



2. Rosace D, Gomez-Casado C, Fernandez P, et al. Profilin-mediated food-induced allergic reactions are associated with oral epithelial remodeling. *J Allergy Clin Immunol.* 2018;18:30611.
3. Obeso D, Mera-Berriatua L, Rodríguez-Coira J, et al. Multi-omics analysis points to altered platelet functions in severe food-associated respiratory allergy. *Allergy* 2018;73:2137-2149.
4. Barber D, de la Torre F, Feo F, et al. Understanding patient sensitization profiles in complex pollen areas: a molecular epidemiological study. *Allergy* 2008;63:1550-1558.
5. Blanco C, Quiralte J, Castillo R, et al. Anaphylaxis after ingestion of wheat flour contaminated with mites. *J Allergy Clin Immunol.* 1997;99:308-313.
6. Van Bruaene N, Derycke L, Perez-Novo CA, et al. TGF- β signaling and collagen deposition in chronic rhinosinusitis. *J Allergy Clin Immunol.* 2009;124(2):253-259.
7. Ribatti D, Crivellato E. Immune cells and angiogenesis. *J Cell Mol Med.* 2009;13:2822-2833.
8. Morita H, Arae K, Unno H, et al. An interleukin-33-mast cell-interleukin-2 axis suppresses papain-induced allergic inflammation by promoting regulatory T cell numbers. *Immunity* 2015;43:175-186.
9. Sugita K, Steer CA, Martinez-Gonzalez I, et al. Type 2 innate lymphoid cells disrupt bronchial epithelial barrier integrity by targeting tight junctions through IL-13 in asthmatic patients. *J Allergy Clin Immunol.* 2018;141(1):300-310.
10. Popescu F-D. Cross-reactivity between aeroallergens and food allergens. *World J Methodol.* 2015;5:31.

SUPPORTING INFORMATION

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The potential role of CD16^{high}CD62L^{dim} neutrophils in the allergic asthma

To the Editor:

Asthma is a chronic disease, characterized by chronic inflammation, airway obstruction and airway hyperresponsiveness. Neutrophils and eosinophils both contribute to disease development and aggravation, as they are found in the airways and lungs of patients with asthma.

Previous research has emphasized the importance of eosinophils in allergic asthma, whereas neutrophils have not been extensively studied. This is likely because the neutrophils are present within the lung at all times, even in asymptomatic patients. Nevertheless, blood neutrophils have been shown to increase during the first hours after allergen bronchial challenge test¹ as well as during the late asthmatic airway response (LAR).² The role and perception of neutrophils changed when Pillay et al³ identified three subsets of neutrophils. In this study, neutrophils were subdivided based on their expression of CD16 and CD62L, each subset representing a different stage of maturity and activity. Neutrophils released from the bone marrow express low levels of CD16 and high levels of CD62L; this CD16^{dim}CD62L^{high} subset is considered to represent the immature neutrophil. These neutrophils are absent in the circulation in healthy donors, but appear rapidly after an lipopolysaccharide (LPS) challenge, and are even more pronounced in severely injured patients. Neutrophils expressing high levels of CD16 and CD62L (CD16^{high}CD62L^{high}) are mature, but nonactivated, whereas the CD16^{high}CD62L^{dim}-expressing neutrophils are believed to be mature and activated and play an important role in systemic inflammation.³ We have recently shown a role for these subsets in allergic rhinitis⁴ and an in vitro model of asthma.⁵ In the latter, the CD16^{high}CD62L^{dim} subset (produced by LPS in vitro stimulation) was found to enhance the response to bradykinin in both human bronchioles and murine

tracheae. No such effects were obtained for the other neutrophil subsets. The response was due to an upregulation of bradykinin receptor 2, through release of TNF α by the neutrophils.⁵

Using flow cytometry, we examined the presence and the distribution of the CD16^{high}CD62L^{high} and the CD16^{high}CD62L^{dim} subsets in peripheral blood from patients with allergic asthma during an exacerbation induced by an inhaled allergen bronchoprovocation test (Figure 1A). The bronchial challenge test presently used is an exacerbation model of allergic asthma common in research settings.⁶ Regional Ethical Review Board (2012/800) approved the study, and all participants provided written informed consent.

