ORIGINAL ARTICLE

Extracellular DNA, Neutrophil Extracellular Traps, and Inflammasome Activation in Severe Asthma

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Abstract

Rationale: Extracellular DNA (eDNA) and neutrophil extracellular traps (NETs) are implicated in multiple inflammatory diseases. NETs mediate inflammasome activation and IL-1β secretion from monocytes and cause airway epithelial cell injury, but the role of eDNA, NETs, and IL-1β in asthma is uncertain.

Objectives: To characterize the role of activated neutrophils in severe asthma through measurement of NETs and inflammasome activation.

Methods: We measured sputum eDNA in induced sputum from 399 patients with asthma in the Severe Asthma Research Program-3 and in 94 healthy control subjects. We subdivided subjects with asthma into eDNA-low and -high subgroups to compare outcomes of asthma severity and of neutrophil and inflammasome activation. We also examined if NETs cause airway epithelial cell damage that can be prevented by DNase.

Measurements and Main Results: We found that 13% of the Severe Asthma Research Program-3 cohort is "eDNA-high," as defined by sputum eDNA concentrations above the upper 95th percentile value in health. Compared with eDNA-low patients with asthma, eDNA-high patients had lower Asthma Control Test scores, frequent history of chronic mucus hypersecretion, and frequent use of oral corticosteroids for maintenance of asthma control (all P values <0.05). Sputum eDNA in asthma was associated with airway neutrophilic inflammation, increases in soluble NET components, and increases in caspase 1 activity and IL-1β (all P values <0.001). In *in vitro* studies, NETs caused cytotoxicity in airway epithelial cells that was prevented by disruption of NETs with DNase.

Conclusions: High extracellular DNA concentrations in sputum mark a subset of patients with more severe asthma who have NETs and markers of inflammasome activation in their airways.

Keywords: asthma; extracellular DNA; caspase 1; neutrophil extracellular traps; IL-1β

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At a Glance Commentary

Scientific Knowledge on the Subject: Neutrophil extracellular traps (NETs) and their products, such as extracellular DNA, are implicated in multiple inflammatory and autoimmune diseases. NETs mediate inflammasome activation and IL-1β secretion from various cell types, but the role of extracellular DNA, NETs, and IL-1β in asthma is unknown.

What This Study Adds to the Field: A subset of patients with severe asthma have NETs and high extracellular DNA concentrations in their sputum with markers of inflammasome activation in their airways. Activated neutrophils and inflammasome activation in these patients were associated with disease morbidity: poorer asthma control, history of chronic bronchitis, and more frequent oral steroid use.

Although sputum neutrophils are associated with more severe subtypes of asthma (1), the role of neutrophils in asthma remains unclear. Recent studies have advanced understanding by drawing attention to the possibility of proinflammatory roles for extracellular DNA (eDNA) derived from neutrophils (2, 3) and the association between airway neutrophilia and airway inflammasome activation (4, 5). Neutrophil-derived DNA is released in chromatin filaments that form weblike structures decorated with granular proteins called neutrophil extracellular traps (NETs), and NETs are established as mediators of a wide range of immune, inflammatory, and metabolic diseases (6). The inflammasome is an intracellular molecular scaffold that supports autocatalytic cleavage of caspase-1 to drive processing and secretion of the proinflammatory cytokines IL-1β and IL-18 (7). IL-1β is a prototypic proinflammatory cytokine (8, 9) and a plausible mediator of acute and chronic airway inflammation.

To explore the role of eDNA, NETs, and the inflammasome in severe forms of asthma, we measured multiple outcomes related to neutrophils, neutrophil activation, eDNA, and the inflammasome in sputum from patients in the Severe Asthma Research Program (SARP)-3. We explored the relationship between sputum eDNA and asthma control outcomes, including outcomes related to mucus symptoms. In cell culture studies, we also explored the effect of NETs on airway epithelial cells (AECs). Some of the results of the eDNA studies were previously presented in abstract form (10).

Methods

Subjects

SARP-3 is a 3-year longitudinal cohort study in which 60% of subjects have severe asthma (11). Severe asthma was defined according to the European Respiratory Society/American Thoracic Society consensus definition (12). Data reported in the present study are from 399 adult participants with asthma who provided a sample of induced sputum that passed sputum quality assurance measures during two baseline visits. Healthy control subjects included 35 subjects recruited by SARP-3 and 59 healthy subjects recruited at the University of California, San Francisco (UCSF). Patients with chronic obstructive pulmonary disease were excluded from the study through exclusion of current tobacco smokers and those with significant past smoking history (>5 pack-years if they were <30 yr of age and >10 pack-years if they were >30 yr of age) and requirement for a positive methacholine challenge test or demonstration of bronchodilator reversibility to albuterol of at least 12%.

UCSF healthy subjects. Fifty-nine healthy control subjects had been recruited to research studies between 2005 and 2014. They had no history of pulmonary disease, atopic disease, or allergic rhinitis, and they had normal methacholine responses.

SARP-3 healthy subjects. Thirty-five healthy control subjects had no history of pulmonary disease, atopic disease, or allergic rhinitis, and they had normal methacholine responses.

Chronic Mucus Hypersecretion

This was defined using the American Thoracic Society/World Health Organization definition of chronic bronchitis, which assesses chronic cough and sputum production in the preceding 2 years (13). The specific question was, Have you had cough and sputum production on most days for at least 3 months per year for at least 2 consecutive years?

Induced Sputum

Induced sputum (entire expectorate) was homogenized using methods previously described (14, 15) and centrifuged to yield a supernatant that was aliquoted for storage at −80°C. Each center shipped two aliquots to the UCSF SARP-3 center for measurement of analytes.

Total and Differential Cell Counts

Cytocentrifuged sputum cells were stained with Diff-Quik (Siemens Healthcare LTD) and shipped from each SARP-3 center to the sputum cytology core (Wake Forest University), where the sputum cells were counted by light microscopy.

eDNA

eDNA was measured by first diluting the sputum supernatant sample 1:10 in 1× Tris–ethylenediaminetetraacetic acid buffer. Next, double-stranded DNA (dsDNA) was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific).

Gene Transcripts for IL-4, IL-5, and IL-13

The combination of the gene expression levels of IL-4, IL-5, and IL-13 in a single quantitative metric ("Th2 gene mean") measures airway type 2 inflammation, and we recently reported the T-helper cell type 2 (Th2) gene mean data in SARP-3 (16).

Myeloperoxidase

Induced sputum supernatant was diluted 1:2,500 in 1× Tris–ethylenediaminetetraacetic acid buffer, and myeloperoxidase (MPO) was quantified using the Human Myeloperoxidase DuoSet ELISA (DY3174; R&D Systems).

