Underestimation of Luminal Eosinophilia by Quantitative Sputum Cytometry

Melanie Kjarsgaard
  McMaster University

Adil Adatia
  McMaster University

Anurag Bhalla
  McMaster University

Nicola LaVigne
  SJHH: Saint Joseph's Healthcare Hamilton

Katherine Radford
  SJHH: Saint Joseph's Healthcare Hamilton

Chynna Huang
  St Joseph Hospital

Manali Mukherjee
  McMaster University

Parameswaran Nair (✉ parames@mcmaster.ca)
  McMaster University   https://orcid.org/0000-0002-1041-9492

Abstract

Rationale: On Wright-stained sputum cytospins, eosinophil differential of >1.2% is considered abnormal and ≥3% clinically relevant. We hypothesized that failure to consider free eosinophil granules (FEG), and the re-emergence of eosinophilia (at ≥1.2 and ≥2.3%) underestimate the prevalence of the eosinophilic phenotype.

Methods: This is a retrospective analysis of our Institutional Review Board-approved clinical sputum database. Of the 24,176 examinations, 17,693 had viable cell counts from 9570 patients (6604 on one occasion, 2967 with two or more) with various airway diseases. In all samples, FEGs were semi-quantified to estimate the prevalence of eosinophilia at <1.2 and <2.3%. In those patients with sputum examined on more than one occasion, subsequent results identified re-emergence of eosinophilia at ³1.2 and ³2.3%.

Results: Of those with intact cell counts (n=15,278), 8562 (56.0%) and 9690 (63.4%) would have been classified as clinically non-relevant eosinophilia of <1.2% and <2.3% respectively. Among those with two or more intact cell counts, 1142 (38.5%) and 1083 (36.5%) of samples had re-emergence of eosinophilia (at 1.2 and 2.3%), either when a previous neutrophilia resolved (n= 218/19.1%, n=312/28.8%) or due to a reduction in the dose of corticosteroids (n=634/21.4%, n=666/22.4%) with no significant differences between groups (p=0.018). There was a significant difference between the proportions of eosinophilic versus non-eosinophilic at 1.2 and 2.3%, using the presence of FEG and the non-intact cell count (p<0.001).

Conclusions: A total of 3180 (32.8%) samples identified as non-eosinophilic, had clinically relevant airway eosinophilia that would not have been identified if the phenotypic classification was limited to ≥2.3% intact eosinophils on one occasion. This underestimation is likely to be significantly more if other means of airway eosinophilic activity were included and if mast cell activity and lymphocyte numbers, (not routinely quantified by sputum cytometry), could be contributing to the under-appreciation of airway T2 inflammatory processes.

Introduction

Quantitative cytometry on Wrights (or Giemsa) stained sputum cytospin provides total cellularity and white cell differential count (1). The normal values have been established. For eosinophil differential %, the mean is 0.4%, and the upper limit of normality is 1.1 % (90th centile) or 2.2% (when 2 standard deviations are considered) (2). An eosinophil endotype of asthma is identified when the eosinophil % is greater than 2.2%, and a measurement > 3% is often considered clinically relevant (3). However, these cut-offs take into consideration only intact eosinophils, and not degranulated eosinophils. Free eosinophil granules (FEG) in tissue is an indicator of eosinophilic activity (4). In sputum, this correlates with eosinophil cationic proteins such as eosinophil peroxidase and the presence of moderate and many free granules correlate with clinical severity (5). Additionally, eosinophil % may change over time when any

associated neutrophilia may resolve (6), with exacerbations (7), or when the dosage of corticosteroids are reduced (8).

We hypothesized that the eosinophilic endotype may be underestimated using the current practices. The objective of this study was to evaluate the prevalence of eosinophilia in sputum (using a cut-off of both 1.2% and 2.3%) in a large clinical database when eosinophil granules, multiple sputum samples, and the effects of corticosteroids are taken into consideration.