Nine nonsmoking patients (aged 18-50 years; 6 males and 3 females) with a physician-confirmed diagnosis of allergic asthma (according to GINA⁷) were included. All of them exhibited less than 40% of activated neutrophils in peripheral blood before the bronchial challenge test. All patients had a positive skin prick test and elevated levels of specific IgE to the allergen used for their respective challenge (3 cat, 2 birch, 2 grass pollen and 2 horse allergen (ALK-Abello)). Seven of the patients could be classified as eosinophilic phenotype based on their blood eosinophils (>150 cells/mcL). One patient had a slightly lower number of eosinophils, and one patient cannot be defined due to lack of data. Five patients had only an early asthmatic response following allergen, while four patients presented with both an early and a late response (Table 1). Blood samples were collected in sodium heparin tubes before and 24 hours after completion of the allergen bronchial challenge test. Cells were stained and analysed using flow cytometry. Neutrophils were gated on CD45, CD16 and CD62L (Figure 1B,C). Statistical analyses were performed using GraphPad Prism software (version 6.0, GraphPad

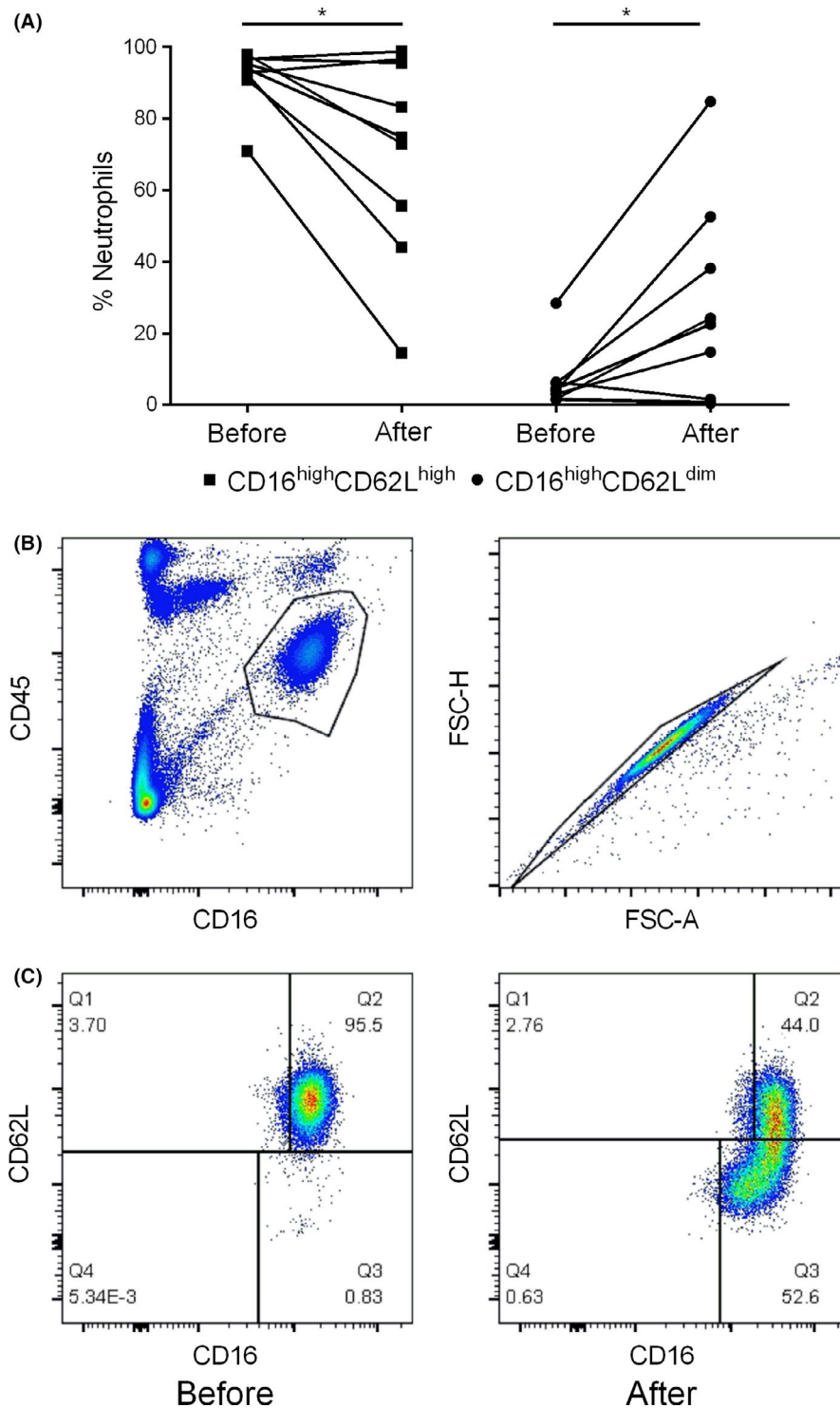


FIGURE 1 Neutrophil subset and allergy challenge. A, The presence of mature (■) CD16^{high}CD62L^{high} and activated (●) CD16^{high}CD62L^{dim} neutrophils in the blood before and after allergen bronchial challenge in the lung (n = 9). B, Gating strategy plots (first and second steps) from the blood samples from the allergen bronchial challenge. C, Gating strategy plot (third step) from blood samples before (left panel) and after (right panel) allergen bronchial challenge. Pared t test was used, *P < 0.05

Software). Between the two groups, paired t test was performed. A P-value of <0.05 was considered statistically significant (*P < 0.05). At 24 hours postallergen, a decrease in nonactivated, mature CD16^{high}CD62L^{high} neutrophils was noted (P = 0.04), which was paralleled by an increase in activated, mature CD16^{high}CD62L^{dim} neutrophils (P < 0.048, Figure 1A). In our in vitro paper, we established that CD16^{high}CD62L^{dim} neutrophils could affect airway hyperresponsiveness.⁵ It has previously been shown that neutrophils increase in blood

