Targeted DNase treatment of obstructive lung disease: a pilot randomised controlled trial

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Short-term recombinant human DNase treatment was able to reduce airway extracellular DNA levels in obstructive airway disease and may represent a novel treatment option for non-T2 related inflammation https://bit.ly/4fW3HAQ

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Abstract

Background Sputum extracellular DNA (eDNA) is associated with disease severity in asthma and COPD and therefore emerging as a potential therapeutic target. The aim of this study was to investigate the effect of 10 days of recombinant human DNase (rhDNase) treatment of eDNA-high asthma and COPD on sputum eDNA levels, neutrophil-related inflammation, lung function and symptoms.

Methods Adults with asthma (n=80) or COPD (n=66) were screened for the presence of high (>20 μg·mL^{-1}) sputum eDNA and those eligible (n=18 asthma, n=17 COPD) were randomised to a two-period crossover controlled trial consisting of daily nebulised rhDNase (2.5 mg/2.5 mL) or placebo (5 mL 0.9% saline) for 10 days, with a 2-week washout period. The primary outcome was sputum eDNA, and secondary outcomes included sputum neutrophil extracellular trap (NET)-related biomarkers, inflammatory cell counts, lung function and respiratory symptoms.

Results At screening, high eDNA was associated with significantly higher sputum total cell count, sputum colour score and inflammation (HNP1-3, LL-37 and interleukin-1β) in both asthma and COPD compared to low eDNA groups. In asthma, participants with high eDNA were older and had poorer lung function and asthma control compared to low eDNA. Administration of nebulised rhDNase significantly reduced sputum eDNA levels in both asthma (median (Q1–Q3) Pre: 48.4 (22.1–74.1); Post: 17.0 (5.0–31.0) μg·mL^{-1}; p=0.022) and COPD (median (Q1–Q3) Pre: 39.3 (36.7–55.6); Post: 25.4 (11.3–38.6) μg·mL^{-1}; p=0.044) compared to placebo. Symptoms, lung function and NET biomarkers remained unchanged. In asthma, there was a reduction in banded blood neutrophils (3.2 (0–7.7) to 0.0 (0.0–1.5); p=0.044).

Conclusion Targeted rhDNase treatment for 10 days effectively reduced sputum eDNA in eDNA-high asthma and COPD.

Introduction

Asthma and COPD are common obstructive airway diseases, affecting millions of people worldwide [1, 2]. Both asthma and COPD negatively impact an individual's health-related quality of life and pose a major burden on the healthcare system. Chronic airway inflammation is a hallmark of obstructive airway diseases and contributes to airway remodelling and airflow limitation [3, 4].

Neutrophils play a major role in the pathogenesis of COPD and severe asthma [5, 6], yet treatments targeting neutrophils directly have not been proven to be efficient in either condition, highlighting the need for alternative treatment approaches.

NETosis, the formation of neutrophilic extracellular traps (NETs), is an efficient mechanism to eliminate pathogens in healthy individuals. Microbes including pathogenic bacteria, viruses and fungi get trapped in the network of the excreted extracellular DNA (eDNA) and are eventually destroyed by neutrophil granule proteins. Excessive NETosis and lack of NET clearance, however, is cytotoxic to the surrounding tissue and has significant implications in chronic airway diseases [7–9]. We have previously demonstrated accumulation of NETs in both COPD and severe asthma, and that sputum eDNA levels negatively correlate with lung function [7]. eDNA increases the viscosity of mucus [10], contributing to the mechanical blocking of airways. Furthermore, it can stimulate innate immune responses, including inflammasome activation and subsequent interleukin (IL)-1β production [11]. Thus, targeting eDNA could potentially be beneficial in asthma and COPD and improve clinical outcomes.

In cystic fibrosis, administration of nebulised recombinant human deoxyribonuclease I/dornase α (rhDNase) mediates mucociliary clearance through the cleavage of eDNA, improving lung function and reducing pulmonary exacerbations [12, 13]. Several well-executed randomised clinical trials in cystic fibrosis have proven the efficacy and safety of daily inhalation of rhDNase, showing that this therapy can slow lung function decline, decrease the frequency of respiratory exacerbations and improve symptoms and general well-being (reviewed in Pressler [14]). Since rhDNase is identical to human DNase, it is well tolerated by most patients with the main side-effects being voice hoarseness, pharyngitis and laryngitis [14]. Treatment of cystic fibrosis patients with rhDNase reduces eDNA load in bronchoalveolar lavage fluid [15]. rhDNase is further used off-label to treat parapneumonic pleural effusion and empyema, and its therapeutic benefit is likely to be attributed to the high level of extracellular DNA in these conditions [16]. Therefore, rhDNase treatment may be beneficial in those with COPD or severe asthma and high levels of eDNA. In asthma, the neutrophil activation marker MPO was found to be strongly correlated with sputum eDNA, suggesting that activated neutrophils are the source of eDNA in the airway [17]. The authors demonstrated in *in vitro* studies that the cytotoxic effect of NETs on airway epithelial cells could be prevented with DNase through the prevention of NET-induced G6PDF release [17]. These findings indicate additional mechanisms and benefits for using rhDNase as a treament.

In the present study we investigated the effect of rhDNase (Pulmozyme) in eDNA-high COPD and asthma. We performed a baseline cross-sectional analysis of high *versus* low eDNA groups in COPD and asthma separately. Those with high eDNA levels were enrolled in a randomised crossover trial. We hypothesised that the administration of nebulised rhDNase reduces eDNA in those with high sputum eDNA levels. Although asthma and COPD share similarities, such as increased NETs in some individuals, they differ in several aspects and manifest as two different diseases. Owing to these underlying differences, the asthma and COPD cohorts were analysed separately.

Material and methods

Study design

We performed a targeted, proof of concept two-period crossover randomised trial in participants with COPD and asthma that had high levels of sputum eDNA. Participants attended a screening visit (V1) where baseline inflammation was assessed and sputum eDNA levels were measured, and eligibility was determined. Eligible participants (>20 $\mu g \cdot mL^{-1}$ eDNA) attended four subsequent visits (V2–5), where they were randomly allocated to nebulised rhDNase treatment (2.5 mg/2.5 mL, 1 ampoule daily) or placebo (5 mL 0.9% nebulised saline daily), for 10 days with a 2-week washout period between treatments (figure 1). The study was conducted at the Hunter Medical Research Institute (HMRI), Australia, and the randomised controlled trial (RCT) was registered on the Australia New Zealand Clinical Trials Register (ACTRN12617000395336). Ethics approval was received from the Human Research Ethics Committees of the Hunter New England Local Health District (15/03/18/3.04) and the University of Newcastle (H-2015-0147). All participants gave written informed consent.