Methods

This is a retrospective study of those patients who attended the Firestone Institute for Respiratory Health for assessment of sputum cell counts between July 2004 to September 2020. The results were entered into FileMaker Pro Version 16 (Claris International, Cupertino, CA), a clinical database maintained for this purpose. The database contained demographics, physician diagnosis, reason for the test, whether the patient was stable or exacerbated (clinical diagnosis), and dose of inhaled and oral corticosteroids. In addition, baseline and post-induction spirometry were also recorded. The sputum differential cell count parameters included total cell count ($x10^6$/g), and percentages of viable cells, squamous cell contamination, neutrophil, eosinophil, macrophage, lymphocyte, and bronchial epithelial cells. The presence of free eosinophil granules was enumerated by quantification of degranulated eosinophil clumps per field of view under 400x magnification. They were recorded as none, 1−2 as few, 2−3 as moderate and > 3 as many. Sputum induction and examination of cell counts were performed as described by Pizzichini et al (9).

Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 8.3.1 (GraphPad Software, USA) and R 2020 (R Core Team, Austria). Continuous variables are presented as mean with standard deviation and categorical variables as percentage. Fisher's exact test was used to compare the proportions between eosinophilic and non-eosinophilic groups based on 1) 1.2 and 2.3% without consideration of FEG, 2) 1.2% with few/moderate/many FEG and 2.3% with moderate/many FEG and 3) addition of non-intact differential count based on specifications in 2). In patients with two or more viable cell counts, detailed database mapping assessed for emergent eosinophilia at both thresholds ($\geq$ 1.2% and presence of few/moderate/many FEG and $\geq$ 2.3% and presence of moderate/many FEG) on subsequent samples from the same patient. One-way ANOVA was used to compare the degree of responses for emergent eosinophilia based on resolution of previous neutrophilia (when total cell count $\geq$ 9.7x10^6/g and neutrophil $\geq$ 64.4%), persistent/new neutrophilia, change in dose of corticosteroids (inhaled and/or oral) and disease flare when there was no change in total cell count, neutrophil %, and corticosteroid dose.

Results

There were 24,176 individual records that include both spontaneously expectorated and induced sputum. Of these, 6483 did not possess adequate sputum volume for analysis. The remaining 17,693 comprise 6604 patients with only cell count and 2967 had two or more cell counts for the remaining 11,088 results. 2415 had excessive cell degeneration and therefore a differential cell count could not be obtained, but 1188 of these exhibited FEGs. The demographic, clinical and sputum characteristics for patients that possessed adequate sputum for analysis is shown in Table 1. The relationship between proportions classified as eosinophilic and non-eosinophilic between cut-off thresholds of 1.2 and 2.3% when considering presence/absence of FEG including non-intact cell differential is shown in Table 2. Using the presence of FEG, the proportion between groups changes by 25.6% with eosinophil cut-off of 1.2% as compared to 11.6% when cut-off is 2.3% (p < 0.001). When a non-intact cell count is included, the proportion between groups with FEG changes to 19.6% for 1.2% threshold and 13.1% for 2.3% threshold (p < 0.001).

Table 1
Demographic, clinical and sputum characteristics for those patients whose sputum was viable for analysis (n = 17,693)

	Female, n (%)	Age, years	Daily dose ICS† (mcg)	On ICS, n (%)	Daily dose OCS (mg)	On OCS, n (%)
	7296 (56.2)	58.0 (30.6)	637.9 (633.1)	13,298 (75.2)	3.4 (16.5)	5713 (32.3)
Differential cell count, 10^6/g (%)						
Viability	Total cells	Neutrophil	Eosinophil	Macrophage	Lymphocyte	Bronchial epithelial
66.2 (31.0)	13.4 (23.7)	61.2 (26.6)	4.9 (12.2)	31.8 (24.1)	1.0 (1.5)	0.9 (2.8)

ICS Inhaled corticosteroids; †Fluticasone equivalent; mcg microgram; OCS oral corticosteroids; mg milligram; g gram. Expressed as mean (standard deviation) unless indicated otherwise.

Table 2
Proportions of eosinophilic versus non-eosinophilic using presence/absence of FEG and non-intact differential cell count.

