

Activity and expression of histone acetylases and deacetylases in inflammatory phenotypes of asthma

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Summary

Background Histone acetyltransferases (HATs) and histone deacetylases (HDACs) regulate gene expression, yet differences in the activity of these enzymes in the inflammatory phenotypes of asthma are unknown. We hypothesized that neutrophilic asthma (NA) would be associated with increased HAT and decreased HDAC activity.

Objective To investigate total HAT/HDAC activity and gene expression in isolated blood monocytes and sputum macrophages from healthy and patients with asthma.

Methods Peripheral blood and induced sputum were collected from adults with asthma ($n = 52$) and healthy controls ($n = 9$). Sputum inflammatory cell counts were performed and asthma inflammatory phenotypes were classified according to sputum eosinophil and neutrophil cut-off's of $> 3\%$ and $> 61\%$ respectively. Peripheral blood monocytes were isolated ($n = 61$) and sputum macrophages were isolated from a subgroup of patients with asthma ($n = 14$), using immunomagnetic cell separation. RNA and nuclear proteins were extracted and quantified. Enzyme activity was assessed using fluorescent assays and gene expression of EP300, KAT2B, CREBBP, and HDACs 1, 2 and 3 were measured by qPCR.

Results There was a significant inverse association between blood monocyte HAT and HDAC activity ($r = -0.58$, $P < 0.001$). NA was associated with increased blood monocyte HAT enzyme activity ($P = 0.02$), decreased HDAC activity ($P = 0.03$), and increased HAT:HDAC ratio ($P < 0.01$) compared with eosinophilic asthma. There were no differences in gene expression of EP300, KAT2B, CREBBP, or HDACs 1, 2 and 3 in blood monocytes from subjects with asthma or inflammatory phenotypes of asthma. There was no effect of inhaled corticosteroid use, poor asthma control, or asthma severity on HAT/HDAC activities. Sputum macrophages had increased expression of KAT2B in eosinophilic compared with paucigranulocytic asthma.

Conclusions and Clinical Relevance Neutrophilic airway inflammation is associated with increased HAT and reduced HDAC activity in blood monocytes, demonstrating further systemic manifestations relating to the altered inflammatory gene transcription profile of neutrophilic asthma.

Keywords asthma, HAT, HDAC, inflammatory phenotypes, monocytes, sputum

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Introduction

Asthma is a chronic heterogeneous inflammatory disorder of the airways, and several different inflammatory phenotypes have now been identified. Although clinically not well distinguished, many aspects of the disease including pathophysiology, treatment and triggers reflect this heterogeneity [1]. Differences in the

recruitment of granulocytes into the airways define three distinct inflammatory phenotypes of asthma [2–4]. Recent work has shown an association of these altered inflammatory states with the corresponding transcriptional profiles in the airways [5, 6]. At the molecular level, there is increased expression of inflammatory genes, involved in the initiation and chronicity of the inflammatory process. Eosinophilic asthma (EA)

is associated with an escalated production of T-helper type 2 (Th2) associated inflammatory cytokines such as (IL-4), (IL-5) and (IL-13) [5, 6] while an increase in IL-8 [4, 7], TNF- α and LTB4 [8] is associated with airway neutrophilia. This characteristic alteration in inflammatory pathways is possibly associated with epigenetic factors, such as changes in chromatin structure. In fact, histone acetyl-transferases (HATs) and histone deacetylases (HDACs), enzymes that regulate acetylation status of chromatin structure, are likely to play a key role in induction of these inflammatory genes.

Acetylation of histones by transcriptional co-activators with intrinsic HAT activity such as CREB-binding protein (CBP), E1A binding protein p300 (p300) and lysine acetyltransferase 2B (KAT2B) results in more open, transcriptionally active chromatin allowing transcriptional factors and RNA polymerase to initiate gene transcription. In contrast, HDAC enzymes including the Class I HDACs, HDAC1, HDAC2 and HDAC3 condense chromatin, forming a more compact, transcriptionally repressive conformation [9]. Alterations in HAT/HDAC protein activity and their mRNA expression therefore have direct consequence on gene transcription. Interestingly, this is a feature of many inflammatory lung diseases. For instance, a significant reduction in total HDAC activity was observed in lung tissues of chronic obstructive pulmonary disease (COPD) patients compared with healthy controls [10]. A concurrent reduction in mRNA expression for HDACs 2, 5, 8 and protein expression of HDAC2 [10] suggests a possible deficiency in certain types of HDACs. Similarly, a decreased activity and expression of HDAC1 and HDAC2 was observed in bronchial biopsies from patients with asthma compared with healthy people [11]. Other than lung tissue, the reduction in HDAC activity was observed in alveolar macrophages (AM) [12] and peripheral blood mononuclear cells (PBMCs) in patients with asthma [13, 14] suggesting possible systemic effects of the airway inflammation. Also using mouse models, certain inflammatory phenotypes such as neutrophilic asthma (NA) has been attributed to reduced HDAC2 activity in lung tissue [15], suggesting possible importance of HAT and HDAC in different inflammatory phenotypes of asthma. A reduction in HDAC activity and concurrent increase in HAT activity can potentiate inflammatory gene transcription and several studies have noted this imbalance in bronchial biopsies, AM and in PBMCs from patients with asthma [11, 12].

In this study, we explored the hypothesis that NA is associated with increased HAT and decreased HDAC activity of peripheral blood monocytes (PBMCs) of human subjects. Studies have previously shown differential activity of HAT and HDAC in the systemic circulation in subjects with asthma and healthy controls [12, 13]. However, to best of our knowledge, no studies have

investigated the activity and expression of these enzymes in purified blood monocytes or in different inflammatory phenotypes of asthma. The objective of this study was therefore to investigate HAT and HDAC activity and gene expression in blood monocytes and sputum macrophages from subjects with asthma and the effects of inflammatory phenotype.

