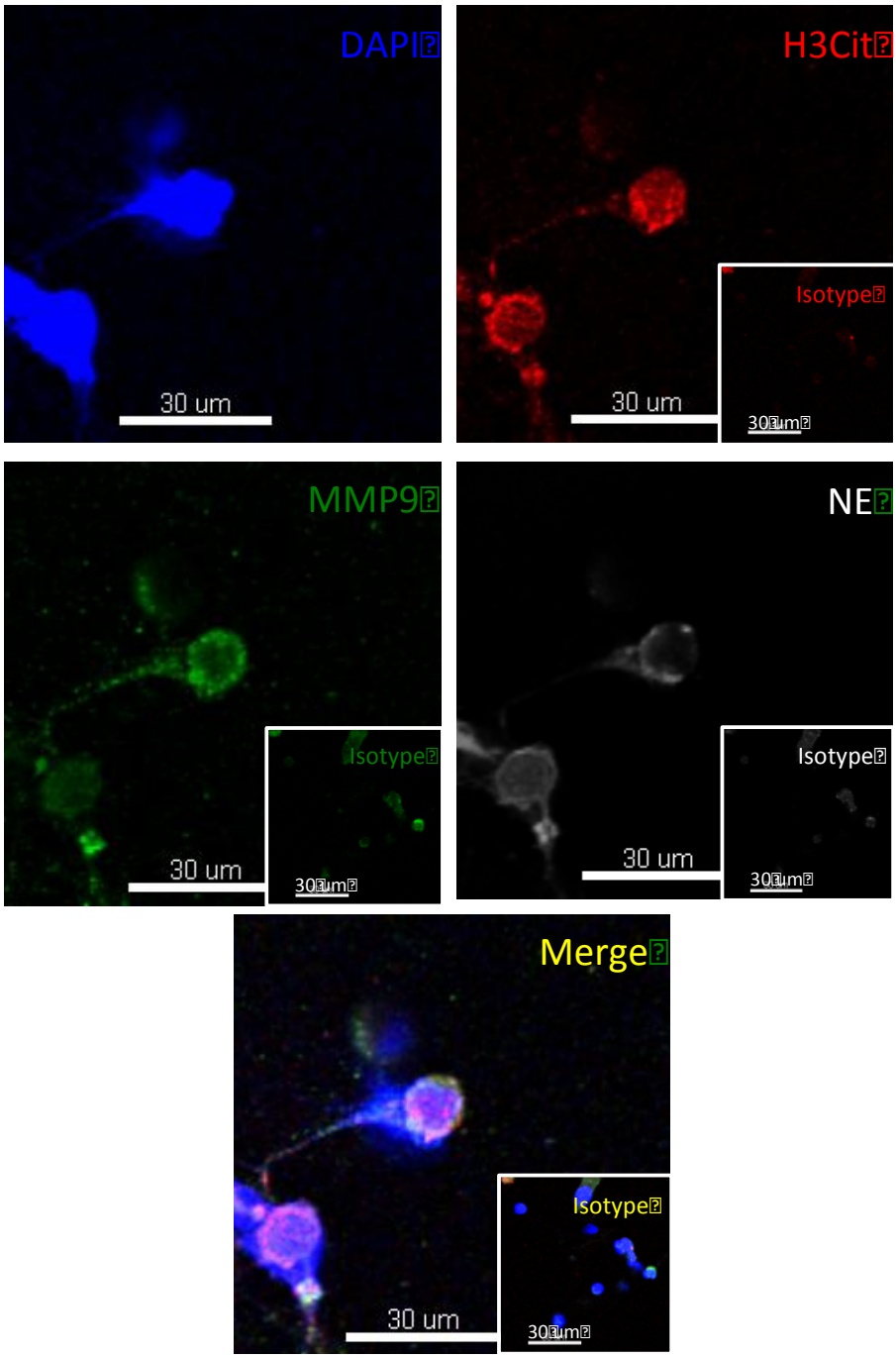
## Figure S1: NET expression

Staining for NET formation is demonstrated in Figure S1. Panel A shows staining for chromatin (DAPI), B staining for citrullinated histone (H3Cit), C staining for MMP9, D staining for NE and E shows the merged image. Insert is isotype control.