Participants

Participants were recruited from the respiratory ambulatory care clinic service of the John Hunter Hospital (Newcastle, Australia) and through the research databases of John Hunter Hospital's Department of Respiratory and Sleep Medicine, and the HMRI volunteer register or by advertisement. We included adults (>18 years) with stable asthma (doctor diagnosed and evidence of variable airflow limitation in the last 10 years (bronchodilator response ⩾12% or 200 mL change, airway hyperresponsiveness, peak flow variability of >12% over at least 1 week of monitoring or forced expiratory volume in 1 s (FEV_1)

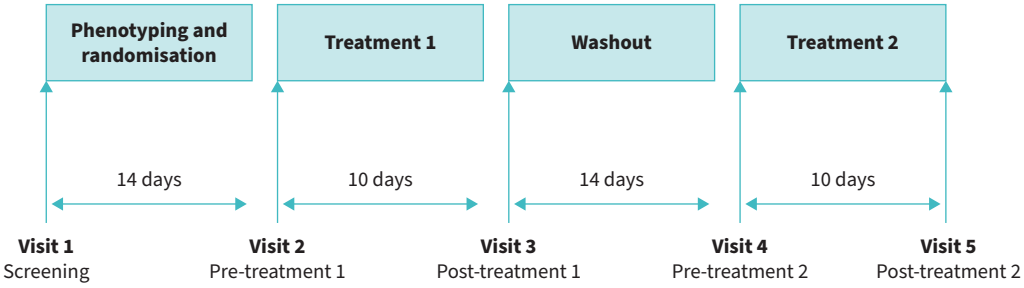

FIGURE 1 Trial schema. Participants attended a screening visit (Visit 1) to assess inflammation and eligibility for the study. Participants who fulfilled the eligibility criteria and sputum NETs (eDNA >20 ng·µL^{-1}) attended a visit 2 weeks later (Visit 2), where they were randomly allocated to either nebulised rhDNase I treatment or placebo, once daily for 10 days. The first treatment sequence was a period of 10 days (Visit 2–3), followed by a 2-week washout period (return for Visit 4) and concluded with the second 10-day treatment sequence (Visit 5). Participants were contacted *via* telephone call midway (Day 5) through each treatment period to assess any adverse events, side-effects of treatment, study treatment adherence, asthma symptoms and any changes in medication. All outcomes were measured at screening visit and at the start and completion of each treatment sequence.

variability of >12% with two values measured within 2 months of each other)) and adults (>18 years) with stable COPD (doctor diagnosed and confirmed evidence of incomplete reversible airflow obstruction (post-bronchodilator FEV_1/forced vital capacity (FVC) <70%) or objective confirmation from chest CT scan or pulmonary function test). Participants were included if they were stable and there was no respiratory infection or exacerbation of their disease or change in therapy within the last month. Participants were excluded if they were unable to attend all study visits, had current malignancy, expected poor prognosis, were currently smoking or pregnant or breastfeeding. Participants were also excluded if they were unable to tolerate nebulisation of 0.9% saline (defined by a drop in lung function >15% during induction on 0.9% saline). For asthma, there was no primary diagnosis of COPD or other respiratory diseases, and for COPD there was no known primary coexisting respiratory disease such as bronchiectasis.

Randomisation

Treatment order was randomly allocated *via* unique computer-generated randomisation codes by an independent statistician. Once a participant was confirmed eligible for the RCT following their screening visit they were randomly allocated to either placebo or active nebulised Pulmozyme treatment for 10 days. Participant and clinician blinding was not possible, as Pulmozyme and placebo ampoules were not identical in appearance, and ampoules were embossed with the drug name.

Intervention

The active treatment consisted of nebulised Pulmozyme (2.5 mg/2.5 mL of recombinant human DNase I (dornase α), Roche), one ampoule once daily for 10 days. The placebo treatment consisted of 5 mL of nebulised saline solution containing 0.9% w/v sodium chloride (Pfizer), one ampoule once daily for 10 days. Participants were provided with a portable nebulising machine (PARI BOY SX compressor in combination with the PARI LC SPRINT Nebuliser, Technipro Pulmomed) to administer the study interventions at home. At the start of the first treatment sequence study participants were shown how to set up the equipment and administer study intervention by research staff. Participants were also given step-by-step written instructions to support the use of their nebuliser at home.

Outcomes

The primary outcome of the RCT was sputum extracellular DNA (eDNA). Secondary outcomes were sputum NETs marker citrullinated histone H3 (CitH3), sputum neutrophil antimicrobial proteins (α-defensins, LL-37), sputum IL-1β, blood and sputum inflammatory cell counts, lung function, health-related quality of life (St. George's Respiratory Questionnaire (SGRQ)) and symptom score (Asthma Control Questionnaire 6 (ACQ6) in asthma; COPD Assessment Test (CAT) in COPD). In our previous study [7], the level of sputum eDNA when log transformed was normally distributed with a standard deviation of 1.97 in asthma and 2.8 in COPD. To detect a change in matched pairs of eDNA equivalent to 1 SD (1.97) we required n=10 subjects to be able to reject the null hypothesis that the response difference is

zero with 80% power and α=0.05. To account for dropouts or failed sample collection we required 15 subjects with asthma. We aimed for the same sample size of participants with COPD.

Clinical assessments

Participants underwent a comprehensive clinical assessment at the screening visit where data regarding demographics, medications, comorbidities and smoking were collected, and were recruited separately to either the asthma or COPD cohort. Participants eligible for the RCT attended a further four visits, pre and post each treatment intervention. At each RCT visit, questionnaires were administered (SGRQ [18], ACQ6 for participants with asthma [19], CAT score for participants with COPD [20], mucus hypersecretion [21], exacerbation history), and spirometry [22] (MedGraphics, CPFS/D and BreezeSuite software, MedGraphics, Saint Paul, MN, USA) and sample collection were performed (sputum and blood). Participants were telephoned midway (Day 5) through each treatment period to assess any adverse events, side-effects of treatment, study treatment adherence, respiratory symptoms and any changes in medication since their last assessment. To monitor adherence to the intervention, participants were asked to complete a medication diary and keep all ampoules (used and unused) and return them to research staff at their treatment completion visits.

Laboratory analysis

Sputum was induced using nebulised 4.5% saline in participants whose FEV_1 was $\geqslant$1 L, using previously described methods [22]. In those with FEV_1 <1 L, 0.9% saline was used. Selected sputum was dispersed using dithiothreitol (DTT), filtered, total cell count performed and cytospins prepared (May–Grünwald–Giemsa stain). A differential cell count was obtained from 400 non-squamous cells, and cell-free supernatant was stored. Sputum colour was assessed by laboratory staff using a 5-point sputum colour chart (BronkoTest; Heredilab Inc, Salt Lake City, UT, USA [23]). Peripheral venous blood was collected into Vacutainer® tubes (BD Worldwide, North Ryde, NSW, Australia) and analysed for full blood counts (CELL-DYN Ruby Haematology analyser, Abott Diagnostics).