Soluble NET Components

Induced sputum supernatant was diluted 1:10 in 1% bovine serum albumin in phosphate-buffered saline (PBS) to measure neutrophil elastase (NE) and citrullinated histone (H3Cit) that are complexed to DNA using immunoassays previously described (17). As capture antibodies for sputum supernatant, we used the C-17 antibody (sc-9520; Santa Cruz Biotechnology) for the NE–DNA complexes and ab5103 antibody (Abcam) for H3Cit–DNA complexes. An

anti–DNA-horseradish peroxidase conjugate (Cell Death Detection ELISAPLUS Kit; Roche) was used for the detection antibody. Luminescence at 450 nm was read using OptiBlaze ELISA femto-solution (G-Biosciences).

Caspase-1 Activity

Caspase-1 was measured by diluting induced sputum supernatant 1:4 in PBS and incubating the sample 1:1 with Caspase-Glo 1 buffer and Z-WEHD-aminoluciferin substrate using the Caspase-Glo 1 Inflammasome Assay (G9951; Promega) (18).

IL-1β

Sputum was diluted 1:5 in RD6-10 calibration diluent, and IL-1β was quantified using the Human IL-1β/IL-1F2 QuantiGlo ELISA Kit (QLB00B; R&D Systems).

IL-18

Sputum was diluted 1:5 in RD1N calibration diluent, and IL-18 was quantified using the Human Total IL-18/IL-1F4 Quantikine ELISA Kit (DL180; R&D Systems).

Western Blots

Sputum supernatant (10 μl) was denatured and run in 4–10% Bis-Tris gels in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer on the NuPAGE system (Life Technologies). Proteins were transferred to polyvinylidene fluoride membranes using iBlot2 (Life Technologies) and blocked for 2 hours at room temperature in 5% nonfat dry milk in Tris-buffered saline mixed with Tween 20 (TBS-T). Membranes were incubated overnight in primary antibody H3Cit (H3 ab5103; Abcam) and with the Inflammasome Antibody Sampler Kit (32961; Cell Signaling Technology), including capsase-1 D7F10, caspase-1 (asp297), IL-1β (D3 UE3), and IL-1β (asp116) at a concentration of 1:1,000 in TBS-T. Membranes were incubated the following day with goat antirabbit horseradish peroxidase 1:500 (The Jackson Laboratory) for 2 hours at room temperature in TBS-T and developed using ECL Prime (GE Healthcare Life Sciences). Membranes were viewed via chemiluminescence on an ImageQuant LAS 4010 imager (GE Healthcare Life Sciences) and sized using MagicMark (Life Technologies) in ImageQuant software.

NETs

Neutrophils were isolated from 40 ml of blood from each of five healthy donors by layering the blood over Axis-Shield Polymorphprep density gradient media (Cosmo Bio USA) and centrifuging. Neutrophils were resuspended in RPMI media with 3% fetal calf serum at a density of 5×10^6 cells per milliliter. Neutrophils were stimulated with phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) at a concentration of 250 ng/ml until NETs were visualized (19).

Stimulation of AECs with NETs

Human bronchial epithelial cells were harvested from cadavers as previously described (20). Cells were plated on rat tail collagen–coated flasks (Corning) and expanded in Rho-associated protein kinase media (21). Cells were plated on human placenta collagen–coated (Sigma-Aldrich) 3460 or 3470 Transwell inserts (Thermo Fisher Scientific) submerged in Chu media (22) until cells formed confluence. After cells reached confluence on inserts (3–7 d), medium was changed to PneumaCult-ALI Medium (STEMCELL Technologies) and maintained at air–liquid interface for a minimum of 3 weeks before experimentation. The apical surface of the cells was exposed to either vehicle control (PBS) or NET solution at a DNA-NET equivalent concentration of 6 μg/ml of DNA for 24 hours. In some experiments, the NETs were disrupted by coincubation for 24 hours with bovine pancreas DN25 (Sigma Aldrich) at a concentration of 10 Kunitz units per 6 micrograms of DNA-NETs.

Glucose-6-Phosphate Dehydrogenase

Glucose-6-Phosphate Dehydrogenase (G6PD) is a cytosolic enzyme and leaks

Table 1. Characteristics of Healthy Subjects and Subjects with Asthma

	Healthy UCSF (*n* = 59)	Healthy SARP (*n* = 35)	Asthma SARP (*n* = 399)
Age, yr*	37.7 ± 12.7	40.1 ± 13.0	47.6 ± 13.9
Female sex, *n* (%)	36 (61.0)	18 (51.0)	263 (65.9)
Body mass index*†, kg/m^2	25.4 ± 5.7	27.1 ± 5.1	32.7 ± 8.6
Sputum cell counts, %			
Eosinophils*†	0 (0–2.2)	0.4 (0–0.8)	0.8 (0.2–3.0)
Neutrophils	44 (28–64)	62 (35–78)	51 (34–74)
Macrophages†	36 (28–50)	25 (13–50)	28 (13–43)
Blood counts, ×10^6/L			
Eosinophils*†	130 ± 106	143.7 ± 79.8	295 ± 279
Neutrophils*†	3,387 ± 1,080	3,269 ± 1,044	4,511 ± 2,149
Serum IgE, IU/ml*†	19 (10–49)	42 (15.6–99.4)	153 (48–363)
$F_{E_{NO}}$, ppb*†	16 (11–21)	16 (11–24)	22 (13–38)
Pack-years smoking history	—	—	0.88 ± 2.13

Definition of abbreviations: SARP = Severe Asthma Research Program; UCSF = University of California, San Francisco.
Data are reported as mean ± SD or median (interquartile range) unless otherwise indicated. Blood counts were not available for two healthy UCSF subjects, five healthy SARP subjects, and one subject with asthma. Serum IgE was not available for two healthy UCSF subjects, five healthy SARP subjects, and two subjects with asthma. $F_{E_{NO}}$ was not measured in 11 healthy UCSF subjects, 3 healthy SARP subjects, and 3 subjects with asthma.
*$P < 0.05$ for comparison between healthy UCSF and asthma SARP.
†$P < 0.01$ for comparison between healthy SARP and asthma SARP.

from cells when plasma membrane integrity is compromised (23). We measured G6PD in the basolateral conditioned media using the Vybrant Cytotoxicity Assay Kit (V23111; Thermo Fisher Scientific).

Gene Transcripts for IL-6 and IL-8 in AECs

cDNA was generated from AEC RNA (20 ng) as previously described (24). Measurement of genes associated with inflammation, remodeling, and inflammasome was done via quantitative PCR using TaqMan Universal PCR Mix (Thermo Fisher Scientific) on a ViiA 7 real-time PCR system and normalized to housekeeping genes as previously described (24, 25). Primer sequences are provided in Table E1 in the online supplement.