after allergen bronchoprovocation test. However, this was before the concept of neutrophil subsets was introduced in the literature.¹ The present study demonstrates, for the first time, that allergens can cause a shift in neutrophils subsets from CD16^{high}CD62L^{high} to CD16^{high}CD62L^{dim} in the peripheral blood of sensitized asthmatics. It has been suggested that neutrophils increased in blood postallergen provocation might be activated and that their subsequent migration to the lung might affect the pathogenesis of the exacerbation of

TABLE 1 Subject characteristics and baseline physiology, data are expressed as a median (interquartile range)

	Allergic asthmatics (n = 9)
Age (years)	25 (22-36)
Specific IgE (kU/L)	2.2 (1.2-11.2)
Exhaled NO ₅₀ (ppb)	17.4 (9.1-52.6)
FEV1 (% of predicted)	95.7 (90.8-114.4)
FVC (% of predicted)	104.5(101.5-114.7)
FEV1 drop early (%)	21.7 (20.9-23.9)
FEV1 max drop after 4-8 h (%)	12.2 (7.8-22.0)

FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; IgE, Immunoglobulin E.

airflow obstruction.⁸ Hence, we postulate that naturally occurring allergens may indirectly activate neutrophils and thereby induce airway hyperresponsiveness in sensitized individuals. The increase in CD16^{high}CD62L^{dim} neutrophils presently seen 24 hours postallergen provocation might be related to the release of cytokines like TNF- α , IL-1 β , G-CSF and IL-6. These mediators are known to induce bone marrow granulopoiesis, followed by release of neutrophils into the blood, and an upregulation of endothelial cell receptors like P-selectin and E-selectin, which facilitate the recruitment of neutrophils into tissues like the lung. These cytokines are found to be elevated in the blood after introducing allergen to the airways, and we believe that the activation occurs in the blood.⁹

The limitation of this study is that it is small, the status in the lung is unknown, and we only observe the neutrophils at one time point in the blood. The optimal time for capturing the activation in the blood may not be the same for all individuals, and therefore, further research is required.