To determine the abundance of NETs in the cell-free sputum supernatant, extracellular DNA was quantitated using the Quant-iT PicoGreen dsDNA Assay Kit (P7589, Invitrogen, Carlsbad, CA, USA) as per manufacturer's instructions. LL-37, α-defensins 1–3 (HNP1–3), IL-1β and CitH3 were measured using commercial ELISA assay kits (LL-37 and HNP1-3: Hycult Biotech, Uden, the Netherlands; IL-1β: DuoSet, R&D Systems, Minneapolis, MN, USA; CitH3: Cayman Chemical, Ann Arbor, MI, USA) as per manufacturer's instructions.

Statistical analysis

Data were analysed using STATA 15.1 (StataCorp, College Station, TX, USA) and GraphPad Prism 10 (La Jolla, CA, USA). Normality of the data was assessed using the D'Agostino & Pearson normality test, and the distribution was reviewed with histograms in Stata. Parametric data are reported as mean±SD and non-parametric data are reported as median (quartile 1 (Q1)–quartile 3 (Q3)). Linear mixed effects modelling (LMM) fit by restricted maximum likelihood was used to assess the difference in change in outcomes from baseline between rhDNase intervention and placebo. Intervention and time (treated as a categorical variable with levels at pre- and post-intervention) were specified as fixed effects with an interaction term. Study ID was included in the model to account for repeated measures. Prior to LMM inclusion, some variables were transformed using logarithmic or cubic root transformations. An independent LMM of period totals by intervention sequence was used to test the assumption of no carry over effects. Within-group changes for each intervention compared to pre-intervention were also analysed using Wilcoxon signed rank tests for nonparametric data and paired t-test for parametric data. Significance was set at $p<0.05$.

Results

Study participants

From June 2017 to February 2020, 80 participants with asthma and 66 participants with COPD were assessed for eligibility (figure 2). The trial was ended as the target was reached, though the Covid pandemic also started at this time. 64 participants with asthma and 48 with COPD were enrolled in the cross-sectional study (n=43 low eDNA and n=21 high eDNA in asthma, n=27 low DNA and n=21 high DNA in COPD). Of those with high eDNA, 18 participants with asthma (n=11 female, n=7 male) and 17 with COPD (n=8 female, n=9 male) were randomised. Analysis of the effects of treatment were performed on 16 participants with asthma and 15 participants with COPD who completed the rhDNase intervention and 15 participants with asthma and 15 participants with COPD who completed the placebo.

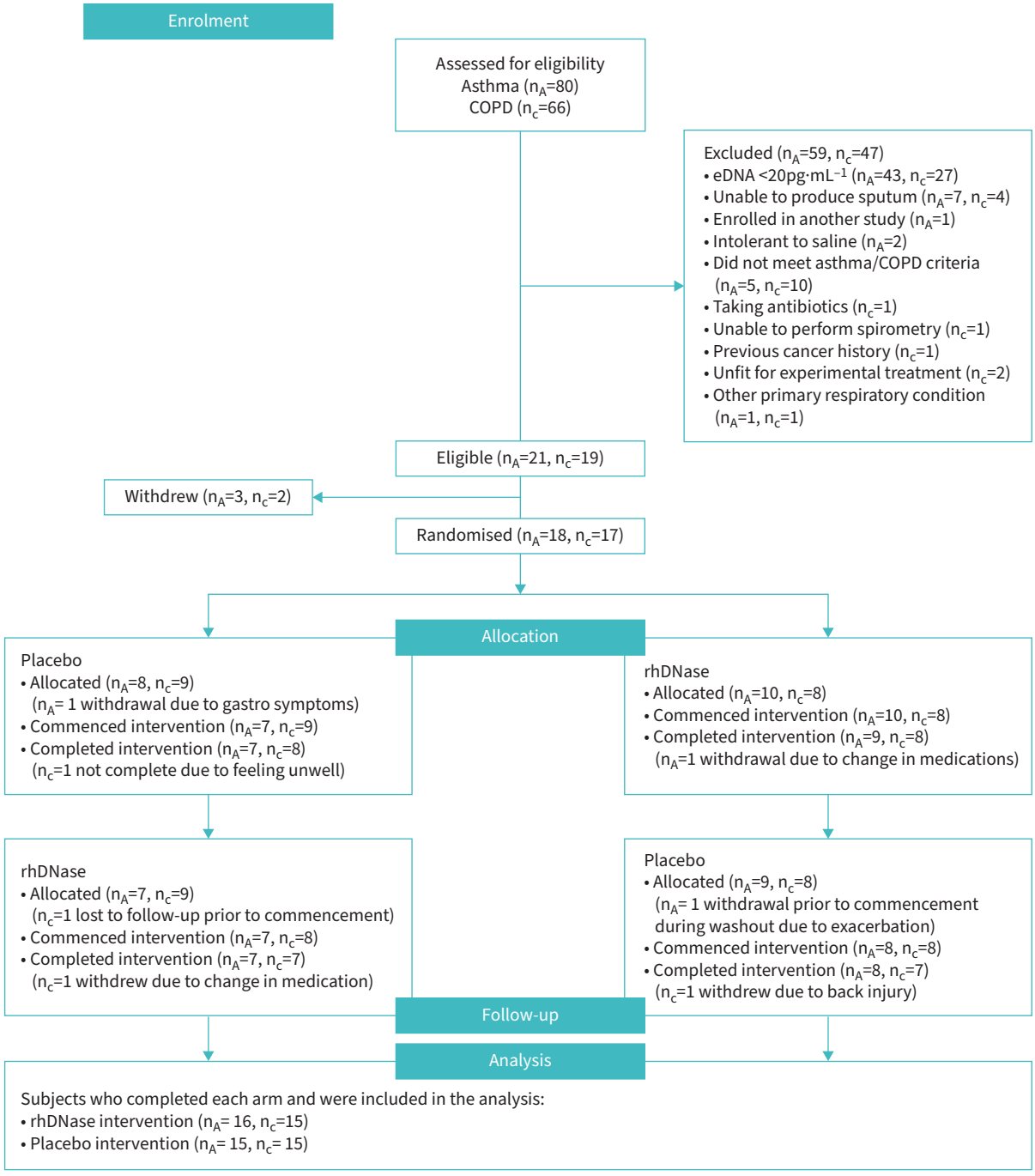

FIGURE 2 Participant CONSORT diagram. eDNA: extracellular DNA; rhDNase: recombinant human DNase. This flow diagram was prepared following the CONSORT 2010 statement [46].