Methods

Participants

Adults with stable asthma ($n = 52$) and healthy controls ($n = 9$) were recruited. Asthma was defined according to ATS guidelines based upon current (past 12 months) episodic respiratory symptoms, doctor's diagnosis (ever) and demonstrated reversible airflow obstruction with a FEV₁ bronchodilator response of $\geq 12\%$ (and > 200 mL) and AHR PD₁₅ < 15 mL. Participants were excluded if they had a recent (past month) respiratory infection, asthma exacerbation, change in maintenance therapy, or a course of oral corticosteroids or antibiotics. Participants were also excluded if they had lung function FEV₁ < 0.5 L, $< 40\%$ predicted, were current smokers or ex-smokers who quit within the last 12 months, if they were morbidly obese (BMI > 40) or had co-morbid conditions such as diabetes mellitus, or cancer, or other respiratory conditions including COPD, emphysema or bronchiectasis.

Subjects attended a visit where spirometry, sputum induction, allergy skin tests, exhaled nitric oxide (FENO) and blood collection were performed, along with information collected regarding demographics, medical history, asthma control [16] and medication use. All participants gave informed written consent and the study was approved by the Hunter New England Health and the University of Newcastle Human Research Ethics Committees.

Induced sputum processing

Induced sputum was processed following the method described previously [17]. Briefly; mucus plugs were selected from saliva and dispersed with 1 in 10 diluted Sputolysin containing 0.1% dithiothreitol (DTT) (Calbiochem, La Jolla, CA, USA). The tube was placed on a rotating mixer (MACS Vario mixer, Miltenyi Biotec, Gladbach, Germany) for at least 30 min at room temperature for cell dispersion. Following this, cells were washed with phosphate-buffered saline (PBS). The suspension was filtered with 60 μ m filter (Millipore, Australia). The filtered cell suspension was centrifuged and the cell pellet was re-constituted with PBS. Total cell count (TCC) and cell viability (trypan blue exclusion) were performed using a haemocytometer while a portion of this cell suspension was used for the preparation of May-Grünwald-Giemsa (MGG) and chromothrope 2R (C2R) cytopins for

differential cell counts, performed on 400 non-squamous cells. Sputum macrophages were isolated from the remainder of the cell suspension using immunomagnetic selection as described below.

Asthma inflammatory phenotype and severity definition

Sputum eosinophilia was defined as $> 3\%$ eosinophils and sputum neutrophilia was classified as $> 61\%$ neutrophils [18, 19]. Severe asthma was defined as asthma needing high doses of Inhaled Corticosteroid (ICS) ($> 1000 \mu\text{g}$ BDP equivalent) in combination with long-acting β_2 agonists (LABA) with poor asthma control ($\text{ACQ} \geq 1$) or $\text{FEV}_1 < 80\%$ predicted and $\text{FEV}_1/\text{FVC} < 70\%$. Mild-to-moderate asthma was divided into controlled ($\text{ACQ} < 1$) or uncontrolled ($\text{ACQ} \geq 1$).

Cell isolations

Peripheral blood monocytes were separated using Ficoll-Hypaque (GE healthcare, Sydney, Australia) as the manufacturer recommended with some modifications. Samples were centrifuged at 400 g for 10 min at 4°C and the plasma fraction was removed. The cellular fraction of the blood was then diluted at least 1 : 1 in PBS, and 30 mL of this was carefully layered on 15 mL of ficoll, and centrifuged at 400 g for 30 min at 18°C . Following this, PBMCs were washed and used for cell separation.

Immunomagnetic cell separation

Monocytes were purified from PBMCs using the Human Monocyte Cell Isolation kit II (Miltenyi Biotec, Sydney, Australia). Airway macrophages were isolated from sputum cell suspensions using Human CD15 cell isolation kit (Miltenyi Biotec). Both these cell isolation kits allowed the negative selection of the desired cells and used the magnetic cell separation system (MACS) (Miltenyi Biotec), LS cell separation columns and MACs buffer (Miltenyi Biotec) as per manufacturer's recommendations. Blood monocyte isolations resulted in an average yield of 8×10^6 cells whereas yields of macrophages from sputum varied depending on sample quantity, quality and subject. We isolated cells with high purity ($> 85\%$ from sputum, $> 95\%$ from blood). Monocytes were divided into two aliquots, one for immediate nuclear protein isolation, and the rest stored at -80°C for gene expression analysis. Sputum macrophages were isolated on a subset of samples, and only stored for gene expression analysis due to the lower cell yield obtained.

Gene expression analysis

Total RNA was extracted using the All Prep Mini kit (Qiagen, Sydney, Australia) according to the manufac-

turer's instructions. RNA concentration and integrity were determined using the NanoDrop 2000 (ThermoFisher Scientific, Victoria, Australia). Real-time quantitative PCR was performed as previously described [5]. A total of 200 ng of RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription kit according to the manufacturer's protocol (Life Technologies, Victoria, Australia). Real-time quantitative PCR (q-PCR) analysis was performed using the Eppendorf Real-Time Detection System (Eppendorf, Sydney, Australia) and standard Taqman methods. The primer/probes for HATs (CBP, p300, KAT2B) and HDACs (HDAC 1, 2, 3) and Taqman gene expression master mix were purchased in kit form from Life Technologies, Australia. The results were calculated using $2^{-\Delta\Delta\text{Ct}}$ relative to both the housekeeping gene (18S rRNA) and expressed as a fold change from the healthy control group.