IL-6 and IL-8 from Conditioned Media

Measurement of IL-6 and IL-8 was performed in 50 μl of basolateral conditioned media or blank cell culture media using the IL-6 and IL-8 Human ProcartaPlex Simplex Kits, Platinum (EPXP010-10213-901 and EPXP010-10204-901; Thermo Fisher Scientific) according to the manufacturer's directions. Samples were read at the UCSF Parnassus Flow Cytometry Core using a Bio-Plex 200 system, and data were generated in Bio-Plex 4.1 software (Bio-Rad Laboratories).

Statistical Methods

Analyses were performed using the JMP 10 (SAS Institute) and Stata 13.1 (StataCorp) software packages. Two-group comparisons between eDNA-high and eDNA-low asthma were made using Student's *t* test for continuous variables with roughly symmetric distributions, Wilcoxon's rank-sum test for continuous variables with skewed distributions, and Pearson's chi-square test for categorical variables. Spearman's correlation was used to assess the relationships between continuous variables. Figures were generated using Prism 7.0 statistical software (GraphPad Software). Box-and-whisker plots were prepared showing the median (marked by a horizontal line), first and third quartiles (box), and extreme

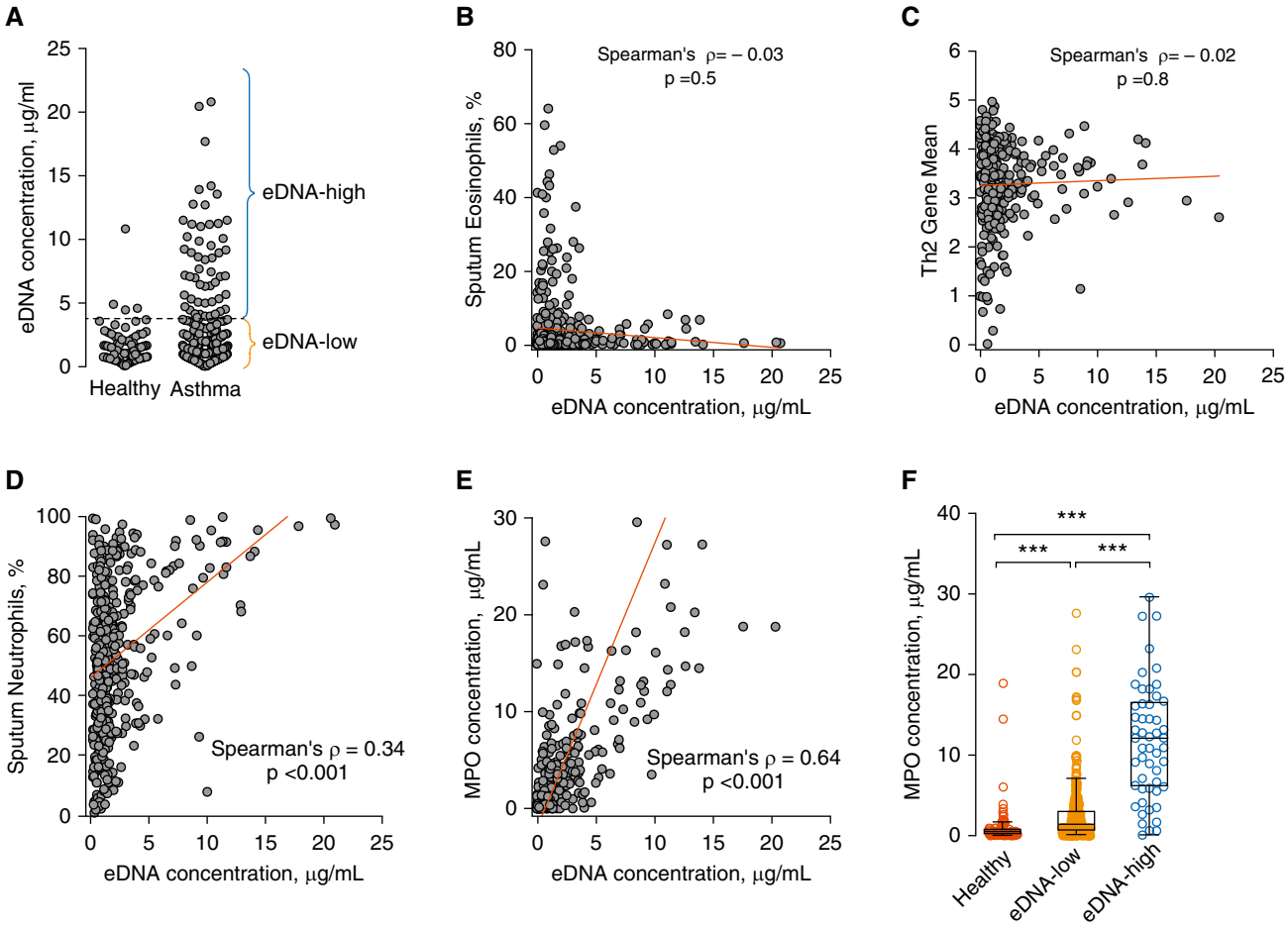

Figure 1. Increased extracellular DNA (eDNA) in sputum from a subset of patients with asthma is associated with increased neutrophil numbers and neutrophil activation. (*A*) A subset of patients with asthma has sputum eDNA concentrations above the 95th percentile value in healthy control subjects. (*B*) Sputum eDNA concentrations are not significantly correlated with sputum eosinophils. (*C*) Sputum eDNA concentrations are not significantly correlated with the T-helper cell type 2 (Th2) gene mean. (*D*) Sputum eDNA concentrations are significantly correlated with sputum neutrophils. (*E*) Sputum eDNA concentrations are significantly correlated with myeloperoxidase (MPO) concentrations. (*F*) Sputum MPO concentrations are significantly higher in eDNA-high asthma than in eDNA-low asthma. ***$P < 0.001$. Circles represent individual data points.

Table 2. Characteristics of Subjects with Asthma, Stratified by DNA Concentration

