The damaging and protective features of eosinophils in healthy individuals and patients with chronic inflammatory respiratory diseases

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There are no mistakes, just happy little accidents - Bob Ross
Chapter 4
Do eosinophils contribute to oxidative stress in mild asthma?

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TO THE EDITOR

Asthma is an obstructive airway disease that involves chronic inflammation of the bronchial mucosa. In asthmatics, reactive oxygen species (ROS) production is elevated and cannot be countered effectively by antioxidant mechanisms, leading to increased oxidative stress levels compared to healthy subjects. ROS likely originate from inflammatory cells (eosinophils, neutrophils and macrophages) and mitochondria and their deleterious activity can result in lipid peroxidation products, modified proteins and oxidative DNA damage.

Asthma patients suffer from periodic acute worsening of symptoms (exacerbations or loss of control when milder), predominantly triggered by respiratory virus infections and allergen exposure. This is characterized by increased activation and recruitment of inflammatory cells to the airways, in which eosinophils are considered key players, and further enhanced oxidative stress. Eosinophils produce ROS upon exposure to, for example, viruses and allergens and by the concomitant release of eosinophil peroxidase (EPO) are able to brominate the amino acid tyrosine. A link between eosinophils and oxidative stress during asthma exacerbations is thus likely. Indeed, elevated amounts of bromotyrosine in bronchoalveolar lavage fluid were detected after allergen challenge in asthmatics and in patients hospitalized for very severe asthma exacerbations.

Eosinophil formation, maturation, recruitment and survival is regulated by interleukin-5 (IL-5), making this cytokine an important therapeutic target. Attenuation of eosinophils using anti-IL-5 significantly reduced exacerbation rates and corticosteroid dependency in severe asthmatics. Here, we examined the impact of eosinophil depletion on oxidative stress and bromination in stable and virus-induced worsening of asthma. In a recent placebo-controlled study, we attenuated blood and sputum eosinophil numbers and activation with mepolizumab (anti-IL-5) in steroid-naïve mild asthma patients, followed by a challenge with rhinovirus 16 (RV16) to cause loss of asthma control. This study provided a unique opportunity to determine the contribution of eosinophils to oxidative stress and bromination during stable disease and after virus exposure. For this analysis, malondialdehyde (MDA; marker of oxidative stress), dityrosine (marker of oxidative stress), nitrotyrosine (marker of nitrosative stress), chlorotyrosine (marker of myeloperoxidase activity), bromotyrosine (marker of EPO activity) and asymmetric dimethylarginine (ADMA; inhibitor of nitric oxide synthase) were measured in exhaled breath condensate (EBC) and plasma at baseline, after mepolizumab or placebo treatment and after RV16 challenge. Details of the clinical trial
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and technical details of the analyses of biomarkers are available in the Data S1 and in Figure S1.

Dityrosine, chlorotyrosine and bromotyrosine were below the detection limit (0.4, 0.02 and 0.05 nmol/L, respectively) in EBC, while dityrosine and nitrotyrosine were not detected in plasma (detection limits of 2 and 1 nmol/L, respectively). Baseline levels of MDA, nitrotyrosine and ADMA in EBC and MDA, chlorotyrosine, bromotyrosine and ADMA in plasma were not significantly different between the mepolizumab and placebo group and did not change upon treatment (Figure S2). Apparently, blood and sputum eosinophils do not contribute to oxidative stress in stable mild asthma. This is in line with the unaffected bromotyrosine levels in plasma, which like ROS production depend on eosinophil degranulation. These findings may explain why mepolizumab does not improve clinical symptoms in stable mild asthmatics.9

We next determined the impact of the RV16 challenge on oxidative stress in both groups. EBC levels of MDA near-significantly ($P = 0.07$) and levels of nitrotyrosine significantly increased after RV16 exposure in the placebo group, but not in the mepolizumab group (Figure 1A,B). EBC levels of ADMA, which can potentiate oxidative and nitrosative stress, did not change upon virus infection in either group (Figure 1C). After stratification for patients with high (>220/µL blood) and low (≤ 220/µL blood) eosinophil numbers (determined at the first study visit), MDA levels, but not those of nitrotyrosine and ADMA, were significantly increased after RV16 in the placebo group with high eosinophils only (Figure 1D-F). When changes of biomarkers in response to RV16 exposure between patients treated with mepolizumab and placebo were compared, no differences were found (Figure 1G-I). After stratification for eosinophil counts, however, MDA levels increased significantly and nitrotyrosine levels trendwise in the placebo group with high eosinophils as compared to the mepolizumab group with high eosinophils (Figure 1J-L). No significant differences were observed after RV16 for any of the biomarkers in plasma (MDA, chlorotyrosine, bromotyrosine and ADMA) in either group, indicating that systemic markers do not reflect the oxidative status in the airways (Figure S3). Together, our findings suggest that eosinophils may drive local oxidative and, possibly indirectly, nitrosative events during virus-induced loss of asthma control, but independent of ADMA. Given the low patient numbers in each of the stratified groups, these results require verification in larger cohorts.