Preparation of nuclear extracts

Nuclear extracts were derived from blood monocytes using a Nuclear Extract Kit, according to the manufacturer's recommendations (Active Motif, Carlsbad, CA, USA), and stored at -80°C . Nuclear protein was quantified by using the ProStain protein quantification kit (Active Motif), as per the manufacturer's protocol.

Histone acetyltransferase (HAT) activity assay

Measurement of HAT enzyme activity was performed using the fluorescent HAT Assay Kit (Active Motif) according to the manufacturer's recommendations. In brief, the purified recombinant p300 catalytic domain was incubated with acetyl-CoA and 1 μg of total nuclear extracts for 20 min. The developer solution was then added which produced fluorescence by reaction with the free sulphhydryl groups on the CoA-SH, measured at 360 nm excitation and 460 nm emission (FLUOstar Optima, BMG Labtech, Durham, NC, USA). A standard curve of CoA-SH (β -mercaptoethanol) was used to quantify the level of fluorescence in the range from 0 to 35 pmol/min.

Histone deacetylase (HDAC) activity assay

Measurement of HDAC enzyme activity was performed using the fluorescent HDAC Assay Kit (Active Motif) according to the manufacturer's recommendations. In brief, HDAC substrate was incubated with 3 μg of total nuclear extracts and incubated for 60 min. Following the incubation, the developer solution was added and fluorescence was measured at 360 nm excitation and 460 nm emission (FLUOstar Optima, BMG Labtech, Durham, NC, USA). A standard curve was used to quantify

the level of florescence and ranged from 0 to 16 pmol/min.

Statistical analysis

Statistical analysis was performed using PRISM version 5 (GraphPad Software, San Diego, CA, USA) and Stata/IC 11.0 (StataCorp, College Station, TX, USA). Parametric and non-parametric data are presented as means (SD) and medians (quartile 1, quartile 3) unless otherwise stated. Categorical data were compared using the χ^2 test or Fisher's exact test. For comparison of two continuous variables the two-sample Student's *t*-test was used for parametric data and the Mann–Whitney *U*-test for non-parametric data. Multiple comparisons of continuous variables (inflammatory phenotypes) were analysed using One-way ANOVA followed by the Bonferroni test for parametric data and the Kruskal–Wallis test for the non-parametric data. The strength of the relationship between HAT and HDAC activities were determined by Pearson correlation. Statistical significance was defined as $P < 0.05$.

Results

Clinical characteristics of asthma participants

Sixty-one eligible participants including 52 patients with asthma and nine healthy controls were recruited into this study. The participant's clinical characteristics are detailed in Table 1. We achieved a good gender balance between the groups. However, those with asthma were older compared with the control group ($P = 0.04$). Atopy was prevalent among the asthmatics (75.5%, $P = 0.02$) who also had airflow obstruction, with reduced post β_2 FEV₁% predicted ($P = 0.04$) and post β_2 FEV₁/FVC ($P = 0.006$). Many of the asthmatics (86.5%) were using ICS with a median dose of 500 (BDP equivalent) mcg/day. Induced sputum TCC ($P = 0.01$) and percentage and absolute count of airway neutrophils were elevated significantly in asthma ($P < 0.02$). Absolute eosinophil counts were also increased in asthma ($P = 0.007$).

HAT and HDAC activity and expression in asthma

Sufficient blood monocytes were obtained from 49 asthmatics for RNA and 48 asthmatics for HAT/HDAC enzyme activity and nine healthy controls for all measurements. HAT and HDAC activities were inversely correlated ($r = -0.58$, $P < 0.0001$; Fig. 1), consistent with the reciprocal effects of these enzymes on gene activation. Total HAT and HDAC enzyme activity in blood monocytes was similar between subjects with asthma and controls (Table 2). HATs (EP300, KAT2B, CREBBP) and HDACs (HDAC1, HDAC2, HDAC3) were selected for

gene expression analysis based on their localization to the cell nucleus as well as existing evidence of importance to inflammation in the literature. HAT and HDAC gene expression of in blood monocytes was similar between asthma and healthy controls (Table 2).

Clinical characteristics of inflammatory phenotypes of asthma

Subjects with asthma were classified into different inflammatory phenotypes using induced sputum cell counts (Table 3). Participants with NA were significantly older compared with both EA and paucigranulocytic asthma (PGA), ($P = 0.015$). More than half (67%) of NA patients were ex-smokers. There was no significant difference in atopic status, BMI or FENO within the subgroups. Patients with NA had the lowest post β_2 FEV₁% predicted, significantly different ($P = 0.001$) from EA and PGA. Post β_2 FVC% predicted and post β_2 FEV₁/FVC followed a similar trend with the largest decline of lung function in NA ($P < 0.01$). There were also significant differences in ACQ scores ($P = 0.02$) which were highest in NA. A significant increase in TCC ($P = 0.03$), an increase in proportion and number of airway neutrophils ($P < 0.001$) as expected and a decrease in proportion and number of macrophages featured in NA. In contrast to this, as expected subjects with EA had significant increases both proportion and number of airway eosinophils ($P < 0.001$).

HAT and HDAC activity and expression in monocytes in inflammatory phenotypes of asthma

Total HAT activity was significantly different between the groups ($P = 0.021$) and post hoc testing showed significantly higher activity in NA compared with EA (Fig. 2a). Total HDAC activity was also significantly different between the groups ($P = 0.028$) and post hoc testing showed a significantly lower activity in NA compared with both EA and PGA (Fig. 2b). The differences in HAT and HDAC activities further emphasized by significantly altered HAT: HDAC activity ratio between the groups ($P = 0.006$) and post hoc testing showed a significantly increased HAT: HDAC ratio in NA compared with both EA and PGA (Fig. 2c).