Baseline clinical and inflammatory characteristics of high and low eDNA groups

Of the 64 participants with asthma and 48 with COPD who met the disease inclusion criteria, 21 (32.8%) and 21 (43.8%), respectively, had high levels of sputum eDNA (⩾20 µg·mL^{-1}) and were eligible for the RCT. The clinical details of participants with asthma and COPD with high and low eDNA are detailed in table 1. In the asthma population, participants with high sputum eDNA were significantly older than those with low sputum eDNA. Lung function parameters including FEV_1 % predicted, FVC % predicted and FEV_1/FVC ratio were all significantly lower in participants with high sputum eDNA than in those with low eDNA. Participants with high sputum eDNA also had poorer asthma control when compared to their

TABLE 1 Demographics and clinical characteristics of high *versus* low sputum eDNA in asthma *versus* COPD at baseline screening visit

Parameters	Asthma			COPD		
	High eDNA[#]	Low eDNA[¶]	p-value	High eDNA[#]	Low eDNA[¶]	p-value
n	21	43		21	27	
Age years, mean±SD	66.2±9.5	58.1±16.6	**0.040**	71.3±21.0	66.1±15.3	0.159
Sex (female), n (%)	13 (61.9)	23 (53.5)	0.598	10 (47.6)	11 (40.7)	0.771
BMI kg·m^{-2}, mean±SD	32.1±6.2	31.2±7.9	0.628	32.0± 8.2	28.9±5.7	0.139
Ex-smokers, n (%)	9 (42.9)	15 (34.9)	0.589	19 (90.5)	25 (92.6)	1.000
Pack-years, median (IQR)	3.5 (1–29)	5 (1–14)	0.596	41 (20–68)	47 (35–58)	0.314
Late disease onset (adult), n (%)	10 (47.6)	15 (35.7)	0.419	17 (81.0)	21 (77.8)	1.000
Post-bronchodilator FEV_1 % pred, mean±SD	69.9±17.8	86.2 ±19.4	**0.001**	65.9±16.8	65.1±22.6	0.897
Post-bronchodilator FVC % pred, mean±SD	83.3±13.5	93.0 ±15.6	**0.018**	87.5±17.0	92.9±20.5	0.336
Post-bronchodilator FEV_1/FVC ratio, mean±SD	0.6±0.1	0.7±0.1	**0.005**	0.6±0.1	0.53±0.14	0.445
Maximum % fall from baseline FEV_1, median (IQR)	8.9 (5.1–13.9)	9.7 (3.8–14.9)	0.721	11.8 (8.9–15.9)	14 (5.9–18.0)	0.568
ICS use, n (%)	21 (100)	36 (88.4)	0.126	13 (100%)	19 (70.4%)	0.454
ICS total daily dose µg·day^{-1}, median (IQR)[+]	800 (500–1200)	800 (400–1000)	0.336	800 (720–1100)	800 (400–1440)	0.846
Exacerbation prone, n (%)[§]	7 (33.3)	9 (20.9)	0.360	8 (38.1)	8 (30.8)	0.758
ACQ6, median (IQR)	1.7(1.2–2.7)	1.2 (0.5–2.0)	**0.043**	N/A	N/A	N/A
AQLQ, median (IQR)	5.8 (4.5–6.0)	5.6 (5.0–6.3)	0.637	N/A	N/A	N/A
CAT total score, mean±SD	N/A	N/A	N/A	18.7±7.8	17.5±7.6	0.619
SGRQ, mean±SD	N/A	N/A	N/A	43.9±16.2	47.3±19.3	0.528

t-test was used for parametric data and Wilcoxon rank sum for nonparametric data. For proportions, the two-sample test of proportions was used. Bold type for p-values denotes statistical significance. eDNA: extracellular DNA; BMI: body mass index; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; ICS: inhaled corticosteroids; ACQ6: Asthma Control Questionnaire-6; AQLQ: Asthma Quality of Life Questionnaire; CAT: COPD Assessment Test; SGRQ: St. George's Respiratory Questionnaire. [#]: participants with eDNA >20 µg·mL^{-1}; [¶]: participants with eDNA <20 µg·mL^{-1}; [+]: ICS dose calculated as beclomethasone equivalents where 1 µg of beclomethasone=1 µg budesonide=0.5 µg fluticasone; §: exacerbation prone = ⩾2 exacerbations in the previous year.

low sputum eDNA counterparts. In COPD, there were no significant differences in clinical parameters between the high and low sputum eDNA groups.

Inflammatory cell counts from induced sputum and whole blood for high and low eDNA groups are detailed in table 2. Sputum total cell count and the sputum colour score were significantly higher in the high eDNA group in both asthma and COPD. We did not observe any differences in the proportion of sputum immune cells. There were no differences in blood cell counts between high and low eDNA neither in asthma nor in COPD (table 2).

The presence of NETs was investigated by the assessment of eDNA and antimicrobial proteins in the sputum supernatant (table 3). In asthma, the median eDNA in sputum supernatant from the high eDNA group was 35.6 (27.0, 46.7) µg·mL^{-1}, five times higher than the low eDNA group (7.1 (3.6–11.1) µg·mL^{-1}). Similarly, eDNA levels in COPD were significantly higher in the eDNA-high group (45.2 (33.5–62.0) *versus* 7.2 (4.9–12.6) µg·mL^{-1}, table 3). Levels of the antimicrobial LL-37, HNP1-3 and IL-1β were all significantly elevated in the high eDNA group in both asthma and COPD. Although CitH3 levels were higher in the high eDNA group, the difference was not statistically significant (table 3).

Response to rhDNase treatment

Administration of nebulised rhDNase for 10 days was able to significantly reduce sputum eDNA levels in both asthma (46.3±29.6 µg·mL^{-1} to 18.4±13.3 µg·mL^{-1}, p=0.022; table 4 and figure 3a) and COPD (from 39.3 µg·mL^{-1} (36.7–55.6) to 25.4 µg·mL^{-1} (11.3–38.6), p=0.044; table 4 and figure 3b). Administration of nebulised rhDNase for 10 days was safe, with no severe adverse events in the asthma group and one event of shortness of breath in the COPD cohort, which was unlikely to be treatment related (supplementary tables S1 and S2).

In asthma, there were no significant baseline differences between treatment groups with respect to lung function (table 4), asthma control (table 4) or sputum cell counts (table 5). Interestingly, there were significant changes in the proportion of segmented and banded neutrophils in peripheral blood after rhDNase treatment. The proportion of segmented neutrophils was significantly higher after treatment with rhDNase (p=0.035, table 5), although this change was not significant compared to placebo. Whereas the

TABLE 2 Sputum inflammatory cell and blood cell counts: high *versus* low sputum eDNA groups in asthma (n=64) *versus* COPD (n=48) at baseline