	All (*n* = 399)	eDNA-Low (*n* = 346)	eDNA-High (*n* = 53)
Age, yr*	47.6 ± 13.9	46.6 ± 13.8	54.0 ± 12.3
Female sex, *n* (%)†	263 (65.9)	238 (68.8)	25 (47.2)
Body mass index, kg/m^2	32.7 ± 8.6	32.4 ± 8.3	34.3 ± 10.2
Maintenance corticosteroid use, *n* (%)			
Inhaled, any dose	355 (89.0)	358 (89.5)	44 (95.7)
Inhaled, high dose	246 (61.7)	245 (61.3)	32 (69.6)
Systemic†	66 (16.5)	52 (15)	14 (26.4)
Severe asthma, *n* (%)	246 (61.7)	211 (61.0)	35 (66.0)
Exacerbations in last 12 mo, *n* (%)			
ER visits in last 12 mo	94 (23.6)	84 (24.3)	10 (18.9)
Hospitalizations in last 12 mo	43 (10.8)	38 (11.0)	5 (9.4)
Exacerbation prone†	94 (23.7)	73 (21.1)	21 (39.6)
Spirometry			
FEV_1% of predicted volume	72.4 ± 19.2	72.9 ± 19.2	68.7 ± 19.3
FVC% of predicted volume*	84.7 ± 16.5	85.7 ± 16.6	78.2 ± 14.4
FEV_1/FVC	0.84 ± 0.12	0.84 ± 0.12	0.86 ± 0.14
FE_{NO}, ppb	22 (13–38)	22 (14–38)	20 (13–38)
Blood			
Neutrophil count, $\times 10^6$/L‡	4,511 ± 2,149	4,397 ± 1,970	5,248 ± 2,990
Eosinophil count, $\times 10^6$/L	295 ± 279	299 ± 292	268 ± 180
IgE, IU/ml	153 (48–363)	154 (50–368)	129 (39–320)
Sputum			
Neutrophil count, $\times 10^6$/L*	476 (199–1,240)	407 (173–921)	1,553 (867–5,200)
Neutrophils, %*	52.1 (34–74)	49.5 (32–68)	79.4 (56–90)
Eosinophil count, $\times 10^6$/L‡	7 (0.6–49)	6 (0.3–41)	30 (2–62)
Eosinophils, %	0.8 (0.2–3.0)	0.8 (0.2–3.1)	0.7 (0.2–2.6)
Macrophage count, $\times 10^6$/L‡	244 (113–529)	231 (109–480)	384 (152–873)
Macrophages, %*	27.6 (13–43)	29.2 (15–45)	14.5 (5–30)
Pack-years smoking history	0.88 ± 2.13	0.85 ± 2.02	1.07 ± 2.75

Definition of abbreviations: eDNA = extracellular DNA; ER = emergency room; FE_{NO} = fractional exhaled nitric oxide.
Data are reported as mean ± SD or median (interquartile range) unless otherwise indicated. Exacerbations were defined as taking a short course of oral corticosteroids for asthma (minimum, 3 d). Exacerbation prone was defined as three or more exacerbations in the last 12 months. Exacerbation data were missing for three DNA-low patients. FE_{NO} measurements were missing for two DNA-low patients and one DNA-high patient. Blood counts were missing for one DNA-low patient. Serum IgE measurements were missing for one DNA-low patient and one DNA-high patient.
*$P < 0.001$ for comparison between DNA-low and DNA-high groups.
†$P < 0.01$ for comparison between DNA-low and DNA-high groups.
‡$P < 0.05$ for comparison between DNA-low and DNA-high groups.

values as far as 1.5× interquartile range beyond the limits of the box (whiskers). Data points farther than 1.5× interquartile range beyond the limits of the box are plotted as outliers. Tests were considered statistically significant with *P* values represented as $P < 0.05$, $P < 0.005$, and $P < 0.0001$.

Results

Increased eDNA in Sputum from a Subset of Patients with Asthma Reflects Neutrophil Activation

The patients with asthma were older and heavier than the healthy control subjects (Table 1). To calculate a reference interval for sputum supernatant eDNA in health, we followed the guidelines of the national committee for clinical laboratory standards (26). First, we logarithmically transformed sputum eDNA values in healthy subjects to normalize the distribution and to determine the upper 95th percentile value as the upper limit of normal (Figure 1A). This approach yielded 3.8 μg/ml as the cutoff value. We found that 13% of the patients with asthma had sputum eDNA concentrations above 3.8 μg/ml, and we classified this asthma subgroup as "eDNA-high." We classified the remaining patients with asthma with sputum eDNA below 3.8 μg/ml as "eDNA-low."

We considered the most likely cellular source of DNA in the sputum to be neutrophils because the neutrophil is the most abundant granulocyte in sputum (27). Indeed, among patients with asthma, we found no significant correlation between sputum eDNA and sputum eosinophils (Figure 1B) or between sputum eDNA and other measures of airway type 2 inflammation, such as the sputum Th2 gene mean (28) (Figure 1C). In contrast, sputum eDNA was significantly and positively correlated with sputum neutrophil percentage (Figure 1D), although the correlation was only moderate in strength. We reasoned that we would find a stronger relationship if we examined a marker of neutrophil activation. To explore the relationship between eDNA and neutrophil activation in sputum, we measured MPO concentrations. MPO is a peroxidase enzyme abundantly expressed by neutrophils and secreted upon neutrophil activation by a wide variety of stimuli (29). We found that sputum MPO concentrations strongly correlated with sputum eDNA concentrations (Figure 1E) and that sputum MPO concentrations were significantly higher in eDNA-high patients than in eDNA-low patients (Figure 1F). Taken together, these data indicate that activated neutrophils are the cellular source of eDNA in the asthma airway.

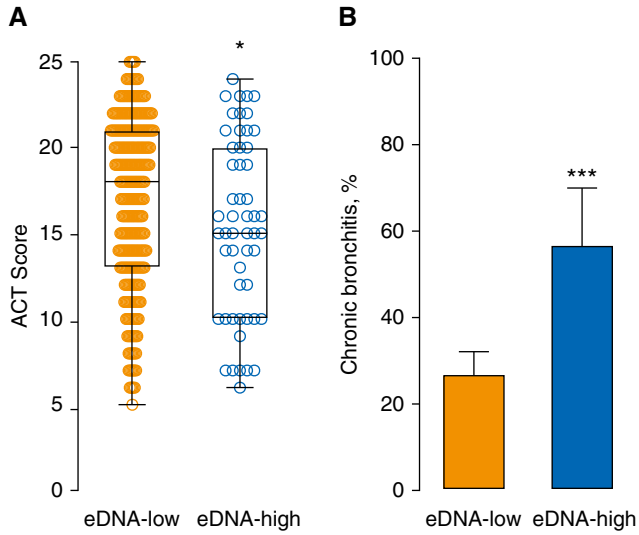

Figure 2. Extracellular DNA (eDNA)-high asthma is associated with poor asthma control and symptoms of chronic mucus hypersecretion but not with airway mucus plugging. (*A*) The Asthma Control Test (ACT) score is significantly lower in eDNA-high asthma than in eDNA-low asthma. (*B*) Chronic mucus hypersecretion (also called "chronic bronchitis") is more prevalent in eDNA-high asthma than in eDNA-low asthma. Chronic mucus hypersecretion data were available for 297 DNA-low patients and 40 DNA-high patients. $^{*}P < 0.05$ and $^{***}P < 0.001$. Circles represent individual data points.

eDNA-High Asthma Is Characterized by Multiple Clinical Indicators of Asthma Severity

We explored differences in the clinical features of the eDNA-high and -low asthma subgroups. Compared with eDNA-low asthma, we found that the demographic features of eDNA-high asthma were notable for older age and male predominance (Table 2). In comparing the clinical features of eDNA-high and -low patients, we noted that the eDNA-high subgroup had significantly lower Asthma Control Test scores (Table 2 and Figure 2A) and that the majority of eDNA-high patients had symptoms of chronic mucus hypersecretion (Table 2 and Figure 2B). We also found that FVC was worse in eDNA-high asthma and that the frequency of daily oral corticosteroid use in the eDNA-high subgroup was almost twice that in the eDNA-low subgroup (Table 2). Although the proportion of patients with three or more exacerbations in the previous year (i.e., exacerbation-prone patients) was higher in the eDNA-high subgroup than in the eDNA-low subgroup, the frequency of hospitalizations or emergency department visits for asthma in the prior year was not higher in the eDNA-high subgroup (Table 2). Regression analyses showed that age and sex did not significantly confound these relationships between sputum eDNA concentrations and clinical outcomes (Table E2).