Criteria for assessing eosinophilic inflammation	Sample size	Eosinophilic	Non-eosinophilic	Fisher's exact test P-value
1. Intact eosinophils as cut-off				
1.2% cut-off	n = 10,495	1933/18.4%	8562/81.6%	< 0.001
2.3% cut-off		805/7.7%	9690/92.3%	
2. Presence of FEG with intact eosinophils				
1.2% cut-off with few/moderate/many FEG	n = 15,278	6716/44.0%	8562/56.0%	< 0.001
2.3% cut-off with moderate/many FEG		2952/19.3%	12,326/80.7%	
3. Presence of FEG and/or intact eosinophils (including those without differential cell count)				
1.2% cut-off with few/moderate/many FEG and non-intact cell differential)	n = 17,693	6716/38.0%	10,977/62.0%	< 0.001
2.3% cut-off with moderate/many FEG and non-intact cell differential		3674/20.8%	14,019/79.2%	

When the definition of eosinophilia ≥ 1.2 and 2.3% and the presence of FEG, 4373/65% and 2081/44% samples were classified as Th2. (Fig. 1,2). If only intact eosinophil counts were considered, 1933/29% samples had eosinophils ≥ 1.2% and 2587/55% samples (29%) had eosinophils ≥ 2.3%. Upon the consideration of any free granules and/or eosinophils ≥ 1.2 and 2.3% to identify samples with eosinophilic inflammation, an additional 410/6% and 66/1% samples were captured for total of 6716 and 4734. From the 11,088 samples from patients who provided two or more sputum samples, there were no significant differences between 1.2 and 2.3% threshold groups for re-emergent eosinophilia (p = 0.018). Comparisons between both thresholds were analysed for episodes of emergent eosinophilia (1142, 1083) with associated events per patient (0.38, 0.37), initial neutrophilia (19.1, 28.8), disease flare (57.8, 60.5), treatment of neutrophilic bronchitis (31.4, 27.3), decreased corticosteroid use (26.5, 28.9) and concomitant treatment of neutrophilic bronchitis with decreased corticosteroid dose (9.2, 8.7).

Discussion

This retrospective study using a large clinical database demonstrates that eosinophilia in sputum may be underestimated by as much as 28% if free eosinophil granules are not taken into consideration. Limiting examination to a single time point assessment that does not consider the changes in eosinophilia associated with exacerbations, resolution of concomitant neutrophilia or change in corticosteroid dosages may lead to further underestimation by as much as 60%. This has important clinical relevance,

not only to endotype for selection of patients into clinical trials, but also to make therapeutic decisions about escalating or decrease steroid dosage and initiation of eosinophil-specific biologic therapies.

It is important, for number of reasons, to recognize free eosinophil granules in sputum. Eosinophil cytolysis and release of cationic proteins are a marker of severity and contribute to bronchial epithelial injury and impairs repair (10). Eosinophil peroxidase contributes to the bromination of tyrosine residues (11) and is associated with epithelial dysfunction. Peroxidase activity also triggers an autoimmune reactive process in the airway (12) that has implications for disease severity and response to treatment of asthma with biologics (13). Further, cytolysis and release of extracellular traps may lead to crystal formation within the airways (14, 15). Indeed, recent evidence suggests that Charcot Leyden Crystals, that are the products of auto-crystallization of galectin-10 is regulated independent of IL-5 (16), and the failure to recognize this in sputum may further underestimate the eosinophilic activity in the airways. This needs further investigation. A further underestimation of airway eosinophilic activity may result from failure to recognize these granular proteins within airway macrophages (17).

A second important reason for underestimating airway eosinophilia is when conclusions are drawn from a single time point assessment. The three most clinically relevant factors that might affect eosinophil % are whether sputum was sampled during an exacerbation, the dose of corticosteroids at the time of sampling, and if there was a concomitant neutrophilia that may mask an underlying eosinophilia. Certain airway infections may also directly induce an eosinophilic response that may resolve over time (18). Particularly in patients with COPD, neutrophilic and eosinophilic exacerbations may interchange over time (19).