	Asthma			COPD		
	High eDNA[#]	Low eDNA[¶]	p-value	High eDNA[#]	Low eDNA[¶]	p-value
Participants n	21	43		21	27	
Sputum cell counts						
N	21	43		19	25	
Total cell count x10^6·mL^{-1}	5.2 (3.0–7.6)	2.7 (1.6–4.7)	**0.003**	7.2 (2.8–8.6)	2.6 (1.3–6.3)	**0.016**
Neutrophils %	33.5 (10.5–58.5)	34.8 (13.8–56.3)	0.926	58.8 (30.3–67.8)	42.8 (24.3–59.5)	0.250
Eosinophils %	0.8 (0.5–2.0)	1.5 (0.5–5.3)	0.187	0.5 (0.5–3.3)	1.5 (0.8–3.5)	0.183
Macrophages %	44 (35.0–65.5)	46 (32–64)	0.937	29.3 (24.0–49.0)	37.8 (26.5–57.5)	0.297
Lymphocytes %	0.8 (0.0–1.8)	2.3 (0.3–3.3)	0.060	1.8 (0.8–3.5)	1.5 (1.0–5.5)	0.537
Columnar epithelial cells %	3.8 (2.3–10.5)	3.3 (1.8–9.5)	0.881	2.5 (1.0–20.0)	2.5 (0.8–10.0)	0.585
Sputum colour score	3 (3–3)	2 (2–3)	**<0.001**	3 (3–3)	2 (2–3)	**0.005**
Blood cell counts						
N	19	36		19	20	
Total white blood cells 10^9·L^{-1}	7.2±1.9	7.4±2.1	0.731	7.9±2.0	8.7±1.8	0.176
% neutrophils	56.7±8.3	58.8±8.8	0.415	60.5±7.0	63.8±8.1	0.192
segmented neutrophils	53.7±8.7	54.8±9.5	0.651	55.3±9.6	59.3±10.0	0.211
% neutrophil banded	1.3 (0.0,4.8)	0.1 (0.0–5.4)	0.956	2.9 (0.0–6.7)	1.2 (0.0–2.7)	0.311
% neutrophils IG	0.6 (0.2–0.9)	0.6 (0.4–0.7)	0.684	0.6 (0.5–1.0)	0.8 (0.5–1.1)	0.196
% lymphocytes	30.8±8.0	28.2±7.0	0.216	26.4±6.6	24.4±7.5	0.370
% monocytes	8.2±2.1	8.2±2.7	0.954	9.2±2.2	8.1±2.1	0.103
% eosinophils	2.8 (2.1–3.6)	2.4 (3.4–4.3)	0.335	2.3 (1.5–3.3)	2.5 (1.2–3.4)	0.704
% basophils	1.3±0.4	1.2±0.4	0.512	1.3 (1.0–1.6)	1.0 (0.9–1.2)	0.089
Red blood cells 10^{12}·L^{-1}	4.7±0.6	4.7±0.5	0.638	4.9±0.7	4.8±0.5	0.662
Haemoglobin g·L^{-1}	141.6±11.8	141.1±13.5	0.887	147.3±17.5	148.0±13.4	0.899
Platelets 10^9·L^{-1}	256.8±49.3	249.3±57.3	0.629	244.7±69.8	258.9±71.1	0.533

Data are presented as mean±SD or median (IQR). *t*-test was used for parametric data and Wilcoxon signed rank test for nonparametric data. Bold type for p-values denotes statistical significance. eDNA: extracellular DNA; IG: immature granulocytes. [#]: participants with eDNA >20 µg·mL^{-1}; [¶]: participants with eDNA <20 µg·mL^{-1}.

proportion of banded peripheral blood neutrophils was significantly reduced following rhDNase *versus* placebo (p=0.044, table 5), reducing from 3.2 (0–7.7) to 0.0 (0.0–1.5) post rhDNase treatment. Other sputum NET-associated biomarkers remained unchanged with rhDNase treatment (table 5).

In COPD, the CAT score was significantly reduced post-treatment with rhDNase (17.3±7.2 *versus* 14.7±6.7; paired t-test p=0.012), although this change was not significant relative to placebo (p=0.214, table 4). There was a significant reduction in lung function (FEV_1 % predicted and FEV_1/FVC ratio) in the placebo group; however, this change was not significant relative to the rhDNase group. There was an increase in the proportion of sputum neutrophils in the rhDNase treatment group (p=0.039, table 5), although this change was not significant relative to placebo. There were no significant changes in peripheral blood cells or other sputum NET-associated biomarkers with rhDNase treatment of COPD (table 5).

TABLE 3 NETs and inflammatory markers in high *versus* low sputum eDNA groups in asthma (n=64) *versus* COPD (n=48) at baseline

	Asthma			COPD		
	High eDNA[#]	Low eDNA[¶]	p-value	High eDNA[#]	Low eDNA[¶]	p-value
Participants n	21	43		21	27	
eDNA µg·mL^{-1}	35.6 (26.9–49.8)	7.1 (3.6–11.1)	**<0.001**	45.2 (32.2–66.3)	7.2 (4.9–12.6)	**<0.001**
CitH3 ng·mL^{-1}	32.8 (12.6–684.6)	19.7 (7.8–54.8)	0.092	27.0 (18.9–95.6)	22.6 (16.4–64.8)	0.425
α-defensins 1–3 µg·mL^{-1}	55.4 (34.4–59.7)	7.8 (5.0–18.3)	**<0.001**	43.8 (33.1–57.0)	16.9 (7.5–34.2)	**<0.001**
LL-37 ng·mL^{-1}	749.6 (629.2–1502.7)	217.8 (137.7–370.7)	**<0.001**	864.6 (664.2–1441.0)	322.5 (203.1–546.9)	**<0.001**
IL-1β pg·mL^{-1}	934.5 (522.1–1665.0)	468.2 (340.1–900.1)	**0.009**	845.1 (460.9–1806.9)	357.3 (283.3–493.3)	**<0.001**

Data are presented as median (IQR). Wilcoxon signed rank test was used. Bold type for p-values denotes statistical significance. eDNA: extracellular DNA; CitH3: citrullinated histone H3; IL-1β: interleukin-1β. [#]: participants with eDNA >20 µg·mL^{-1}; [¶]: participants with eDNA <20 µg·mL^{-1}.

TABLE 4 Treatment effect on sputum eDNA and clinical outcomes in asthma *versus* COPD

Outcome	Asthma				COPD			
	Pre	Post	p-value[#]	p-value[¶]	Pre	Post	p-value[#]	p-value[¶]
eDNA µg·mL^{-1}								
rhDNase	48.4 (22.1–74.1)	17.0 (5.0–31.0)	**<0.005**	**0.022**	39.3 (36.7–55.6)	25.4 (11.3–38.6)	**0.042**	**0.044**
Placebo	35.6 (18.2–73.5)	24.1 (18.2–60.6)	0.184		37.8 (22.9–50.5)	42.5 (35.1–62.9)	0.359	
Post-bronchodilator FEV_1 % pred								
rhDNase	73.6±19.9	74.6±19.3	0.235	0.616	67.0±17.8	65.5±16.8	0.587	0.071
Placebo	71.7±18.1	73.4±18.7	0.060		68.5±17.1	66.8±16.7	**0.006**	
Post-bronchodilator FVC % pred								
rhDNase	85.3±11.4	86.0±12.4	0.488	0.362	85.6±18.6	84.9±17.6	0.810	0.376
Placebo	82.6±11.1	85.1±14.2	0.179		84.9±18.3	84.7±16.2	0.158	
Post-bronchodilator FEV_1/FVC ratio								
rhDNase	65.1±13.5	65.4±12.9	0.674	0.900	0.58 ±0.11	0.58 ±0.12	0.593	0.159
Placebo	65.6±12.4	66.0±14.1	0.653		0.60±0.11	0.59 ±0.12	**0.030**	
ACQ6								
rhDNase	1.6±0.9	1.5±0.9	0.990	0.561	N/A	N/A	N/A	N/A
Placebo	1.6±0.9	1.5±0.8	0.438		N/A	N/A	N/A	N/A
AQLQ								
rhDNase	5.4±0.9	5.5±1.1	0.165	0.410	N/A	N/A	N/A	N/A
Placebo	5.5±0.9	5.4±0.9	0.617		N/A	N/A	N/A	N/A
CAT								
rhDNase	N/A	N/A	N/A	N/A	17.3±7.2	14.7±6.7	**0.012**	0.214
Placebo	N/A	N/A	N/A		17.1±6.8	16.5±6.1	0.657	
SGRQ total								
rhDNase	N/A	N/A	N/A	N/A	40.7±18.5	38.9±19.2	0.272	0.520
Placebo	N/A	N/A	N/A		40.5±16.8	38.1±16.7	0.354	