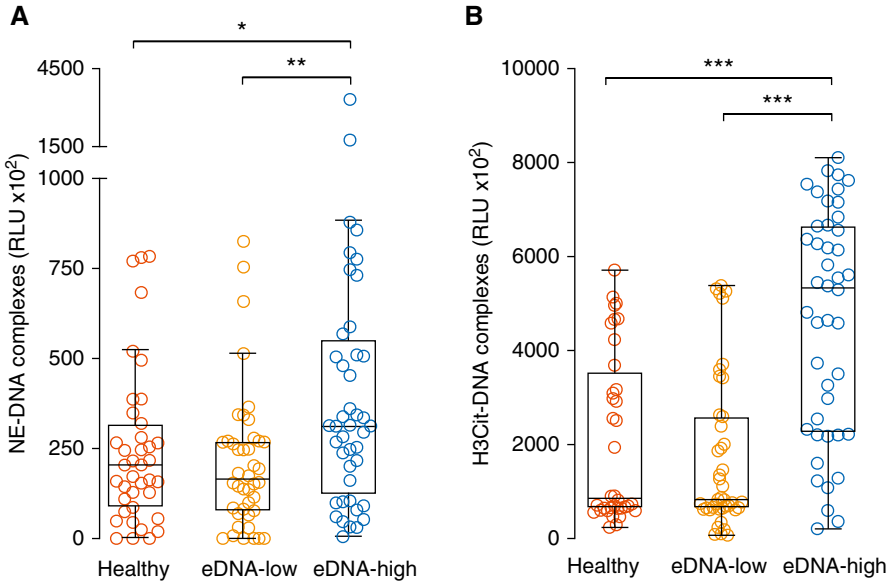

Figure 3. Soluble neutrophil extracellular trap complexes are higher in extracellular DNA (eDNA)-high asthma than in eDNA-low asthma. (*A*) Neutrophil elastase (NE)–DNA complexes are significantly higher in eDNA-high asthma than in eDNA-low asthma. (*B*) Citrullinated histone H3 (H3Cit)–DNA complexes are significantly higher in eDNA-high asthma than in eDNA-low asthma. Healthy, $n = 35$; eDNA-low, $n = 42$; eDNA-high, $n = 44$. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Circles represent individual data points. RLU = relative luminometer units.

Soluble NET Components Are Increased in the eDNA-High Asthma Subgroup

We next returned to analyses of sputum and to measures of NETs. To quantify NETs, we measured NE–DNA and H3Cit–DNA complexes using ELISAs recently described (17). For these studies, we analyzed sputum from 44 eDNA-high patients, 42 randomly selected eDNA-low patients, and the 35 SARP healthy control subjects. We found that both NE–DNA and H3Cit–DNA complexes are increased in eDNA-high patients but not in eDNA-low patients (Figures 3A and 3B).

Increased Caspase-1 Activity and IL-1β in Sputum in eDNA-High Asthma

To explore if caspase-1 activity is increased in sputum from eDNA-high patients, we assayed for caspase-1 using immunoblots and a recently described bioluminescence assay (18). Caspases are inactive zymogens, and caspase maturation into active caspase involves homodimerization and proteolytic processing to remove the proregion and split the large subunit from the small subunit (9). Using immunoblots, we found that increased full-length and activated caspase-1 proteins (p20 and p10 bands, as indicated) were increased in eDNA-high patients (Figure 4A). Using the bioluminescence activity assay, we found