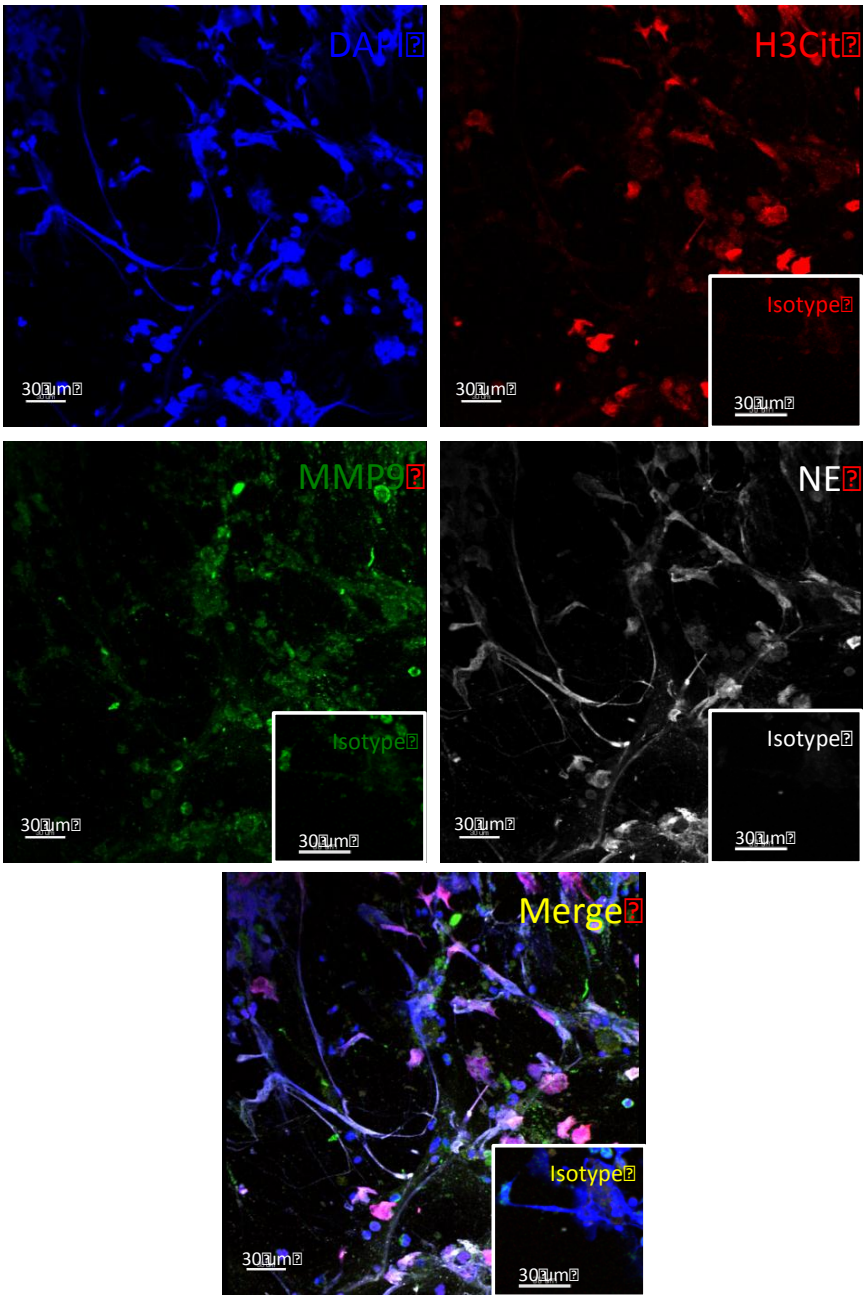
## Figure S2: MET expression

Staining for MET formation is shown in Figure S2. Panel A shows staining for chromatin (DAPI), B staining for H3Cit, C staining for MMP9, D staining for NE and E shows the merged image. Insert is isotype control.