These differences remained significant when the analysis was restricted to ICS users only (Table 4). Despite the significant differences in total HAT and HDAC activities, the relative gene expression of the selected HATs and HDACs in blood monocytes did not differ between asthma inflammatory phenotypes (Table 5). This suggests post-translational effects on HAT/HDAC activity in the different asthma phenotypes.

Compared with blood monocytes, the levels of HAT and HDAC gene expression in airway macrophages

Table 1. Demographic and clinical characteristics

	Asthma	Healthy control	<i>P</i> value
<i>N</i>	52	9	
Age (years), median (Q1, Q3)	51.5 (32.5, 63.0)	32.0 (29.0, 50.0)	0.044
Gender (M/F)	21/31	4/5	1.0
Ex-smoker, <i>n</i> (%)	15 (28.9)	0	0.097
Pack years, median (Q1, Q3)	0.8 (0.1, 10.4)	N/A	
Atopy, <i>n/N</i> (%)	37/49 (75.5)	3/9 (33.3)	0.020
BMI, mean (SD)	29.14 (6.47)	25.63 (4.80)	0.127
FENO p.p.b., median (Q1, Q3)	13.85 (7.5, 26.8)	9.8 (5.8, 12.1)	0.072
Post β2 FEV ₁ % predicted, mean (SD)	82.6 (19.1)	97.6 (12.3)	0.036
Post β2 FVC% predicted, mean (SD)	91.1 (17.0)	97.3 (13.4)	0.328
Post β2 FEV ₁ /FVC, median (Q1, Q3)	71.6 (11.0)	83.0 (4.5)	0.006
ACQ7 score, mean (SD)	1.16 (0.80)	N/A	
ICS, <i>n</i> (%)	45 (86.54)	N/A	
ICS dose (BDP equivalent mcg/day)*, median (Q1, Q3)	500 (500, 1600)	N/A	
Sputum cell counts			
Total sputum cell count × 10 ⁶ /mL, median (Q1, Q3)	3.87 (2.97, 6.03)	1.63 (1.08, 3.56)	0.006
Neutrophils%, median (Q1, Q3)	29.38 (15.1, 45.75)	13.25 (6.8, 23.0)	0.021
Neutrophils × 10 ⁴ /mL, median (Q1, Q3)	125.48 (54.94, 280.8)	23.86 (5.4, 53.91)	0.001
Eosinophils%, median (Q1, Q3)	1.5 (0.75, 5.75)	0.75 (0.63, 1.75)	0.141
Eosinophils × 10 ⁴ /mL, median (Q1, Q3)	10.40 (2.97, 25.86)	1.86 (0.68, 5.06)	0.007
Macrophages%, median (Q1, Q3)	58.1 (44.0, 69.75)	70.63(62.5, 80.65)	0.080
Macrophages × 10 ⁴ /mL, median (Q1, Q3)	207.97 (145.35, 277.02)	110.92 (75.78, 257.13)	0.151
Lymphocytes%, median (Q1, Q3)	1.0 (0.25, 1.75)	1.75 (1.0, 3.75)	0.057
Lymphocytes × 10 ⁴ /mL, median (Q1, Q3)	3.53 (1.48, 9.92)	3.17 (1.18, 7.82)	0.688
Viability%, mean (SD)	68.3 (20.2)	45.54 (18.2)	0.006
CEP%, median (Q1, Q3)	1.13 (0.25, 4.0)	3.13 (1.4, 18.63)	0.097
Squamous%, median (Q1, Q3)	6.32 (2.92, 11.5)	9.99 (7.0, 18.23)	0.092

Data are presented as mean ± SD or median (interquartile range), unless otherwise specified. Results that are significant at *P* < 0.05 are shown in bold.

*ICS dose is calculated as beclomethasone dipropionate (BDP) equivalents, where 1 µg of beclomethasone = 1 µg budesonide = 0.5 µg fluticasone. ACQ, asthma control questionnaire; BMI, body mass index; FENO, fractional exhaled nitric oxide; p.p.b., parts per billion; FEV₁, forced expiratory volume in 1s; % pred, %predicted; FVC, forced vital capacity; ICS, inhaled corticosteroids.

were significantly higher in subjects with asthma (Table 6). In airway macrophages, KAT2B expression differ significantly between the groups (*P* = 0.024; Table 6) and was highest in the EA compared with PGA (*P* = 0.018, post hoc testing). There were no statistically significant differences in HDAC expression in airway macrophages due to inflammatory phenotype of asthma (Table 7).

Effects of severity, ICS use and age on HAT and HDAC activity

There were no differences in HAT and HDAC enzyme activity between asthmatics using maintenance ICS (*n* = 43) and those not using ICS (*n* = 7; Table 8). When subjects were grouped by severity of their asthma symptoms, there was also no difference detected in

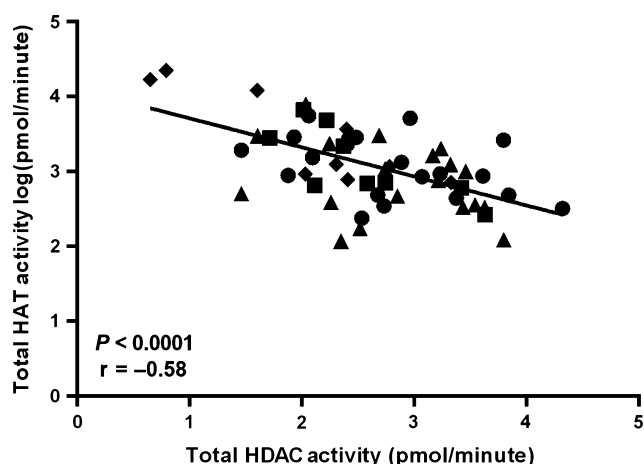


Fig. 1. Correlation of total histone acetyltransferases and histone deacetylases enzyme activities in peripheral blood monocytes. Triangles denote subjects with eosinophilic asthma, circles denote subjects with paucigranulocytic asthma, diamonds denote subjects with neutrophilic asthma and squares denote healthy controls.