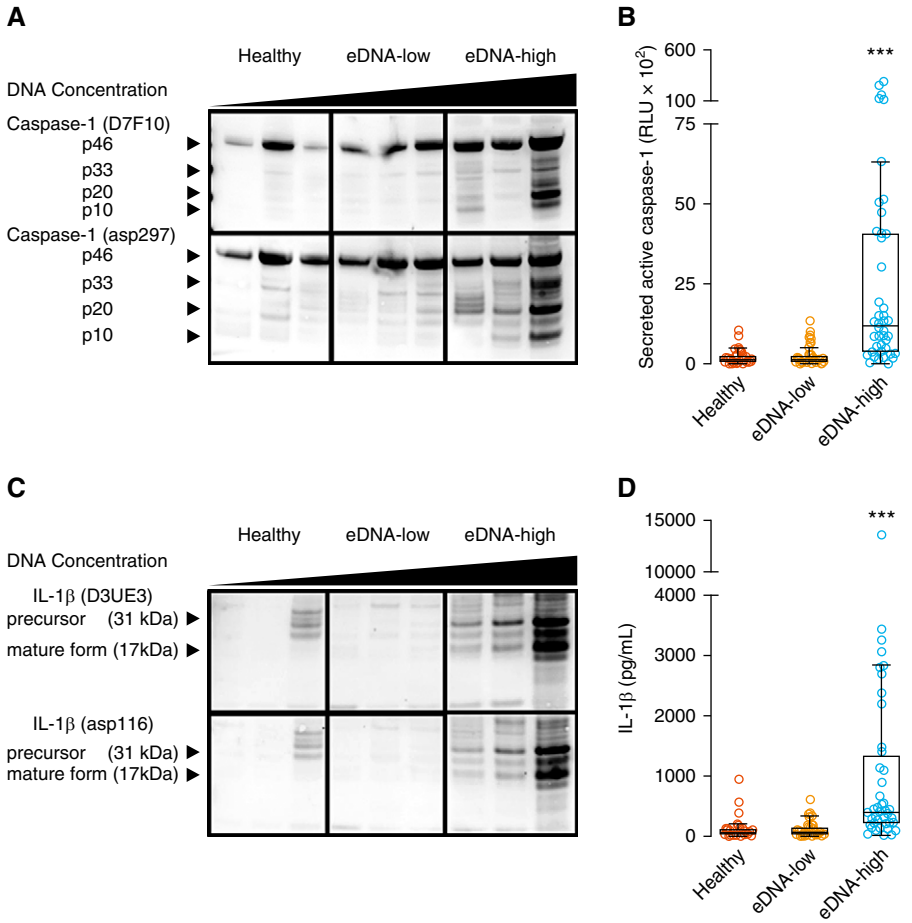

Figure 4. Caspase-1 activity and IL-1β concentrations are higher in extracellular DNA (eDNA)-high asthma than in eDNA-low asthma. (*A*) Western blot data for caspase 1 in sputum from three healthy control subjects, three patients with eDNA-low asthma, and three patients with eDNA-high asthma. The bands show caspase 1 zymogen and cleavage at Asp297 yielding the activated protease bands p20 and p10. (*B*) Bioluminescence data for caspase 1 in sputum showing significantly higher concentrations in eDNA-high asthma ($n = 44$) than in eDNA-low asthma ($n = 42$) or healthy control subjects ($n = 35$). (*C*) Western blot data for IL-1β in sputum from three healthy control subjects, three patients with eDNA-low asthma, and three patients with eDNA-high asthma. The data show cleavage of IL-1β at Asp116 in eDNA asthma. (*D*) ELISA data for IL-1β in sputum showing significantly higher concentrations in eDNA-high asthma ($n = 44$) than eDNA-low asthma ($n = 42$) or healthy control subjects ($n = 35$). ***$P < 0.001$. Circles represent individual data points. RLU = relative luminometer units.

very low caspase activity in sputum from healthy control subjects ($n = 35$) and eDNA-low patients ($n = 42$) but increased caspase activity in eDNA-high patients ($n = 44$) (Figure 4B). To determine how much of the caspase activity detected in the bioluminescence assay reflects caspase-1, we measured caspase activity in four sputum samples in the presence of Ac-YVAD-CHO, a specific inhibitor of caspase-1. We found that Ac-YVAD-CHO decreased the caspase signal by more than 60% (Figure E1), indicating that caspase-1 is the dominant source of the bioluminescent signal but that some other caspases also contribute.

Increased IL-1β in Sputum in eDNA-High Asthma

Because caspase-1 activity cleaves proforms of IL-1β to active mature forms, we looked for IL-1β in the sputum from eDNA-high patients. Using immunoblots to probe sputum supernatant from a limited number of subjects in each subgroup, we found increased IL-1β precursor and a cleaved mature form of IL-1β in eDNA-high patients (Figure 4C). We also measured IL-1β using a high-sensitivity ELISA. For these studies, we analyzed sputum from the 44 eDNA-high patients, 42 selected eDNA-low patients, and 35 SARP healthy control subjects. We found very low IL-1β concentrations in sputum from healthy control subjects and eDNA-low patients, but we easily detected markedly increased IL-1β protein in eDNA-high patients (Figure 4D). We noted that the concentration of IL-1β in sputum varied widely in the asthma subgroup, so we compared clinical variables among patients with asthma with sputum IL-1β concentrations above and below the median split value. We found that the IL-1β–high asthma subgroup had more severe asthma than the IL-1β–low subgroup. Specifically, compared with the IL-1β–low asthma subgroup, the IL-1β–high asthma subgroup had a higher frequency of chronic bronchitis (29.6% vs. 61.4%; $P = 0.009$), a higher frequency of being exacerbation prone (21.2% vs. 46.9%; $P = 0.02$), a lower FEV_1 percent predicted (69.0% vs. 85.0%; $P < 0.001$), and a lower FVC percent predicted (78.0% vs. 94.0%; $P < 0.001$).

Low Concentrations of IL-18 in Sputum in eDNA-High Asthma

IL-18 is a member of the IL-1 superfamily, and, like IL-1β, it is activated by caspase-1 in the inflammasome. In a subset of sputum samples from patients from the UCSF SARP-3 center, we assayed for IL-18 in sputum from healthy ($n = 14$), eDNA-low ($n = 18$), and eDNA-high ($n = 19$) subjects. We found that the concentrations of IL-18 were a log order lower than the concentrations of sputum IL-1β and were not significantly different in the eDNA-high and -low patients (Figure E2). Accordingly, we did not extend these IL-18 analyses to the full sputum samples set of eDNA-high and -low patients.

NETs Cause Toxicity in AECs

In considering the pathogenic role of NETs in neutrophilic asthma, we hypothesized that NETs could produce airway injury by activating AECs. To explore this possibility, we first generated NETs by activating neutrophils from freshly collected whole blood (five healthy donors) with PMA. We then exposed AECs in air–liquid interface culture to NETs and found that they induced upregulation of IL-6 and IL-8 mRNA as well as secretion of these

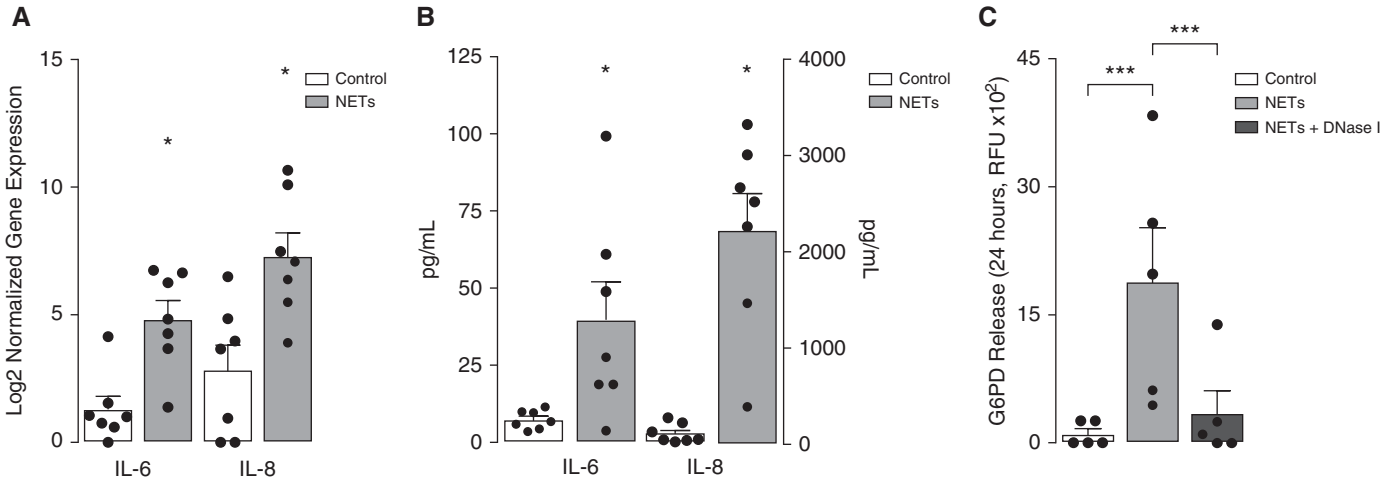

Figure 5. Neutrophil extracellular trap (NET)-mediated injury to airway epithelial cells (AECs). (*A*) Apical exposure of AECs grown at air–liquid interface (ALI) to NETs (6 μg/ml) for 24 hours causes upregulation of gene expression for IL-6 and IL-8 (data are from epithelial cells from seven donors). (*B*) Apical exposure of AECs grown at ALI to NETs (6 μg/ml) for 24 hours causes release of IL-6 and IL-8 protein into the conditioned media (data are from epithelial cells from seven donors). (*C*) Glucose-6-phosphate dehydrogenase (G6PD) release into conditioned media of AECs in ALI culture after exposure to NETs (6 μg/ml) at the apical surface for 24 hours in the presence or absence of DNase I cotreatment (data are from epithelial cells from five donors). Error bars represent SEM. $^{*}P < 0.05$ and $^{***}P < 0.0001$. Circles represent individual data points. RFU = relative fluorescence units.