Data are presented as mean±SD for parametric data or median (IQR) for nonparametric data. Bold type for p-values denotes statistical significance. eDNA: extracellular DNA; rhDNase: recombinant human DNase; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; ICS: inhaled corticosteroids; ACQ6: Asthma Control Questionnaire-6; AQLQ: Asthma Quality of Life Questionnaire; CAT: COPD Assessment Test; SGRQ: St. George's Respiratory Questionnaire. [#]: change within intervention arm analysed by Wilcoxon signed rank for nonparametric data and paired t-test for parametric data; [¶]: difference in variable change compared to placebo, analysed by mixed model regression accounting for repeated measures.

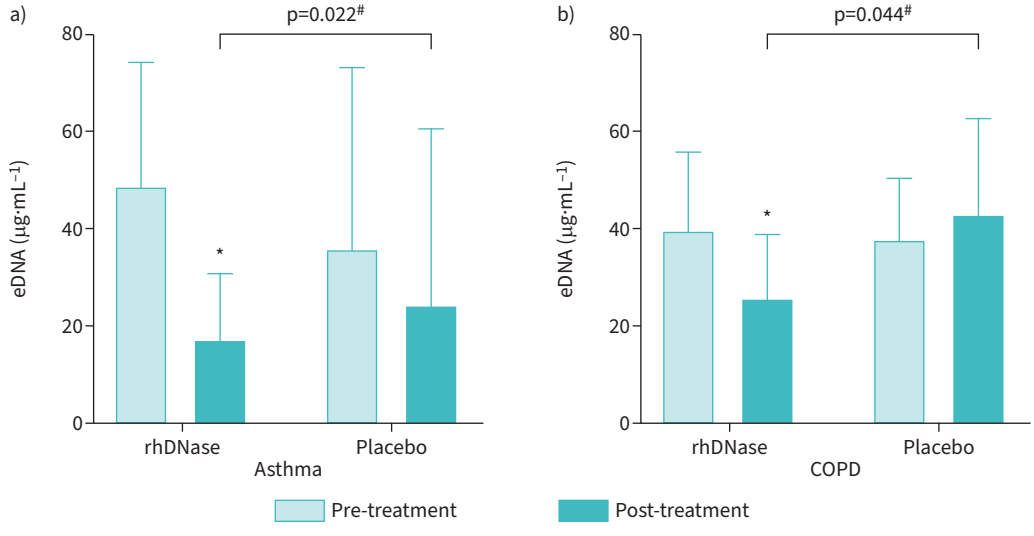

FIGURE 3 eDNA levels are reduced with rhDNase treatment in a) asthma and b) COPD. Bars are medians with the upper quartile as the error bar. eDNA: extracellular DNA; rhDNase: recombinant human DNase. *p<0.05 in pre- *versus* post-treatment analysed by Wilcoxon signed rank test; [#]: pre- *versus* post-treatment relative to placebo, analysed by mixed model regression accounting for repeated measures.