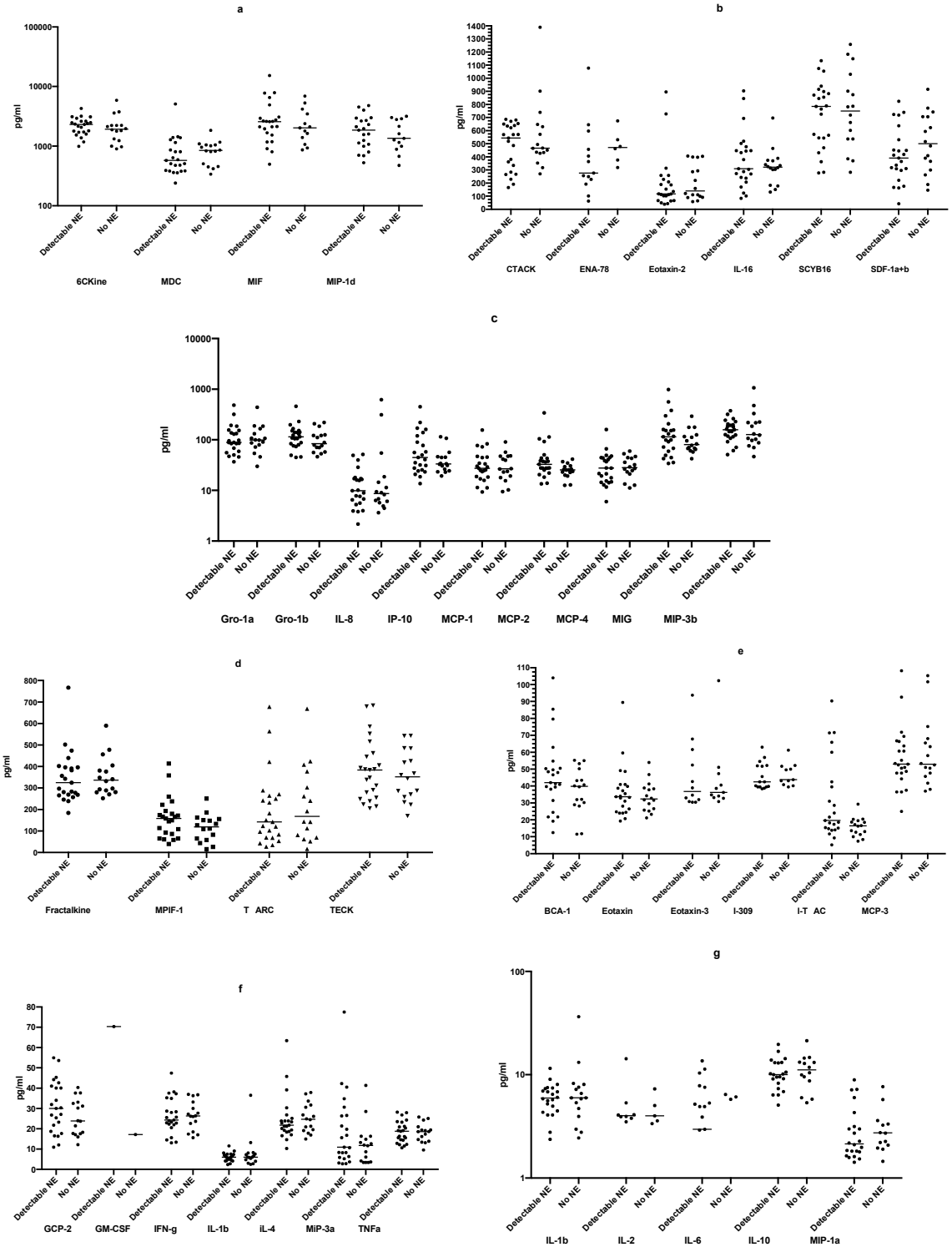
## Figure S3: Extracellular trap expression (low power)

Staining for NET/MET formation is shown using a lower magnification field in Figure S3. Panel A shows staining for chromatin (DAPI), B staining for MMP9, C staining for H3Cit, D staining for NE and E shows the merged image. Insert is isotype control.



# Figure S4: Serum inflammatory mediators and NE levels

A bioplex assay was used to assess if there was a relationship between detectable NE levels. There was no significant association in any of the 40 mediators measured.



## Figure S5: Serum IL-6 Levels

Levels of IL-6 were measured in serum by ELISA in the CF and non-CF groups.