cytokines into the basolateral media (Figures 5A and 5B). We also noted a large increase in G6PD release (Figure 5C). To control for the possible effect on AECs of PMA in the NET preparations, in preliminary experiments, we included PMA as a control and found that it had no effect on G6PD release from epithelial cells (data not shown). We further found that the NET structure is required for this effect, because disrupting NETs with DNase (DNase I) largely prevented the NET-induced G6PD release (Figure 5C).

Discussion

We report that approximately 13% of the SARP-3 cohort had increased concentrations of eDNA in their sputum. We found strong evidence that this eDNA originates from neutrophils, including significant positive correlations between eDNA concentrations and sputum neutrophil percentages and between eDNA concentrations and MPO concentrations. Thus, eDNA in sputum marks neutrophil activation in the airway, and the relatively small but distinct subgroup of eDNA-high patients with asthma are among the more severe patients in the SARP-3 cohort. Specifically, eDNA-high asthma is characterized by poor asthma control and frequent use of oral corticosteroids as maintenance therapy for asthma control. In addition, more than half of the eDNA-high asthma subgroup reported cough and sputum production in the questionnaire instrument used to capture symptoms of chronic mucus hypersecretion (also called "chronic bronchitis").

Neutrophils can release their extracellular chromatin, nuclear protein, and serine proteases to form netlike structures (i.e., NETs) (6, 30, 31). Soluble NET components such as NE–DNA or H3Cit–DNA complexes can be measured using immunoassays, and we found that NE–DNA and H3Cit–DNA complexes were increased in the sputum of the eDNA-high asthma subgroup. NETs are an important component of host defense against microbial infection, but they are known to cause collateral damage to the lung in pneumonia (17). Indeed, NETs cause AEC death and detachment and induce autoantigen production in AECs (32–35). We report that NETs cause AEC injury (G6PD release) and that NET structure is required for this effect, because disrupting NETs with DNase blocks NET-induced G6PD release. This finding raises the intriguing possibility that inhaled DNase could prevent NET-driven epithelial cell damage in the airway and could have therapeutic benefit in eDNA-high patients.

We found that consistent with previous studies (3), IL-1β concentrations were increased in eDNA-high asthma, and other studies in severe asthma have also reported increased sputum IL-1β (15, 36). IL-1β is cleaved to its secreted cytokine isoform by caspase-1 that is activated in the inflammasome (7). In the present study, we show that caspase-1 concentrations are increased in eDNA-high patients and thus provide direct evidence for inflammasome activation in at least a subset of patients with asthma that is marked by high sputum concentrations of eDNA. Taken together, our data suggest that a subset of patients with severe asthma have neutrophil activation in their airways, including NETs, and that NETs may activate the inflammasome in resident cells such as monocytes or macrophages to cause secretion of IL-1β (37). The reasons for neutrophil activation and inflammasome activation in eDNA-high asthma are not revealed by our study, but airway infection should be considered, especially because this inflammation signature (airway neutrophilia, inflammasome activation, and IL-1β responses) is present in a mouse model of infection-mediated steroid-resistant asthma (4). An alternative possibility is that the caspase signal we detected in eDNA-high asthma is indicative of airway pyroptosis that could promote gasdermin D–mediated NETosis (38, 39). Although mitochondrial DNA (mtDNA) is a source of eDNA in NETs, we did not

investigate the role of mtDNA in our study. Viable eosinophils and neutrophils release mtDNA in a reactive oxygen species–dependent manner in the absence of nuclear proteins and independently of cell death (40, 41). The presence of H3Cit suggests that the source of eDNA is from the nucleus. Formation of H3Cit is part of chromatin decondensation in the nucleus during NETosis (42). Given that we observed nuclear proteins and a signature associated with IL-1β/caspase-1 activation in the sputum, our data suggest that the source of eDNA is from the nucleus in activated cells undergoing cell death (43). All participants in SARP-3 were enrolled at a time when they had been free of an airway infection or asthma exacerbation for at least 4 weeks, so it is unlikely that an acute airway infection explains neutrophil activation in their airways. It is possible, however, that these patients have chronic airway infection or microbial dysbiosis (44); our study did not investigate this possibility. However, our findings do suggest novel treatment possibilities for eDNA-high asthma, such as disruption of NETs with inhaled DNase or treatments directed at the inflammasome, including inhibition of IL-1β. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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Supplementary Appendix

Title: Extracellular DNA, Neutrophil Extracellular Traps, and Inflammasome activation in the Airway in Severe asthma

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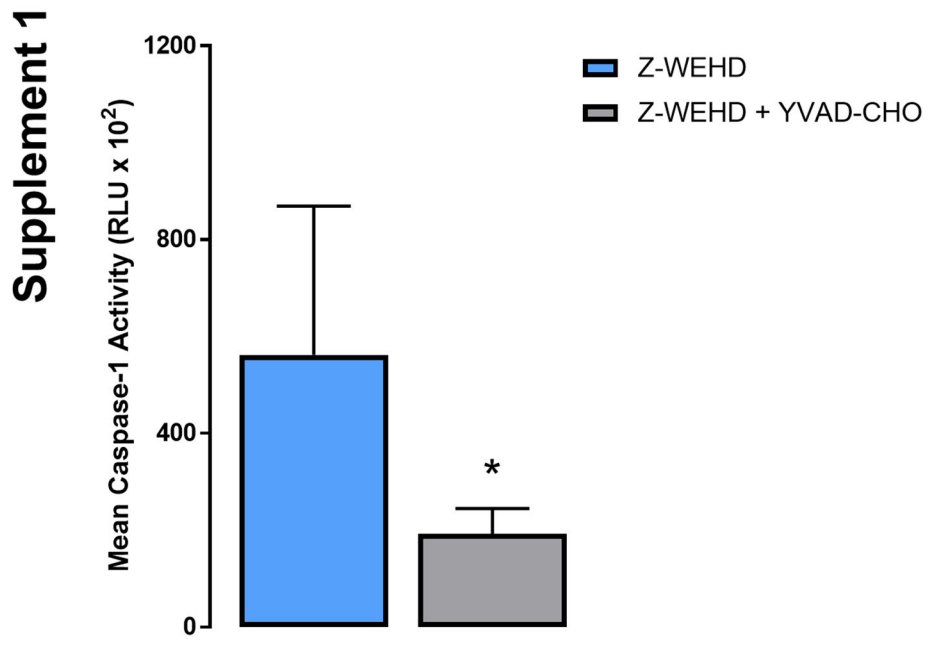
Table E1. Primers for Airway Epithelial TaqMan