To summarize, the neutrophils comprise a heterogeneous group of cells with specified roles in inflammation. The role for the activated neutrophils in allergic asthma becomes clearer as more studies highlight this cell type. The novel findings of a specific neutrophil subset, CD16^{high}CD62L^{dim}, which increases in the blood following an inhaled allergen bronchial challenge bronchoprovocation test, prompt us to re-evaluate the traditional concepts of allergic asthma which may guide us to new treatment strategies. In the worldwide ongoing effort to find endotype-tailored treatments for asthma, the present demonstration of increased neutrophil subgroups in allergic asthma might offer new possibilities and warrants further research.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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REFERENCES

1. Upham JW, Denburg JA, O'Byrne PM. Rapid response of circulating myeloid dendritic cells to inhaled allergen in asthmatic subjects. *Clin Exp Allergy*. 2002;32(6):818-823.
2. Asman B, Strand V, Bylin G, Bergstrom K. Peripheral neutrophils after allergic asthmatic reactions. *Int J Clin Lab Res*. 1997;27(3):185-188.
3. Pillay J, Kamp VM, van Hoffen E, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest*. 2012;122(1):327-336.
4. Arebro J, Ekstedt S, Hjalmarsson E, Winqvist O, Kumlien Georen S, Cardell LO. A possible role for neutrophils in allergic rhinitis revealed after cellular subclassification. *Sci Rep*. 2017;7:43568.
5. Ekstedt S, Safholm J, Georen SK, Cardell LO. Dividing neutrophils in subsets, reveals a significant role for activated neutrophils in the development of airway hyperreactivity. *Clin Exp Allergy*. 2019;49(3):285-291.
6. Stenberg H, Wadelius E, Moitra S, et al. Club cell protein (CC16) in plasma, bronchial brushes, BAL and urine following an inhaled allergen challenge in allergic asthmatics. *Biomarkers* 2018;23(1):51-60.

7. (GINA) GIfA. *Global Strategy for Asthma Management and Prevention (Updated 2014)*. In. Global Initiative for Asthma (GINA) 2014.
8. Mattheyse FJ, van Heerden K, Mattheyse M, et al. Reduced activation of peripheral blood neutrophils after late-phase asthmatic responses but not in mild stable asthma. *Respiration*. 2001;68(5):471-479.
9. Pelikan Z. Delayed type of asthmatic response to allergen challenge and cytokines in the peripheral blood. *Respiration*. 2012;84(5):385-395.

SUPPORTING INFORMATION

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Factors associated with hyperresponsiveness to adenosine 5'-monophosphate in healthy subjects

LETTER TO THE EDITOR To the editor

Airway hyperresponsiveness (AHR) is used to diagnose asthma. AHR can be assessed with a direct (eg, methacholine) or an indirect (eg, adenosine 5'-monophosphate (AMP)) test. Direct tests induce airway constriction by stimulating airway smooth muscle contraction. Indirect tests provoke mast cells and other inflammatory cells to release mediators, that cascade into airway constriction. AHR can be defined as the concentration of the stimulus that causes a 20% drop in forced expiratory volume in the first second (FEV_1)(PC_{20}). Although healthy subjects are expected to be unresponsive to AMP,¹ in a group of 108 healthy controls we observed 8 (7.4%) subjects with AHR to AMP (PC_{20} AMP ≤ 160 mg/mL). We therefore investigated which factors associate with AHR to AMP in these healthy subjects.

The NORM database (approved by the ethical committee of our hospital, NCT00848406, see Data S1) consists of healthy never or current smokers (>10 cigarettes per day). Subjects had no respiratory complaints (ie, cough or dyspnoea) nor any doctor's respiratory diagnosis, in the past or present. They presented with normal lung function ($FEV_1 > 80\%$ predicted, $FEV_1/FVC > 70\%$, and <10% increase in $FEV_1\%$ predicted after 400 μ g salbutamol) and the absence of AHR to methacholine (PC_{20} methacholine >19.8 mg/mL; Table 1). To investigate which factors associate with AHR to AMP, we compared subjects with AHR to AMP to those without (≤ 160 mg/mL vs >160 mg/mL, Table 1) with a chi-square or Mann-Whitney U test. From each group of variables, we entered in a multivariate logistic regression model the variable which increased the model's adjusted- R^2 most (univariate $P < 0.01$ and mutual Spearman's correlation <|0.7|; Table 2, SPSS version 22).