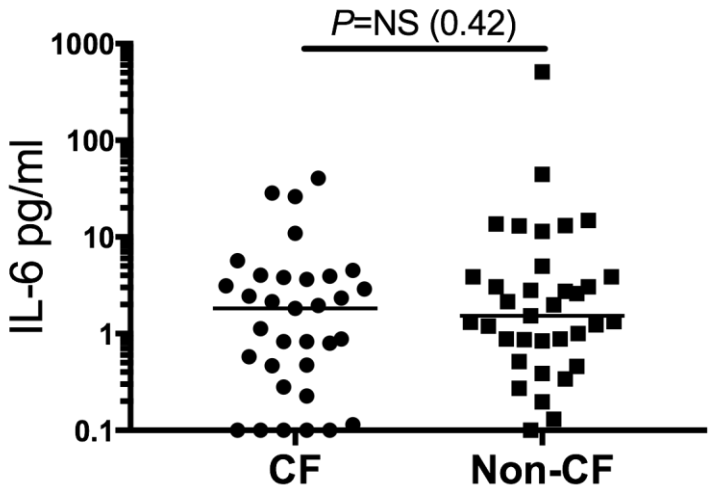
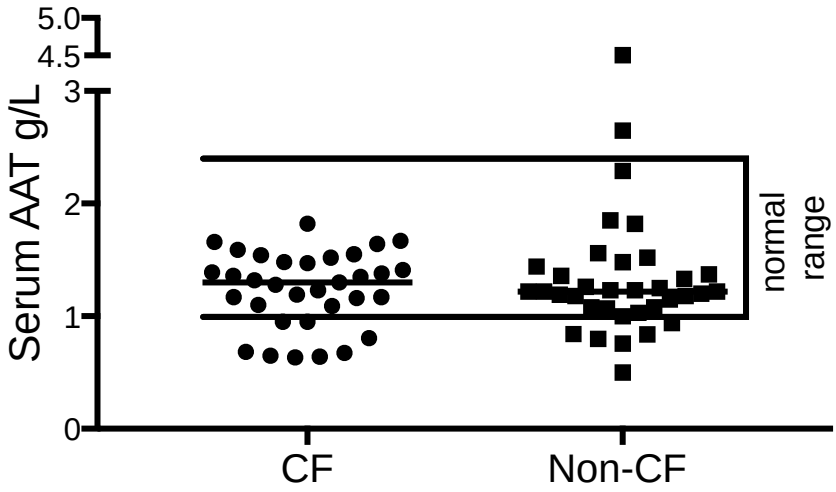