TABLE 5 rhDNase treatment effect on cell counts and other NETs markers

Outcome	Asthma				COPD			
	Pre	Post	p-value[#]	p-value[¶]	Pre	Post	p-value[#]	p-value[¶]
Sputum cell counts								
Total cell count $\times 10^6 \cdot mL^{-1}$								
rhDNase	3.6 (2.6–6.4)	4.3 (2.3–6.0)	0.793	0.919	6.0 (3.2–9.7)	10.2 (4.2–13.9)	0.135	0.538
Placebo	3.7 (2.3–6.9)	3.0 (2.3–5.8)	0.577		5.6 (3.0–8.6)	7.2 (5.9–8.7)	**0.046**	
Neutrophils %								
rhDNase	34.2±26.3	36.8±23.4	0.757	0.484	46.4±24.7	61.9±17.2	**0.039**	0.071
Placebo	37.2±23.8	29.0±24.9	0.458		53.0±25.1	54.9±24.4	0.853	
Eosinophils %								
rhDNase	1.0 (0.5–3.0)	0.5 (0.5–2.2)	0.609	0.561	1.25 (0.75–3.00)	0.75 (0.50–3.25)	0.491	0.596
Placebo	0.9 (0.3–2.1)	0.5 (0.3–1.5)	0.930		0.5 (0.25–4.90)	1.0 (0.5–3.0)	0.246	
Macrophages %								
rhDNase	47.7±20.1	53.5±26.9	0.402	0.721	40.3±19.0	31.9±16.2	0.132	0.137
Placebo	43.4±23.5	50.1±25.7	0.626		34.5±18.5	35.8±22.0	0.779	
Lymphocytes %								
rhDNase	1.0 (0.5–1.8)	1.3 (0.5–2.0)	0.858	0.295	1.00 (0.25–1.50)	1.00(0.0–1.75)	0.199	0.145
Placebo	1.8 (0.0–3.6)	1.5 (0.3–3.8)	0.748		0.5 (0.13–2.00)	1.25 (0.00–2.75)	0.080	
Blood cell counts								
Total white blood cells $10^9 \cdot L^{-1}$								
rhDNase	7.8 (6.2–8.7)	7.8 (6.1–8.8)	0.729	0.925	8.3 (5.8–8.9)	7.0 (6.3–8.5)	>0.999	0.271
Placebo	8.5 (6.6–9.7)	8.3–(7.6–9.3)	0.711		7.6 (6.5–8.4)	8.1 (6.0–9.5)	0.380	
% neutrophils								
rhDNase	56.4±7.9	57.0±7.9	0.567	0.622	59.0±5.7	60.1±6.3	0.526	0.618
Placebo	57.6±7.9	59.3±8.7	0.322		57.7±8.1	60.8±6.2	0.596	
% segmented neutrophils								
rhDNase	50.9±9.6	54.2±7.9	**0.035**	0.296	52.4 (49.1–57.9)	54.9 (50.7–61.4)	0.273	0.351
Placebo	55.1±7.4	55.4±7.8	0.803		53.1 (48.2–59.0)	55.1 (46.2–58.9)	0.459	
% neutrophil banded								
rhDNase	3.2 (0–7.7)	0.0 (0.0–1.5)	0.052	**0.044**	4.3 (0.9–8.9)	0.9 (0.0–7.1)	0.054	0.133
Placebo	0.0 (0.0–2.0)	1.5 (0.0–4.2)	0.380		3.2 (0.0–6.2)	4.5 (1.8–5.7)	0.677	
% neutrophils IG								
rhDNase	0.5 (0.4–1.2)	0.6 (0.5–0.7)	0.940	0.875	0.6 (0.3–0.9)	0.5 (0.4–0.8)	0.685	0.357
Placebo	0.8 (0.5–1.0)	0.6 (0.4–1.1)	0.890		0.7 (0.5–0.9)	0.8 (0.6–1.0)	0.970	
% lymphocytes								
rhDNase	31.2±7.1	30.4±7.8	0.455	0.898	27.9±6.5	26.8±6.7	0.726	0.668
Placebo	29.3±7.6	28.8±8.5	0.716		29.7±7.7	26.7±5.5	0.517	
% monocytes								
rhDNase	9.0 (6.4–10.1)	8.1 (7.5–10.8)	0.900	0.052	8.7 (7.7–11.7)	9.2 (7.1–10.7)	0.273	0.983
Placebo	8.2 (7.6–10.3)	8.0 (6.8–9.5)	0.073		8.6 (6.7–10.8)	7.5 (6.8–10.2)	0.791	
% eosinophils								
rhDNase	2.3 (1.4–3.2)	2.2 (1.5–3.1)	0.464	0.916	2.3 (1.7–3.2)	3.2 (1.4–3.8)	>0.999	0.845
Placebo	2.3 (1.4–3.6)	2.4 (1.6–3.1)	0.169		2.5 (1.8–3.1)	2.7 (1.2–3.7)	0.424	
% basophils								
rhDNase	1.2±0.4	1.1±0.4	0.147	0.380	1.2±0.3	1.4±0.5	0.478	0.181
Placebo	1.2±0.4	1.1±0.4	0.722		1.3±0.4	1.2±0.3	0.709	
Red blood cells $10^{12} \cdot L^{-1}$								
rhDNase	4.7±0.5	4.8±0.5	0.615	0.673	4.5 (4.4–4.9)	4.5 (4.3–4.6)	0.109	0.985
Placebo	4.8±0.4	4.8±0.5	0.899		4.7 (4.4–4.9)	4.6 (4.4–4.8)	0.266	
Haemoglobin $g \cdot L^{-1}$								
rhDNase	142.1±11.2	141.9±11.5	0.930	0.600	142.5 (138.0–147.3)	138.0 (131.0–147.0)	0.419	0.769
Placebo	143.0±10.1	144.1±12.1	0.542		143.0 (138.5–151.0)	144.0 (137.0–151.0)	0.532	
Platelets $10^9 \cdot L^{-1}$								
rhDNase	258.0 (212.5–293.5)	264.5 (213.3–303.3)	0.794	0.424	216.5 (187.5–255.0)	226.0 (173.0–265.0)	0.826	0.676
Placebo	264.0 (215.0–295.0)	273.0 (238.0–305.0)	0.309		223.0 (175.5–281.5)	226.0 (179.0–270.0)	0.624	
Sputum NETs marker								
CitH3 $ng \cdot mL^{-1}$								
rhDNase	19.3 (11.5–530.1)	26.0 (21.8–145.1)	0.808	0.367	46.0 (16.0–73.0)	12.8 (3.8–22.7)	0.079	0.596
Placebo	376.5 (13.2–1210.0)	34.6 (9.1–869.5)	0.092		30.5 (16.6–141.8)	24.2 (7.3–66.7)	0.208	
α-defensins1–3 $\mu g \cdot mL^{-1}$								
rhDNase	45.8±25.6	45.8±21.2	0.761	0.975	54.5±29.3	53.1±30.6	0.803	0.473
Placebo	45.9±24.3	45.8±28.1	0.666		44.6±26.5	48.9±21.6	0.141	

Continued

TABLE 5 Continued

Outcome	Asthma				COPD			
	Pre	Post	p-value[#]	p-value[¶]	Pre	Post	p-value[#]	p-value[¶]
LL-37 ng·mL^{-1}								
rhDNase	895.1±503.4	894.5±530.0	0.925	0.691	958.0 (585.6–1240.0)	981.2 (593.9–1562.0)	0.268	0.975
Placebo	802.3±363.5	744.0±414.3	0.424		1030.0 (482.6–1628.0)	1011.0 (626.8–1544.0)	0.679	
IL-1β pg·mL^{-1}								
rhDNase	1252 (484–1701)	1283 (422–2099)	0.761	0.359	1396 (365–3456)	1524 (485–2194)	0.761	0.273
Placebo	1051 (539–2740)	721 (570–3331)	0.733		862 (280–1660)	1243 (671–2290)	0.135	

Data are presented as mean±SD or median (IQR). Bold type for p-values denotes statistical significance. rhDNase: recombinant human DNase; NETs: neutrophilic extracellular traps; CitH3: citrullinated histone H3; IG: immature granulocytes; IL-1β: interleukin-1β.. [#]: change within intervention arm analysed by Wilcoxon signed rank for nonparametric data and paired *t*-test for parametric data; [¶]: difference in variable change compared to placebo, analysed by mixed model regression accounting for repeated measures.

Discussion

Increased levels of extracellular DNA are associated with reduced lung function in airway obstructive diseases [24–27]. NETs have been suggested as a potential source, and excessive NETs formation is associated with tissue damage and inflammation and is implicated in several lung conditions [10, 28, 29]. In this study we investigated the therapeutic effect of rhDNase treatment on sputum eDNA levels in people with asthma or COPD with high baseline levels of eDNA. We further explored the pathogenic nature and prevalence of NETs by measuring sputum NET markers and their association with clinical outcomes. We found that high eDNA levels were associated with significantly increased sputum total cell count, a higher sputum colour score, and elevated levels of neutrophil and inflammatory markers (HNP1-3, LL-37 and IL-1β) in both asthma and COPD. In asthma, participants with high eDNA were older, and had significantly reduced lung function and asthma control. We demonstrated for the first time *in vivo* that administration of nebulised rhDNase for 10 days could significantly reduce sputum eDNA levels relative to placebo in both asthma and COPD. Despite this reduction, there was no significant beneficial effect on clinical outcomes in either disease group. Interestingly, we did observe changes in the proportion of peripheral blood neutrophil subtypes after rhDNase treatment of asthma, meriting further mechanistic investigations.