A limitation of our study is its retrospective nature that is associated with all the inherent biases of such a study design. However, the strengths include the large sample size, robust laboratory methods, and regular stringent external quality control. Another limitation is that we did not characterize patients into specific disease categories such as asthma, COPD, overlap, bronchiectasis etc. This is because of the likely imprecision in the data coding based on a physician diagnosis. Given the large sample size, scrutiny of individual charts to confirm the physician diagnosis was not possible and we did not have approval from our Research Ethics Board for chart review. However, the purpose of this manuscript is not to relative eosinophilia to a particular disease state, rather to report fallacies in estimation of eosinophilia in sputum analysis. Although our analysis was limited to assessment of eosinophilic activity, there might be other aspect of cellular inflammation that are often not taken into consideration leading to inaccurate characterization as a non-T2 endotype. These include lymphocyte (20) and mast cell numbers (21) in sputum that can be identified particularly with more advanced microscopy, flow cytometry, and mass cytometry. They could also be markers of steroid responsiveness and it remains to be seen how often these endotypes may occur in the absence of eosinophilia.

In summary, we highlight the relevance of recognizing free eosinophil granules, and the importance of multiple examinations to identify sputum eosinophilia. This is likely relevant to select patients for anti-

eosinophil clinical trials, to interpret treatment responses, and to guide the use of corticosteroids to treat eosinophil-responsive airway diseases.

Declarations

Acknowledgements

Authorship

PN, MK and AA designed the study and wrote the manuscript. PN, MM, MK and AA collected and analyzed the data and edited the manuscript. All authors read and approved the final manuscript.

Conflict of Interest Statement

PN reports grants and personal fees from AstraZeneca, grants from Novartis, grants and personal fees from Teva, grants from Sanofi, grants and personal fees from Roche, personal fees from Novartis, personal fees from Merck and personal fees from Equillium, outside the submitted work. MM reports a grant from Methapharm Specialty Pharmaceuticals and personal fees from AstraZeneca and GlaxoSmithKline, outside the submitted work. The other authors declare no competing interests or conflicts of interest.

Funding Statement

Competing interests

The other authors declare no competing interests or conflicts of interest.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This study was approved by the Research Ethics Board of St. Joseph's Healthcare Hamilton (RP#11020), for the collection and maintenance of a sputum cell count database for research.