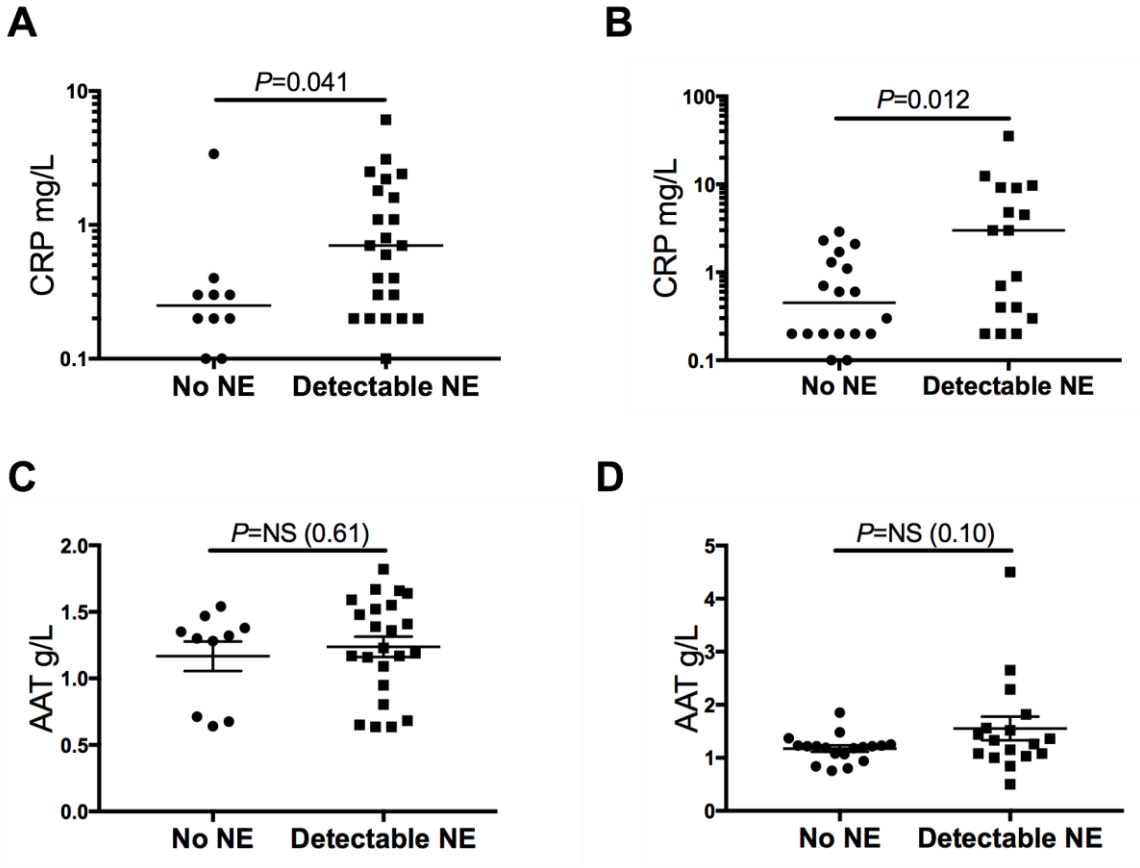
**Figure S6: Serum AAT Levels**

Serum levels of AAT were measured in the CF and non-CF groups



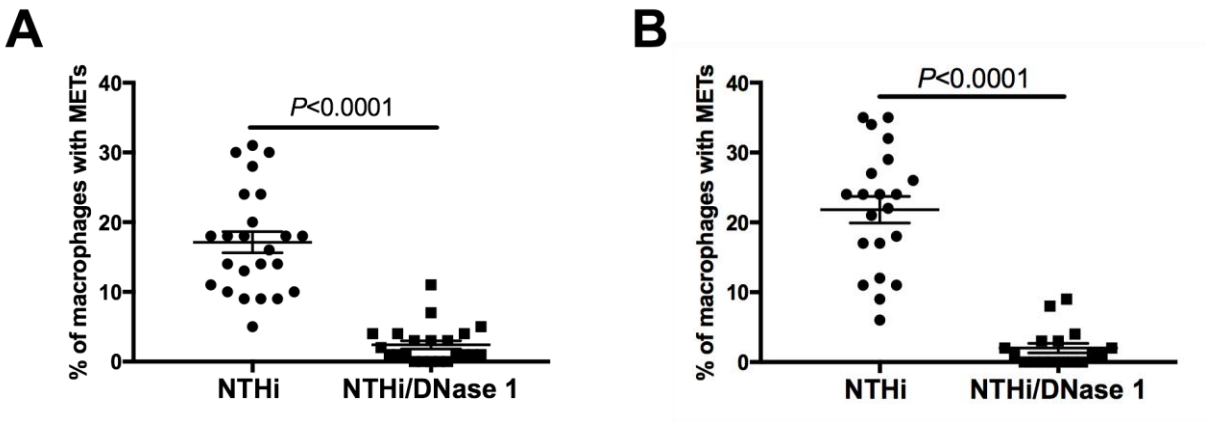
## Figure S7: Serum CRP/AAT levels and NE

Levels of CRP and AAT (measured by nephelometry) in the CF and non-CF groups; results were compared between subjects with undetectable and detectable neutrophil elastase (NE). Levels of CRP in CF (A) and non-CF groups (B). Levels of AAT in CF (C) and non-CF groups (D).