Gene name	Primer type	Sequence (5' to 3')
PPIA	Forward RT	ATGAGAACTTCATCCTAAAGCATACG
PPIA	Reverse RT	TTGGCAGTGCAGATGAAAAACT
PPIA	TaqMan Forward	ACGGGTCCTGGCATCTTGT
PPIA	TaqMan Probe	ATGGCAAATGCTGGACCCAACACA-FAM(BHQ)
PPIA	TaqMan Reverse	GCAGATGAAAAACTGGGAACCA
EEF1A1	Forward RT	TGCTAACATGCCTTGGTTCAAG
EEF1A1	Reverse RT	TTGGACGAGTTGGTGGTAGGAT
EEF1A1	TaqMan Forward	CCTTGGTTCAAGGGATGGAA
EEF1A1	TaqMan Probe	CACTGGCATTGCCATCCTTACGGG-FAM(BHQ)
EEF1A1	TaqMan Reverse	GCCTCAAGCAGCGTGGTT
RPL13A	Forward RT	GGACCGTGCGAGGTATGCT
RPL13A	Reverse RT	TTCAGACGCACGACCTTGAG
RPL13A	TaqMan Forward	TATGCTGCCCCACAAAACC
RPL13A	TaqMan Probe	CAGAGCGGCCTGGCCTCGCT-FAM(BHQ)
RPL13A	TaqMan Reverse	TGCCGTCAAACACCTTGAGA
IL6	Forward RT	GGTGTTGCCTGCTGCCTT
IL6	Reverse RT	GTGCCTCTTTGCTGCTTTCAC
IL6	TaqMan Forward	CCCCCAGGAGAAGATTCCA
IL6	TaqMan Probe	AGATGTAGCCGCCCCACACAGACAG-FAM(BHQ)
IL6	TaqMan Reverse	TCAATTCGTTCTGAAGAGGTGAGT
IL8	Forward RT	ACTCCAAACCTTTCCACCCC
IL8	Reverse RT	CATCTTCACTGATTCTTGGATACCAC
IL8	TaqMan Forward	CCACACTGCGCCAACACAGAAATTATTG
IL8	TaqMan Probe	AAGCTTTCTGATGGAAGAGAGCTCTGTC-FAM(BHQ)
IL8	TaqMan Reverse	GCCCTCTTCAAAAACTTCTCCACAACCC

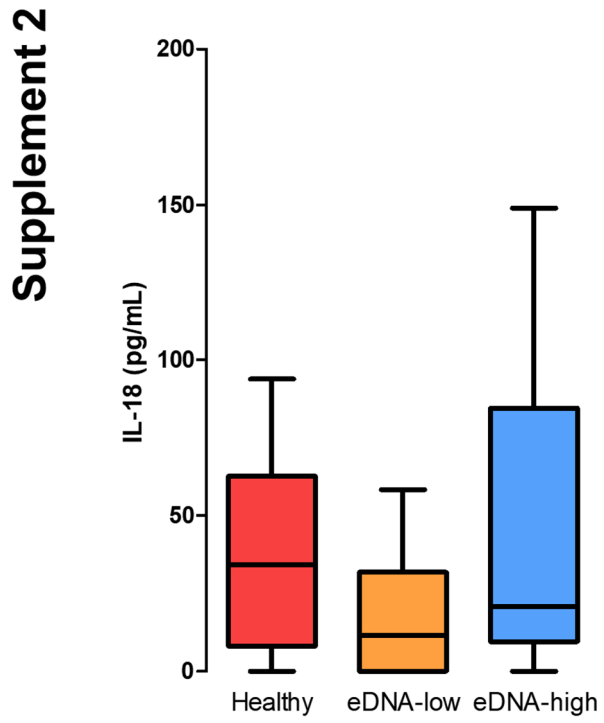
Table E2. Regression models for clinical outcomes in asthma

Outcome variable	Predictor variable	Model 1	Model 2	Model 3	Model 4
Asthma Control Test score	eDNA concentration	-0.21 (-0.36, -0.05) p=0.012	-0.23 (-0.39, -0.07) p=0.006	-0.23 (-0.39, -0.07) p=0.004	-0.26 (-0.43, -0.1) p=0.002
Chronic Mucus Hypersecretion	eDNA concentration	1.19 (1.10, 1.29) p<0.001	1.16 (1.07, 1.26) p<0.001	1.18 (1.09, 1.28) p<0.001	1.16 (1.06, 1.26) p=0.001
FVC, % predicted	eDNA concentration	-1.12 (-1.66, -0.59) p<0.001	-0.80 (-1.32, -0.27) p=0.003	-1.12 (-1.66, -0.57) p=<0.001	-0.77 (-1.31, -0.24) p=0.005
Oral corticosteroid use	eDNA concentration	1.12 (1.04, 1.21) p=0.003	1.11 (1.03, 1.20) p=0.008	1.13 (1.05, 1.21) p=0.002	1.12 (1.03, 1.21) p=0.006
Exacerbation prone	eDNA concentration	1.11 (1.04, 1.19) p=0.003	1.10 (1.03, 1.18) p=0.008	1.12 (1.04, 1.20) p=0.002	1.11 (1.03, 1.19) p=0.005

Asthma Control Test and FVC outcome variables were analysed using linear regression models. Chronic mucus hypersecretion, oral corticosteroid use, and exacerbation prone outcome measures were analysed using logistic regression models. Data are presented as a ß coefficient or odds ratio, with 95% confidence interval and p value.
Model 1 – unadjusted model
Model 2 – model adjusted for age at enrolment
Model 3 – model adjusted for gender
Model 3 – model adjusted for age at enrolment and gender



Supplement Figure E1: Caspase-1 activity in sputum is inhibited by the Ac-YVAD-CHO inhibitor. Ac-YVAD-CHO decreased the caspase bioluminescent signal (n=4). Bars represent S.E.M. *Indicates $p<0.05$



Supplement Figure E2: IL-18 levels in sputum in asthma subgroups. IL-18 levels in sputum are low and not significant different among eDNA-high asthma (n=19), eDNA-low asthma (n=18) or healthy control (n=14) subgroups.