Table 2. Protein enzyme activity and gene expression of selected HATs and HDACs in blood monocytes from subjects with and without asthma

	Asthma	Healthy controls	P value
Protein enzyme activity			
N	48	9	
Total HAT activity	3.06 (0.522)	3.11 (0.475)	0.810
Total HDAC activity	2.66 (0.795)	2.53 (0.640)	0.664
Gene expression			
N	49	9	
EP300 (E1A binding protein p300) mRNA	0.84 (0.292)	1.05 (0.377)	0.071
KAT2B (lysine acetyltransferase 2B) mRNA	0.97 (0.319)	1.04 (0.324)	0.525
CREBBP (CREB-binding protein) mRNA	0.90 (0.279)	1.04 (0.292)	0.197
HDAC1 (histone deacetylases 1) mRNA	0.96 (0.323)	1.07 (0.446)	0.395
HDAC2 (histone deacetylases 2) mRNA	0.91 (0.304)	1.06 (0.409)	0.200
HDAC3 (histone deacetylases 3) mRNA	0.94 (0.342)	1.06 (0.383)	0.354

Data (mean $\pm$ SD). Gene expression is calculated relative to 18S rRNA and presented as the fold change compared to the mean of the healthy control group. Total HAT activity expressed as log (pmol/min), total HDAC activity (pmol/min).

HAT, histone acetyltransferases; HDACs, histone deacetylases.

HAT or HDAC enzyme activity (Table 9). Similarly gene expression of HATs and HDACs did not alter in blood monocytes due to ICS or severity of asthma. In addition, there was no correlation of HAT ($r = 0.097$; $P = 0.51$ and HDAC ($r = 0.035$; $P = 0.81$) activity and age in asthma.

Discussion

In this study, we found that NA was associated with increased HAT activity and decreased HDAC activity in isolated PBMCs compared with both EA and PGA. Consequently, the HAT: HDAC activity ratio was greatly increased in NA. Interestingly, these differences in activity were not reflected in gene expression of selected HATs including EP300, KAT2B, CREBBP or HDACs including HDAC1, HDAC2 or HDAC3, which were not different in asthma compared with healthy controls or between inflammatory phenotypes of asthma. However, we observed an increased expression of KAT2B in airway macrophages in EA compared with PGA. Furthermore, HAT/HDAC activities were not altered by ICS use or asthma severity in this population. There was no association between HAT or HDAC activities and age in asthma. The results indicate that histone acetylation/de-acetylation of PBMCs may be a relevant mechanism resulting in neutrophilic airway inflammation.

The reduction in HDAC activity was associated with increased HAT activity in our cohort of asthmatics, suggesting possible co-regulation of these enzymes. The difference in the ratio of HAT:HDAC was greater between NA and EA, suggesting these inflammatory phenotypes may have a determinant effect in such co-regulation. The underlying reasons for this are not yet completely understood. However, it has been suggested that this may be a result of inactivation of HDACs due to oxidative and nitrosative stress [20]. In particular, oxidative stress is present in asthma [21] and may be elevated in certain inflammatory phenotypes such as NA [22], possibly resulting in greater impact on HDAC activity. In addition, reduced HDAC activity in smokers [23] and in COPD [24] also suggests that oxidative stress may be a possible mechanism. A recent study observed a reduction in HDAC2 in COPD compared with healthy controls only during exacerbations [25], suggesting that the lung environment may have a determinant effects.

We have selected non-smoking subjects with stable disease for this study. Therefore, current smoking or current asthma exacerbations are unlikely to be the cause for the reduction in HDAC activity observed in NA. Also, there was no difference in HAT or HDAC activities according to asthma severity. This would imply that the differences in HAT/HDAC activity may be an important feature of NA.

At a molecular level, the extent of histone acetylation determines relative accessibility of DNA to transcription factors [26]. For instance, hyperacetylation of core histones by HAT enzymes causes unwinding of the chromatin structure facilitating the binding of transcription factors. Binding and activation of transcription factors such as nuclear factor- κ B (NF- κ B) and activator protein 1

Table 3. Demographic and clinical characteristics in inflammatory phenotypes of asthma