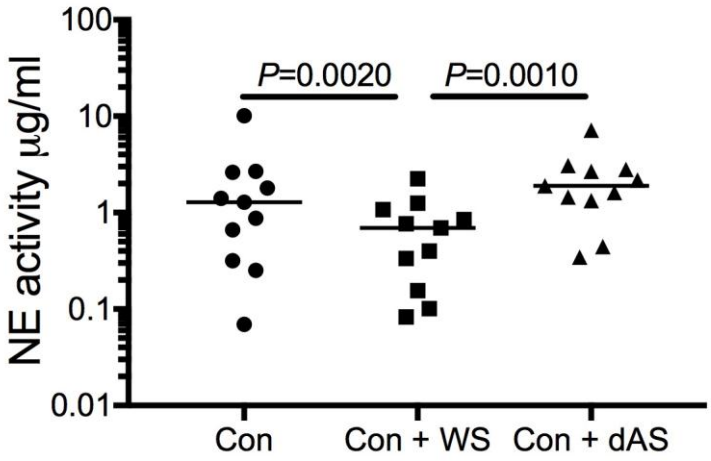
## Figure S8: Effect of DNase 1 on MET expression

The effect of DNase 1 on the MET expression of macrophages stimulated with the bacterium NTHi was assessed. Results expressed as % of macrophages expresssing METs. MET expression in CF (A) and non-CF groups (B).



## Figure S9: Extra AAT experiments

The effect of whole serum (WS) on NE activity was assessed as was the effect of WS that had been depleted of AAT (dAS). The results showed that WS decreased NE activity when compared to control, but dAS had increased NE activity when compated to WS. Results assessed in CF subjects



# Table S1: CF Group Data

| ID | Age | Sex | Dornase alfa | BAL pathogen | CFTR mutation | | % neutrophils | NET Activity | MET/NET |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Mutation 1 | Mutation 2 | | | |
| 1 | 8.0 | M | Y | *Staphylococcus aureus, Aspergillus fumigatus* | deltaF508 | R533X | 52 | Y | Y* |
| 2 | 8.6 | F | Y | *Streptococcus pneumoniae, S. aureus, Penicillium sp.* | deltaF508 | deltaF508 | 23 | Y | Y |
| 3 | 7.2 | M | Y | N | deltaF508 | deltaF508 | 8 | N | Y |
| 4 | 1.0 | M | N | *Haemophilus influenzae, S. aureus* | deltaF508 | G551D | 68 | Y | Y |
| 5 | 4.0 | M | N | *Stenotrophomonas maltophilia* | deltaF508 | deltaF508 | 27 | N | Y |
| 6 | 5.4 | F | N | *Pseudomonas aureginosa, Aspergillus sp.* | deltaF508 | deltaF508 | 60 | Y | Y |
| 7 | 9.2 | M | Y | Rhinovirus (RV) | deltaF508 | deltaF508 | 83 | Y | Y |
| 8 | 3.6 | M | Y | *Moraxella catarrhalis* | deltaF508 | deltaF508 | 1 | N | Y |
| 9 | 6.4 | M | N | *A. fumigatus* | deltaF508 | deltaF508 | 23 | Y | Y |
| 10 | 1.4 | F | Y | *H. parainfluenzae* | c.1521_1523 | c.1521_1523 (delCTT) | 11 | N | Y |
| 11 | 8.4 | M | N | *S. aureus* | deltaF508 | deltaF508 | 26 | Y | Y |
| 12 | 1.7 | F | N | *Sten maltophilia* | C.1521_1523 | (delCTT)/C.1521_1523 (de | 86 | Y | Y |
| 13 | 1.4 | M | N | *Haemophilus sp.* | deltaF508 | deltaF508 | 14 | N | Y |
| 14 | 5.2 | M | N | *H. parainfluenzae, Escherichia coli, Penicillium sp.* | deltaF508 | deltaF508 | 10 | N | Y |