In asthma, high eDNA levels were associated with significantly reduced lung function and asthma control, which is consistent with literature reporting a significant negative correlation of NET components with pulmonary function and symptom control [7, 10, 30]. We did not observe significant differences in lung function between eDNA-high *versus* eDNA-low in our COPD cohort. While previous publications indicated that eDNA could serve as a biomarker for NETs [17], our findings suggest that eDNA might not be an appropriate surrogate. Although eDNA and neutrophils correlated in our previous study [7], we did not observe a significant difference in neutrophil proportions and CitH3 (a more specific NETosis biomarker) between high and low eDNA groups in this study. Further, NETs have been demonstrated to correlate with sputum neutrophils in COPD and other chronic airway diseases [30, 31], and our populations did not feature notably higher proportions of sputum neutrophils. These findings suggest that the detected eDNA may have not been entirely NET derived.

Despite a reduction of eDNA post-treatment with rhDNase, we did not observe a significant improvement of clinical outcomes in either disease group. The lack of a beneficial effect could be due to the persistence of neutrophil granular proteins. LL-37 and IL-1β have previously been found to be inversely correlated with lung function in COPD and asthma, respectively [32–34], and these protein levels were unaffected by the administration of rhDNase for 10 days in this study. Concerns have been raised that rhDNase may increase airway inflammation through releasing neutrophil mediators bound to DNA within NETs. Shah *et al.* [35] found an increase in sputum neutrophil elastase activity after one dose of rhDNase; however the levels subsequently dropped after following doses, and the authors showed that longer term rhDNase therapy resulted in lowered elastase activity and CXCL8. Subsequent studies were unable to reproduce the findings of increased inflammation, with no effect on the level of inflammatory cell proportions [36], but reductions in sputum and plasma elastase activity [37], and MMP levels [38]. It is possible that the treatment duration was too short to promote changes. However, in cystic fibrosis administration of the same dose significantly improved FEV_1 within the first 2 weeks [11]. It is noteworthy that we observed a significant reduction in the CAT symptom control score in COPD pre *versus* post rhDNase treatment, suggesting that the treatment had some effect on symptom control in COPD. However, this effect was not

significant relative to placebo. Since the CAT score is self-reported, and the participants were not blinded, this value could potentially be subject to bias. It would be interesting to further investigate why rhDNase did improve lung function in cystic fibrosis and, potentially, in COPD, but not in asthma, and if a longer administration could lead to reduced neutrophil granular proteins and significantly improved symptoms.

Previous studies of asthma have shown rhDNase was effective in reversing mucus plugging in children [39] and adults [40] with life-threatening asthma refractory to conventional treatments. During an acute exacerbation, a single dose of rhDNase treatment had no obvious improvement in lung function or symptoms in children [41] or adults [42]. These trials did not stratify patients according to phenotype or presence of high eDNA. However, when Silverman *et al.* [42] evaluated patients with more severe airway obstruction, those receiving rhDNase tended to have a greater improvement in lung function, indicating a variation in response. Further studies are needed to clarify the role of rhDNase in treating neutrophil-mediated chronic airways diseases including severe asthma, COPD and bronchiectasis.

Interestingly, we observed changes in systemic and sputum neutrophil proportions and subtypes post-treatment. In asthma, segmented neutrophils were increased, whereas banded neutrophils were reduced in blood after rhDNase administration. COPD featured a similar trend of reduced banded blood neutrophils. These systemic changes of neutrophil subtype proportions suggest that rhDNase may affect inflammatory cytokines, altering neutrophil trafficking and maturation, and possibly reducing systemic stimulation of the bone marrow release of immature neutrophils into the blood stream [43]. COPD further featured an increased proportion of sputum neutrophils post rhDNase treatment, indicating that rhDNase and subsequent eDNA degradation may have differential effects in different diseases and the corresponding pathophysiological contexts. Neutrophil heterogeneity has been previously shown in obstructive lung diseases, and more research is needed to determine the functional consequences of these observations [44]. However, further investigation in a larger cohort is necessary to confirm these findings. It would also be of interest which effect a shift from the immature, banded neutrophil subtype to the segmented subtype has on clinical outcomes, and to explore their corresponding pro-inflammatory or immunomodulatory functions.

Our study has strength and limitations. A strength is the biomarker guided approach. The investigated cohort was well selected based on high sputum eDNA levels, and the administration of rhDNase clearly demonstrated a reduction of sputum eDNA, meeting our primary end-point. Yet, no statistically significant effect on clinical outcomes could be observed. Potentially, the treatment period was too short to promote changes, or the investigated population size too small to achieve adequate power to detect changes. The lack of changes in more specific NETs markers suggests that rhDNase either has no effect on neutrophil granular proteins or neutrophil activation, and that sputum eDNA is not a specific enough marker of NETs. To further investigate the potential of rhDNase to reduce NETs, follow-up studies with a larger cohort and longer treatment duration could be performed. Participants could further be screened for more specific NETs markers such as CitH3 or PAD4, although the feasibility of such screening is clinically challenging. Alternatively, reduced lung function could be considered as a screening marker in addition to high eDNA levels, as Silverman's study in asthma demonstrated that administration of rhDNase was only beneficial in those with reduced baseline lung function [42]. A combination of rhDNase and antibodies targeting PAD4 [45] could further be beneficial in chronic airway diseases through the prevention of NETosis in addition to the reduction of NET-derived eDNA.

In summary, our study demonstrated that 10 days of nebulised rhDNase treatment effectively reduced sputum eDNA in both asthma and COPD, without significantly improving clinical outcomes or modulating other NET-related biomarkers. There were changes to systemic neutrophil phenotypes after rhDNase treatment in asthma, suggesting lowering of systemic neutrophil-related inflammation. Further research is necessary to identify easily detectable, more specific NETs markers and/or a more suitable therapeutic target to reduce NETs and to prevent their formation in chronic airways disease.

Provenance: Submitted article, peer reviewed.

Data availability: This study commenced in July 2017, prior to the required statement of data sharing provisions.

This study is registered at https://www.anzctr.org.au with identifier number ACTRN12617000395336.

Ethics statement: Patients provided informed consent for their voluntary participation in this crossover randomised controlled trial. At all times, their rights were protected and the data generated were de-identified. They were informed that they could cease participation and their safety was priority at all times throughout the study.

Conflict of interest: V.M. McDonald reports support for the present manuscript from NHMRC; and grants or contracts received from NHMRC and Medical Research Futures Fund, consulting fees received from GSK, payment or honoraria received for lectures, presentations, speakers' bureaus, manuscript writing or educational events from GSK, Boehringer Ingelheim and the Menarini Foundation, and support received for attending meetings and/or travel from GSK and Boehringer Ingelheim, outside the submitted work; and is a board member of the TSANZ COPD X guideline committee, outside the submitted work. K.J. Baines reports support for the present manuscript from NHMRC, Australian Lung Foundation and John Hunter Charitable Trust. The remaining authors have nothing to disclose.

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