	Asthma			P value
	Neutrophilic	Eosinophilic	Paucigranulocytic	
N	9	21	22	
Age (years), median (Q1, Q3)	69 (55, 73)	48* (39, 59)	46* (27, 63)	0.015
Gender (m/f)	5/4	9/12	7 15	0.460
Ex-smoker, n (%)	6 (66.7)	5 (23.8)	4 (18.2)	0.029
Pack years, median (Q1, Q3)	5.7 (3.0, 19.0)	0.45 (0, 10.4)	0.2 (0, 1.0)	0.080
Atopy, n/N (%)	5/8 (62.5)	15/21 (71.4)	17/20 (85.5)	0.367
BMI, mean (SD)	29.2 (7.9)	28.6 (5.4)	29.6 (7.1)	0.881
FENO p.p.b., median (Q1, Q3)	11.9 (3.4, 18.1)	19.1 (10.9, 42.2)	13.9 (6.3, 25.5)	0.147
Post β 2 FEV ₁ % predicted, mean (SD)	62.2 (14.9)	85.9* (19.8)	87.9* (14.5)	0.001
Post β 2 FVC% predicted, mean (SD)	76.6 (9.4)	92.5 (19.1)	95.8* (14.3)	0.01
Post β 2 FEV ₁ /FVC, mean (SD)	61.0 (13.0)	73.9* (10.9)	73.7* (7.6)	0.004
ACQ7 score, median (Q1, Q3)	1.67 (1.28, 2.0)	0.86* (0.78, 1.36)	0.69 (0.3, 1.7)	0.017
ICS, n (%)	9 (100.0)	20 (95.2)	16 (72.7)	0.065
ICS dose (BDP equivalent mcg/day), median (Q1, Q3)	1000 (500, 2000)	650 (500, 1300)	500 (400, 1300)	0.456
Total cell count $\times 10^6$ /mL, median (Q1, Q3)	7.2 (5.18, 11.89)	3.51 (2.7, 4.59)	3.87 (2.88, 5.76)	0.032
Viability%, mean (SD)	89.16 (8.82)	62.78* (22.35)	66.20 (17.27)	0.034
Neutrophils%, median (Q1, Q3)	83.25 (66.0, 90.75)	25.75* (13.5, 35.25)	25.88* (14.25, 43.0)	0.0001
Neutrophils $\times 10^4$ /mL, median (Q1, Q3)	572.4 (390.80, 964.29)	93.56* (43.88, 146.88)	103.28* (954.34, 158.62)	0.001
Eosinophils%, median (Q1, Q3)	1.25 (0.75, 2.25)	6**† (3.75, 11.25)	0.88 (0.25, 1.0)	0.0001
Eosinophils $\times 10^4$ /mL, median (Q1, Q3)	9.19 (2.88, 12.41)	27.13**† (15.84, 42.3)	2.97 (1.06, 4.05)	0.0001
Macrophages%, median (Q1, Q3)	12.0 (6.75, 31.75)	58.5* (50.5, 67.75)	66.63* (55.5, 81.0)	0.0001
Macrophages $\times 10^4$ /mL, median (Q1, Q3)	91.27 (66.67, 165.24)	208.98* (166.16, 265.07)	261.22* (164.83, 296.66)	0.011
Lymphocytes%, median (Q1, Q3)	0.25 (0.25, 0.9)	0.75 (0.25, 1.25)	1.25 (0.5, 2.0)	0.080
Lymphocytes $\times 10^4$ /mL, median (Q1, Q3)	3.33 (0.19, 12.70)	2.84 (0.94, 6.84)	7.2 (1.67, 10.58)	0.317
Columnar epithelial cells%, median (Q1, Q3)	0.7 (0, 0.75)	2.0 (0.5, 3.75)	1.38 (0.25, 6.0)	0.097
Squamous%, median (Q1, Q3)	5.2 (1.72, 7.19)	8.68 (4.53, 13.61)	4.99 (2.68, 11.15)	0.124

Data are presented as mean $\pm$ SD, n (%) or median (interquartile range), unless otherwise specified. Results that are significant at $P < 0.05$ are shown in bold.

*post hoc significant vs neutrophilic asthma ($P < 0.011$).

†post hoc significant vs. paucigranulocytic asthma ($P < 0.011$).

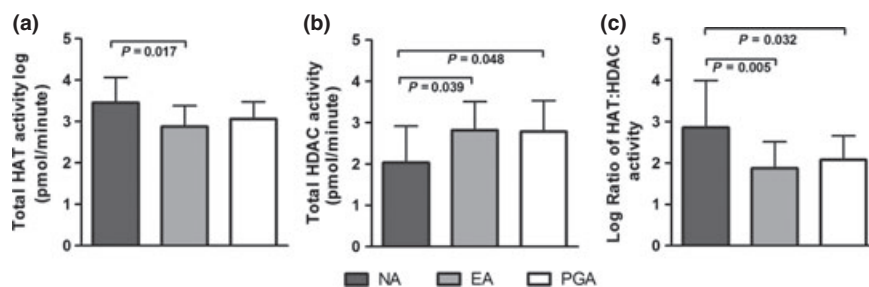


Fig. 2. Total histone acetyltransferases (HAT) (a), histone deacetylases (HDAC) (b) enzyme activities and their ratio (HAT: HDAC) (c) in peripheral blood monocytes in inflammatory phenotypes of asthma. Graphs show mean $\pm$ SD and significance (Bonferroni post hoc comparisons after ANOVA).

(AP-1) then induces multiple inflammatory genes [27]. The regulation of gene expression is then achieved by opposing actions of HDACs. The HDAC family consists of 11 distinct deacetylases [28], involved in rewinding of chromatin, thereby silencing gene transcription. In fact, decreased HDAC activity in NA may favour increased acetylation leading to increased expression of inflammatory genes.

Existing literature indicates an activation of the innate immune system [29] and possible dysfunction in

certain innate immune components such as airway macrophages may be important in development of non-eosinophilic asthma [30]. Consistent with this, we observed a reduction in absolute number of airway macrophages in subjects with NA. As macrophages are phagocytic cells and modulators of inflammation, the low availability of these may result inefficient phagocytosis leading to escalation of the innate immune response. Also a recent study found impaired efferocytosis in airway macrophages in non-eosinophilic

Table 4. HAT and HDAC enzyme (protein) activity in blood monocytes from subjects with asthma inflammatory phenotypes who were taking ICS

	Neutrophilic	Eosinophilic	Paucigranulocytic	P value
N	9	18	15	
Log HAT	3.45 (0.611)	2.89* (0.514)	2.98 (0.407)	0.028
HDAC	2.03 (0.882)	2.85* (0.694)	2.73 (0.766)	0.036
Log ratio	2.86 (1.138)	1.87* (0.658)	2.01* (0.601)	0.009

Data are presented as mean $\pm$ SD.

*post hoc significant vs. neutrophilic asthma ($P < 0.037$).

HAT, histone acetyltransferases; HDACs, histone deacetylases.