Besides attenuation of eosinophil numbers and activation, mepolizumab also prevented RV16-induced neutrophil recruitment and activation.8 As a result, even though eosinophils generally possess a more potent respiratory burst,4,5 the effects
**FIGURE 1** Exhaled breath condensate (EBC) levels of MDA (A, D, G and J), nitrotyrosine (B, E, H and K) and asymmetric dimethylarginine (ADMA) (C, F, I and L) before and after RV16 challenge and corresponding delta values in patients treated with placebo (black dots) or mepolizumab (grey triangles). Dots/triangles represent patient individuals; bars and whiskers represent mean ± SEM. Paired or unpaired *t*-tests: *P* < 0.05
described here may not be fully attributed to eosinophils. Also, a role for basophils cannot be excluded, since these cells express the IL-5 receptor as well and therefore may be affected by mepolizumab. Nonetheless, the significantly enhanced (delta) MDA EBC levels in the high eosinophilic placebo group point towards a contribution of particularly eosinophils to the virus-induced increase in oxidative stress. It is likely that more pronounced effects on oxidative stress by eosinophils will occur in severe eosinophilic asthmatics or during severe virus-induced exacerbations.

In conclusion, we have demonstrated for the first time that in mild asthma eosinophils contribute to oxidative and nitrosative stress after RV16-induced loss of asthma control, but not in stable disease. Thus, targeting oxidative stress should be considered as treatment option during asthma exacerbations.
REFERENCES


SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.
METHODS

Study design
The study from which this secondary analysis originates was a double blinded, randomized and placebo-controlled study of 28 steroid-naïve mild asthma patients. In short, subjects received one high dose of mepolizumab (750 mg) or placebo and after two weeks were subjected to experimental RV16 infection (Figure S1). The study was approved by the AMC Medical Ethics Committee and all participants provided written informed consent. The study was registered at clinicaltrials.gov (NCT01520051).

Sample collection
EBC and plasma were collected at baseline (day 0), after treatment (day 11 and 14, respectively) and after RV16 challenge (day 19 and 21, respectively) and stored at -80°C until further analysis. EBC was collected using the ECoScreen1 (Jaeger, Germany) following the European Respiratory Society Methodological Recommendations Task Force. Venous blood was collected in EDTA tubes.

UPLC-MS/MS and UPLC-HRMS measurements
In deproteinized samples of 250 µl EBC and 50 µl plasma, butylated derivatives of dityrosine, 3-nitrotyrosine, chlorotyrosine, bromotyrosine and ADMA were determined by UPLC-MS/MS and UPLC-HRMS using $^{2}$H$_{3}$-3-nitrotyrosine and $^{2}$H$_{4}$-tyrosine as internal standards. Samples were injected on an analytical Acquity HSS T3, 100 × 2.1 mm, 1.8 µm column and analyzed in positive ESI mode on respectively a Xevo TQ MS hyphenated to an Acquity UPLC I-Class (Waters) in MRM mode and a Q-Exactive plus Orbitrap hyphenated to an UltiMate 3000 UHPLC in targeted SIM, exact mass, mode (Thermo Fisher Scientific).

After alkaline hydrolyzation, deproteinization and derivatization with DNPH (2,4-dinitrophenylhydrazine), total MDA was determined on a Quattro Premier XE in positive ESI and MRM mode, hyphenated to an Acquity UPLC system (Waters) equipped with an LC-18-DB analytical column (250 × 4.6 mm, 5 µm particles, Supelco).

Statistical analysis
Statistical analysis was performed using GraphPad Prism 7. P-values <0.05 were considered statistically significant.
REFERENCES


FIGURE S1. Schematic overview of the study.
EBC was collected at day 0, 11 and 19, plasma was collected at day 0, 14 and 21.
FIGURE S2. EBC and plasma levels of MDA (A and D), nitrotyrosine (B), ADMA (C and G), chlorotyrosine (E) and bromotyrosine (F) in patients before and after treatment with placebo (black dots) or mepolizumab (grey triangles). Dots/triangles represent patient individuals; bars and whiskers represent mean ± SEM. Paired or unpaired t-tests: *p<0.05
FIGURE S3. Plasma levels of MDA (A and E), chlorotyrosine (B and F), bromotyrosine (C and G) and ADMA (D and H) before and after RV16 challenge in patients treated with placebo (black dots) or mepolizumab (grey triangles). Dots/triangles represent patient individuals; bars and whiskers represent mean ± SEM. Paired or unpaired t-tests: *p<0.05.