We expected a relation with allergies based on a skin prick test ($P = 0.59$) and self-reported allergic rhinitis ($P = 0.91$), but this relation was absent. Two samples had sputum eosinophils above the commonly used threshold of 3%. Therefore, we analyzed the percentage of sputum eosinophils categorized as <1%, $\geq 1\%$, and missing (only 74 subjects were able to expectorate sputum). AHR was significantly associated with sputum eosinophils (Table 1). The multivariate model (Table 2) shows that AHR is more likely in subjects with $\geq 1\%$ sputum eosinophils compared to subjects with <1% sputum eosinophils, but

equally likely in subjects with a missing sputum sample compared to those with <1% sputum eosinophils. This indicates that $\geq 1\%$ sputum eosinophils in a healthy subject associates with AHR to AMP. This aligns with earlier observations in asthma and allergic rhinitis that sputum eosinophils predict AHR to AMP.² We now show the association in healthy subjects.

Strikingly, all subjects with AHR to AMP were current smokers. Smoking status was significantly different ($P < 0.01$) between subjects with and without AHR and smokers with AHR smoked more cigarettes per day than smokers without AHR ($P < 0.01$). Previously reported healthy subjects with AHR to AMP were also all current smokers.³ Furthermore, smoking is associated with AMP sensitivity in patients with chronic obstructive pulmonary disease (COPD),³ as COPD smokers had a significantly lower PC_{20} AMP compared to COPD nonsmokers. Based on these findings, we speculate that AHR to AMP in healthy subjects is affected by cigarette smoke-induced presence of mast cells, the primary target for AMP. Cigarette smoke irritates epithelial cells, initiating the release of pro-inflammatory cytokines.⁴ These trigger mast cell infiltration and proliferation, resulting in increased numbers of mast cells in the airway's submucosa.⁵ Cigarette smoke also causes the reduction of epithelial integrity which facilitates the cigarette smoke to reach the mast cells.⁶ As AMP provocation initiates bronchoconstriction through mediators released by mast cells,⁷ healthy smokers may have AHR to AMP.

Next to smoking, AHR to AMP strongly associated with small airways function. Univariately, AHR to AMP associated with lower forced expiratory flow at 50%, 75%, and 25%-75% of the forced vital capacity (FVC) (FEF_{50} , FEF_{75} , and FEF_{25-75} , respectively), a higher resistance in the small airways (difference in resistance to 5 Hz and 20 Hz (R_{5-20})), and lower lung compliance (increased resonance frequency (F_{res}) and reactance area (AX); all $P < 0.03$, Table 1). Multivariately, small airways function, in terms of R_{5-20} , was independently associated with AHR (Table 2). Previously, small airways function has been suggested to explain why some patients present with respiratory complaints, despite a normal FEV_1 . It is possible that small airways anomalies are already important before respiratory complaints arise, as AHR to AMP might occur earlier than AHR to methacholine. Michils *et al*⁸ proposed that a discrepancy in the

SUPPLEMENTARY TABLE

Table 1. Subject characteristics and baseline physiology, data are expressed as a median (interquartile range).

	Allergic asthmatics (n=9)
<i>Age (years)</i>	25 (18-50)
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<i>Exhaled NO₅₀ (ppb)</i>	17.4 (3.9-116.5)
<i>FEV1 (% of predicted)</i>	95 (86-120)
<i>FVC (% of predicted)</i>	105 (88-130)
<i>FEV1 drop early (%)</i>	21.7 (20.1-27.2)
<i>FEV1 max drop after 4-8 h (%)</i>	22.0 (20.2-36.3)

IgE=Immunoglobulin E, FEV1=forced expiratory volume in 1 second, FVC=forced vital capacity.