Table 5. Elative gene expression of selected HATs and HDACs of blood monocytes in inflammatory phenotypes of asthma

	Asthma			P value
	Neutrophilic	Eosinophilic	Paucigranulocytic	
N	8	19	22	
EP300	0.83 (0.214)	0.83 (0.260)	0.87 (0.347)	0.902
KAT2B	0.93 (0.304)	0.99 (0.303)	0.96 (0.350)	0.917
CREBBP	0.92 (0.256)	0.90 (0.246)	0.90 (0.323)	0.977
HDAC1	0.95 (0.350)	0.98 (0.338)	0.96 (0.316)	0.974
HDAC2	0.90 (0.336)	0.93 (0.263)	0.90 (0.338)	0.946
HDAC3	0.92 (0.312)	0.98 (0.344)	0.91 (0.362)	0.807

Gene expression data (mean $\pm$ SD) is calculated relative to 18S rRNA and presented as the fold change compared to the mean of the healthy control blood monocytes.

HAT, histone acetyltransferases; HDACs, histone deacetylases.

Table 6. Elative gene expression levels of three HATs and three HDACs in airway macrophages compared to peripheral blood monocytes in asthma

Gene	Blood monocytes	Airway macrophages	P value
N	49	14	
EP300	0.81 (0.64, 1.00)	1.34 (0.92, 2.61)	<0.001
KAT2B	0.89 (0.75, 1.08)	2.66 (1.76, 5.73)	<0.001
CREBBP	0.86 (0.68, 1.02)	1.26 (0.92, 2.38)	0.001
HDAC1	0.90 (0.71, 1.17)	3.15 (2.04, 7.24)	<0.001
HDAC2	0.83 (0.73, 1.04)	1.90 (0.97, 3.82)	<0.001
HDAC3	0.84 (0.66, 1.10)	3.05 (1.80, 5.39)	<0.001

Gene expression data [median (Q1, Q3)] is calculated relative to 18S rRNA and presented as the fold change compared to the mean of the healthy control blood monocytes.

HAT, histone acetyltransferases; HDACs, histone deacetylases.

asthma [30], possibly associating with HAT/HDAC activities. To this end, it may be desirable to assess HAT/HDAC activities in airway macrophages. However, we were unable to proceed in this study due to the insufficient quantities of cells.

The reduction in HDAC activity with increased HAT activity in blood monocytes is highly favourable for activation of transcription factors including NF- κ B.

Consequently, elevation of inflammatory gene transcription in peripheral blood may result inflammation at the systemic level. Up regulation innate immunity-related genes such as IL-1 and TNF- α /NF- κ B have been suggested in pathogenesis of NA [5]. A microarray study by Baines *et al.* [31] has established a substantial link between NA and systemic up regulation of neutrophil α -defensins and serine proteases including neutrophil elastase. Wood *et al.* [32] found increased systemic inflammation including plasma IL-6 and C-reactive protein in NA.

Transcriptional activation of many these pathways are intricately linked to NF- κ B or AP-1 which may be regulated by histone acetylation status. For instance, the TNF- α /NF- κ B pathway elicits IL-8 expression in lung through chromatin remodelling [33] whereas IL-8 gene silencing is associated with increased HDAC activity [34]. Likewise TNF- α stimulated expression of IL-6 depends on NF- κ B and its intrinsic HAT activity [27]. In fact, our observation of depleted HDAC activity in monocytes together with their capacity for cytokine synthesis suggests a possible role of monocytes in these systemic effects.

Previous studies have suggested diminished corticosteroid sensitivity in PBMCs in severe asthma which associates with reduction in HDAC activity [13]. Our analysis of HAT and HDAC activities according to asthma severity did not indicate a difference in HAT or HDAC activity. However, the requirement of high ICS doses in NA may be related to reduce HDAC activity in blood monocytes. Many inhaled corticosteroids restore the balance of HAT and HDAC. However, the repression of HAT activity may be the predominant action of steroids compared with gene induction of HDACs [11]. This agrees with our observations of the effects of ICS use towards HDAC activity in blood monocytes. Also other studies noted that ICS do not contribute to changes in HDAC activity in PBMCs [13].

Other than differences in total activity, there may be differences in the expression of HAT and HDAC genes. Previous studies have suggested a selective reduction in HDAC1 and HDAC2 expression in bronchial biopsies causing decreased total HDAC activity, whereas the expression of pCAF (KAT2B) and CBP (CREBBP) did not account for increased HAT activity [11]. Similarly, a reduction in the expression of HDAC1, but not HDAC2 or HDCA3, was noted in AM from patients with asthma [12]. Interestingly these differences were not seen in PBMCs, suggesting changes occur locally in the airways of asthmatics [12]. Other than in asthma, a selective reduction in HDAC2 expression was observed in severe COPD with lesser reduction in HDAC5 and HDAC8 and normal expression of the other HDACs [10].

In this study, we assessed mRNA expression of EP300 (p300), KAT2B (pCAF), CREBBP and HDAC1, 2 and 3.

Table 7. Elative gene expression of selected HATs and HDACs of airway macrophages in inflammatory phenotypes of asthma

Gene	Asthma			<i>P</i> value
	Neutrophilic	Eosinophilic	Paucigranulocytic	
<i>N</i>	3	6	5	
EP300	1.45 (1.04, 2.89)	1.72 (0.96, 2.52)	0.95 (0.84, 1.61)	0.734
KAT2B	2.88 (2.53, 6.49)	4.76 (2.79, 6.25)*	1.60 (1.30, 1.85)	0.024
CREBBP	1.39 (1.08, 2.50)	1.66 (1.44, 2.35)	0.94 (0.87, 1.33)	0.523
HDAC1	3.54 (2.76, 7.20)	4.62(2.73, 7.37)	2.09 (1.92, 2.19)	0.308
HDAC2	1.94 (0.95, 4.75)	1.90 (0.98, 2.43)	1.86 (1.81, 3.60)	0.926
HDAC3	3.26 (3.21, 5.80)	4.27 (1.83, 7.52)	2.02 (1.71, 2.86)	0.148

Gene expression data [median (Q1, Q3)] is calculated relative to 18S rRNA and presented as the fold change compared to the mean of the healthy control blood monocytes as there were no airway macrophages obtained from the healthy subjects. Kruskal–Wallis test. Results that are significant at $P < 0.05$ are shown in bold.