| 15 | 4.3 | F | N | *H. parainfluenzae, H. influenzae* | deltaF508 | deltaF508 | 21 | Y | Y |
| 16 | 4.1 | F | Y | *H. parainfluenzae, H. parahaemolyticus* | deltaF508 | deltaF508 | 32 | N | Y |
| 17 | 4.1 | F | N | N | p.G551D | p.R117H 5T | 5 | N | Y |
| 18 | 6.2 | F | Y | *S. aureus* | deltaF508 | deltaF508 | 55 | Y | Y |
| 19 | 2.1 | M | N | *Escherichia. coli, S. aureus* , Influenza Virus A (IAV) | deltaF508 | deltaF508 | 17 | N | Y |
| 20 | 6.0 | F | Y | *H. parainfluenzae* | deltaF508 | deltaF508 | 16 | Y | Y |
| 21 | 1.0 | F | N | N | deltaF508 | deltaF508 | 21 | N | Y |
| 22 | 4.0 | F | Y | *S. pneumoniae, M. catarrhalis, H. influenzae* | deltaF508 | deltaF508 | 53 | Y | Y |
| 23 | 1.0 | M | N | *Enterobacter cloacae, H. parainfluenzae, Candida sp.* | deltaF508 | deltaF508 | 20 | Y | Y |
| 24 | 1.0 | M | Y | N | deltaF508 | deltaF508 | 9 | N | Y |
| 25 | 5.9 | M | N | *S. aureus, H. Parainfluenzae, Sten maltophilia* | deltaF508 | p.G551D | 47 | Y | N |
| 26 | 1.9 | M | N | IAV, *Ent. cloacae, H. parainfluenzae, Klebsiella sp.* | deltaF508 | deltaF508 | 18 | Y | N |
| 27 | 3.9 | F | Y | *S. aureus* , IAV | p.Arg1158Ter | p.Arg1158Ter | 56 | Y | N |
| 28 | 1.9 | F | Y | *H. parainfluenzae, E. coli* | c.1521_1523 | c.1521_1523 (delCTT) | 5 | N | N |
| 29 | 3.9 | M | N | *H. parainfluenzae, S. aureus* | p.R553X | p.Phe508del | 22 | Y | N |
| 30 | 6.0 | M | Y | *P. aeruginosa, S. aureus, H. influenzae, Sten. maltophilia* | deltaF508 | deltaF508 | 60 | Y | N |
| 31 | 0.9 | M | N | N | deltaF508 | c.1521_1523 [delCTT];1646 | 4 | N | N |
| 32 | 4.0 | M | N | *H. influenzae* | 2_2657 | 3insA | 41 | N | N |
| 33 | 4.8 | F | N | RV | deltaF508 | deltaF508 | 21 | Y | N |
| 34 | 0.9 | M | N | N | deltaF508 | deltaF508 | 5 | N | N |
| 35 | 5.0 | F | N | *Haemophilus haemolyticus, H. influenza* | deltaF508 | deltaF508 | 11 | Y | N |
| 36 | 4.7 | M | Y | N | deltaF508 | deltaF508 | 23 | Y | N |
| 37 | 4.0 | M | Y | *S.aurus, Candida sp, Sten.maltophilia* | deltaF508 | deltaF508 | 9 | Y | N |
| 38 | 6.0 | M | Y | *H. influenzae* , RV | p.Phe508del | p.Pro99Ala probable path | 80 | Y | N |