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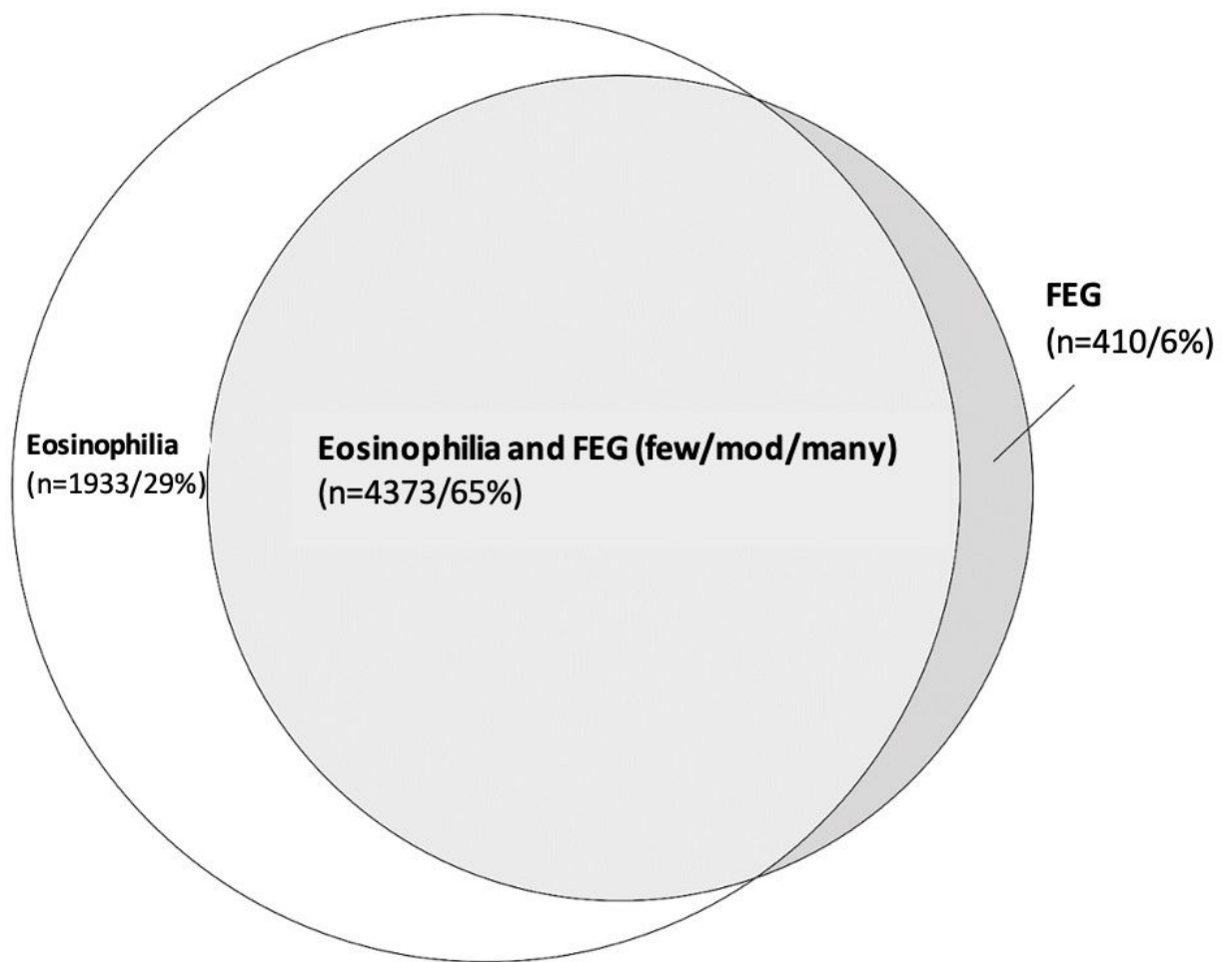
Figures

Eosinophilia
(n=1933/29%)

Eosinophilia and FEG (few/mod/many)
(n=4373/65%)

FEG
(n=410/6%)