*post hoc $P = 0.018$ significance EA vs. PGA, post hoc $P = 0.025$ significance NA vs. PGA.

HAT, histone acetyltransferases; HDACs, histone deacetylases.

Table 8. HAT and HDAC activity in blood monocytes from subjects with and without ICS treatment

	Asthma (ICS used)	Asthma (No ICS used)	<i>P</i> value
<i>N</i>	42	6	
Log HAT	3.04 (0.54)	3.21 (0.41)	0.476
HDAC	2.63 (0.81)	2.84 (0.71)	0.536
Log ratio	2.13 (0.84)	2.19 (0.51)	0.883

Data are presented as mean $\pm$ SD.

HAT, histone acetyltransferases; HDACs, histone deacetylases.

Table 9. HAT and HDAC activity in blood monocytes from subjects with well-controlled asthma, poor asthma control and severe asthma

	Controlled asthma	Uncontrolled asthma	Severe asthma	<i>P</i> value
<i>N</i>	25	12	10	
Log HAT	2.93(2.94)	3.09 (3.07)	3.18(3.31)	0.178
HDAC	2.73 (0.68)	2.68 (0.76)	2.54 (1.13)	0.672
Log ratio	1.81 (1.99)	1.98 (2.09)	2.19(2.54)	0.187

Data are presented as mean $\pm$ SD.

HAT, histone acetyltransferases; HDACs, histone deacetylases.

CREBBP, p300 and pCAF are transcriptional co-activators with intrinsic HAT activity. These HATs may interact with NF- κ B and activator protein 1 (AP-1) causing their activation and consequently inflammatory gene induction. We chose three different Class I HDACs (HDAC1, 2 and 3) which are widely localized in the cell nucleus. These HDACs interact with co-repressor molecules such as NuRD, mSin3 and elicit their specific actions on switching off inflammatory genes [35]. HDAC1 and HDAC2 can interact with NF- κ B leading to de-acetylation and promoting its association with the inhibitor I κ B within the nucleus, thereby terminating inflammatory actions of NF- κ B [36]. Other studies have shown that inhibition of HDAC1 and HDAC2 promotes the activation of NF- κ B leading to inflammatory gene expression such as IL8 [26].

We found no difference in the gene expression of HATs or HDACs in blood monocytes. However, in airway macrophages the expression of KAT2B was increased in EA compared with PGA. This difference in KAT2B gene expression in airway macrophages in EA compared with PGA requires further investigation as KAT2B expression may be central to the regulation of many other cellular processes related to allergic inflammation and autophagy [37]. For instance, the phosphoinositide 3-kinase (PI3K) pathway is implicated in allergic airway inflammation as it involved in many facets of disease development [38–40]. The action of PI3K is regulated by PTEN (Phosphatase and tensin homologue deleted on chromosome 10) [41]. Having both lipid phosphoinositol and protein phosphatase activities, PTEN phosphorylates integral components of the PI3K complex, thereby inhibiting its kinase activity. However, it is known that PTEN itself may undergo acetylation caused by KAT2B [42]. Acetylated PTEN is no longer capable of down regulation of PI3K and therefore the expression of KAT2B may be a master switch in PI3K-mediated inflammatory effects. Other than immunomodulatory effects, PI3K may be important in autophagy [43]. The lipid PI3P is required for nucleation of autophagic membranes and is derived from the PI3K pathway. In fact, inhibition of PI3K negatively affects autophagy, an important function of macrophages.

No difference in gene expression of blood monocytes, despite differences in the total HAT and HDAC activities, suggests that inactivation of these enzymes due to the nature of inflammation may be more likely. The factors other than transcription, possibly translation or simply poor contribution from the HAT or HDAC that were selected to measure towards total activity may also important.

Although this study has emphasized the importance of histone acetylation in NA, there are several limitations and potential improvements that must be considered.

First, we analysed total HAT enzyme activity, and the activities of 11 different HDACs that belong to Class I, II and IV. Sirtuins (SERT 1–7), which belong to Class III, were omitted. However, it may be of interest to include this group in future studies. Second, we were not able to determine activities of individual enzymes in this study. Assaying of this would yield important insights into possible selective inhibition. Similarly, our selection of nuclear HATs HDACs, mainly based on existing literature about their importance in airway inflammation, however, an analysis of a larger panel of HAT and HDAC genes for expression may be useful in future studies. Third, we could not determine enzyme activities in airway macrophages therefore finding any localized differences in enzyme activities. This will be one of the most interesting aspects that future studies may look into, along with further downstream targets of HATs and HDACs. Finally, the sample size in this study is relatively small and our outcomes should be further investigated in a larger study population. In spite of these limitations, this study has made a significant contribution to the advancement of our understanding of disease mechanisms associated with NA.

In summary, NA is characterized by increased total HAT and reduced total HDAC activity in PBMCs compared with eosinophilic asthma. Asthma severity and ICS

medications did not alter HAT or HDAC activity in this cohort. These differences in enzyme activities are not associated with alterations in gene expression of HDAC1, HDAC2, HDAC3, EP300, KAT2B or CREBBP in blood monocytes. Determining these systemic effects of neutrophilic inflammation may help to understand disease mechanisms and yield new therapeutic targets for asthma.

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Conflict of interest

The authors declare no conflict of interest.

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