Age in years. % neutrophils refers to neutrophil % in the BAL. For MET/NET the * in subject 1 means that only METs were measured. N=no, and Y=yes.

# Table S2: Non-CF Group Data

| ID | Age | Sex | BAL pathogen | % neutrophils | NET Activity | MET/NET |
|---|---|---|---|---|---|---|
| | | | | | | |
| 1 | 1.7 | M | N | 5 | N | Y* |
| 2 | 2.7 | M | *M. catarrhalis* | 20 | N | Y* |
| 3 | 1.5 | M | *S. pneumoniae* | 82 | Y | Y |
| 4 | 5.2 | M | *S. pneumoniae* , RV, RSV | 21 | N | Y |
| 5 | 3.1 | M | *S. pneumoniae*, *Staph. aureus* , RV, | 44 | Y | Y |
| 6 | 1.7 | M | N | 1 | Y | Y |
| 7 | 4.3 | M | *H. influenzae* | 13 | Y | Y |
| 8 | 7.3 | M | N | 27 | Y | Y |
| 9 | 1.5 | M | N | 36 | Y | Y |
| 10 | 1.3 | M | N | 20 | N | Y |
| 11 | 2.4 | F | RV | 2 | N | Y |
| 12 | 3.7 | M | *H. influenzae* , Adenovirus | 13 | N | Y |
| 13 | 6.5 | M | N | 12 | Y | Y |
| 14 | 4.0 | M | N | 48 | Y | Y |
| 15 | 2.2 | M | *S. aureus* | 25 | Y | Y |
| 16 | 4.3 | M | RV | 82 | N | Y |
| 17 | 4.8 | M | N | 72 | Y | Y |
| 18 | 4.6 | F | N | 43 | N | Y |
| 19 | 1.6 | F | *S. pneumoniae* | 26 | Y | Y |
| 20 | 3.0 | M | Parainfluenza virus | 94 | Y | Y |
| 21 | 7.4 | F | *H. influenzae*, *S. pneumoniae* | 94 | Y | Y |
| 22 | 9.0 | M | N | 14 | N | Y |
| 23 | 11.1 | F | N | 8 | Y | N |
| 24 | 14.8 | F | *M. catarrhalis* | 31 | N | N |
| 25 | 7.3 | F | *M. catarrhalis* | 12 | N | N |
| 26 | 7.8 | M | N | 35 | N | N |
| 27 | 1.9 | M | *M. catarrhalis* /adenovirus | 44 | N | N |
| 28 | 4.6 | F | *H. influenzae* | 37 | Y | N |
| 29 | 1.3 | M | *H. influenzae* , Adenovirus | 31 | Y | N |
| 30 | 1.1 | m | *M.catarrhalis*, *H. influenzae* , RV | 6 | N | N |
| 31 | 1.7 | F | RV, Adenovirus | 11 | N | N |
| 32 | 3.7 | M | N | 10 | N | N |
| 33 | 9.6 | M | *M. catarrhalis* | 17 | N | N |
| 34 | 2.1 | f | *H. influenzae* | 8 | N | N |
| 35 | 11.7 | F | *S. aureus* | 35 | N | N |
| 36 | 5.9 | F | *S. aureus*, *H. parainfluenzae* | 3 | N | N |
| 37 | 3.6 | F | *H.influenzae* | 2 | N | N |
| 38 | 2.3 | M | *H.influenzae* | 24 | Y | N |

Age in years. % neutrophils refers to neutrophil % in the BAL. For MET/NET the * in subjects 1 and 2 means that only METs were measured. N=no, and Y=yes.

# Table S3: Adult control group

| Characteristic | Study group |
|---|---|
| | No definable lung disease |
| Number of subjects | 21 |
| Age (mean ± SD) | 58 ± 12 |
| Sex | |
| Male | 9 |
| Female | 12 |
| Significant smoking history | |
| Numbers of patients | 2 |
| Subjects with pathogens in BAL (%) | 0/21 (0) |
| Subjects with detectable BAL NE (%) | 0/21 (0) |
| Subjects with > !0% neutrophils in BAL | 0/21 (0) |
| Differential cell count (% of cells) | |
| Macrophages | |
| Median | 91 |
| Interquartile range | 79-94 |
| Lymphocytes | |
| Median | 9 |
| Interquartile range | 5-16 |
| Neutrophils | |
| Median | 1 |
| Interquartile range | 1-5 |
| Eosinophils | |
| Median | 0 |
| Interquartile range | 0-1 |
| Spirometry | |
| $FEV_1$ (% of predicted value) | |
| Median | 105 |
| Interquartile range | 97-105 |
| FVC (% of predicted value) | |
| Median | 107 |
| Interquartile range | 98-118 |
| $FEV_1$/FVC | |
| Median | 80 |
| Interquartile range | 75-83 |

SD = standard deviation, $FEV_1$ = forced expiratory volume in one second, FVC = forced vital capacity

## Table S4: Antibodies used

| Primary Antibody | Isotype control | Concentration | Secondary Antibody |
|---|---|---|---|
| Rabbit anti-human H3Cit (Citrulline R26) (Abcam, UK) | Rabbit IgG (in-house) | 0.02 mg/ml | Chicken anti-rabbit AF 594 (Life technologies, USA) |
| Mouse monoclonal anti-MMP9 (abcam, UK) | Mouse IgG2a (BioLegend, USA) | 0.02mg/ml | chicken anti-mouse AF 488 (Life technologies, USA) |
| Sheep anti-human neutrophil elastase (LSBio, UK, LB-B4244) | Sheep IgG (in-house) | 0.01mg/ml | Donkey anti-sheep AF 647 (Life Technologies, USA) |

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