Figure 1

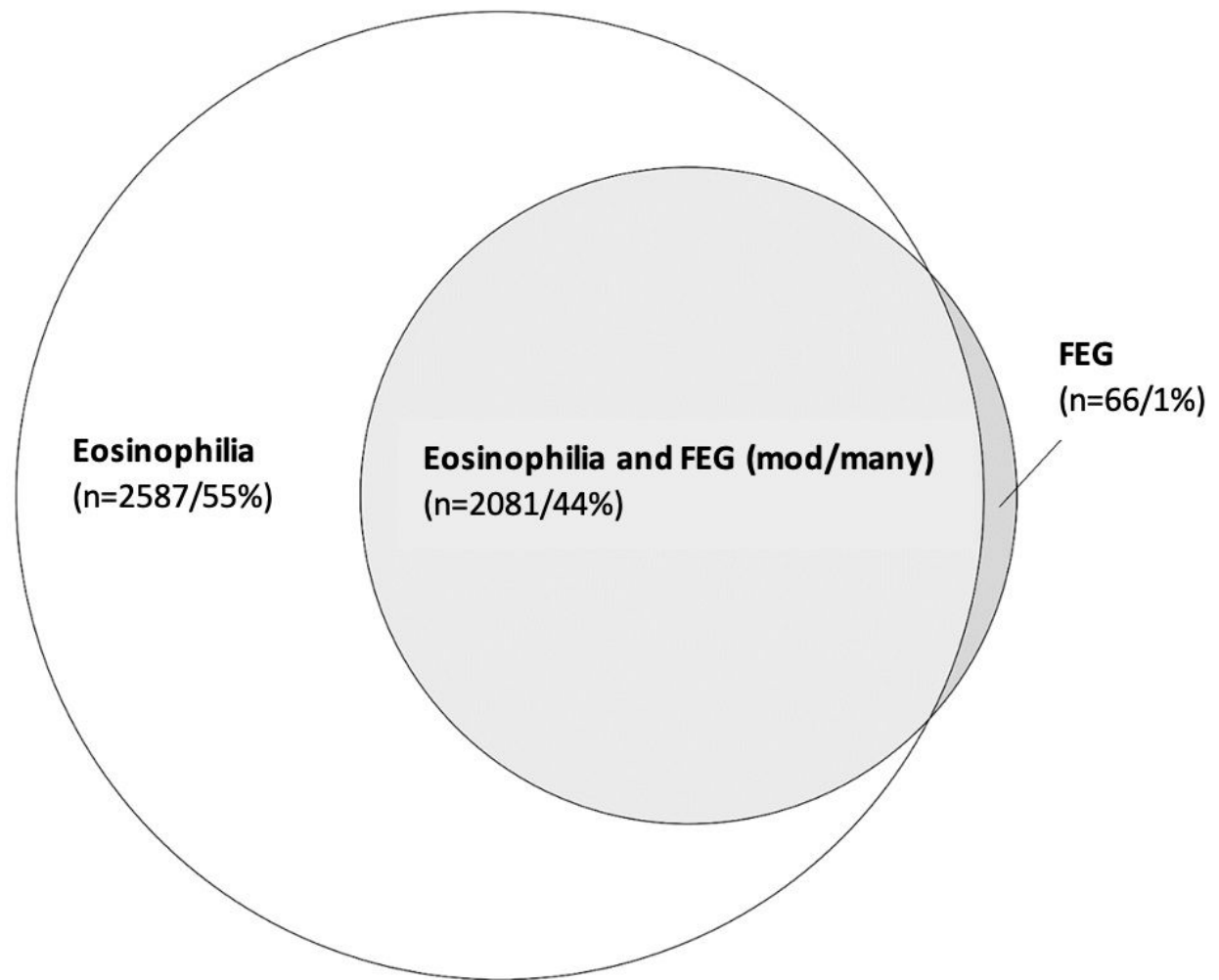Proportions of eosinophilic activity using threshold of 1.2% (n=6716)

Figure 2

Proportions of eosinophilic activity using threshold of 2.3% (n=4734)
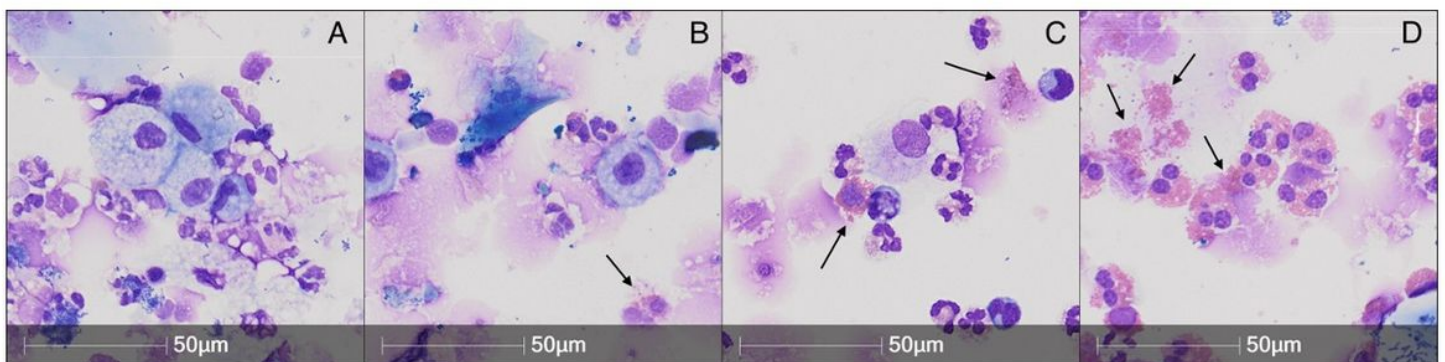

Figure 3

Microscopy images at 40x magnification of sputum cytospins stained with Wright Giemsa demonstrating grading of free eosinophil granules (black arrows); A) none, B) few, C) moderate and D) many(12).