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ORIGINAL ARTICLE

Carbon in airway macrophages from children with asthma

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ABSTRACT

Background Airway macrophage (AM) phagocytosis is impaired in severe asthma. Prostaglandin (PG) E_2 and D_2 are increased in severe asthma and suppress AM phagocytic function in vitro. In this study, we sought evidence for PG-mediated impairment of phagocytosis of inhalable carbonaceous particulate matter (PM) by AM in children with severe asthma compared with mild asthmatics and healthy controls.

Methods AM were obtained from children with asthma and healthy controls using induced sputum. AM carbon area (μm^2) was assessed by image analysis. In a subgroup of asthmatics, urinary PGE_2 and PGD_2 metabolites were measured by high-performance liquid chromatography, and PM exposure at the home address was modelled. Phagocytosis of PM by human monocyte-derived macrophages and rat AM was assessed in vitro by image analysis.

Results AM carbon was 51% lower in children with moderate-to-severe asthma (n=36) compared with mild asthmatics (n=12, $p<0.01$) and healthy controls (n=47, $p<0.01$). There was no association between modelled PM exposure and AM carbon in 33 asthmatics who had a urine sample, but there was an inverse association between AM carbon and urinary metabolites of PGE_2 and D_2 (n=33, rs=−0.40, $p<0.05$, and rs=−0.44, $p<0.01$). PGE_2 10^{-6} M, but not PGD_2 10^{-6} M, suppressed phagocytosis of PM_{10} by human macrophages in vitro ($p<0.05$ vs control). PGE_2 10^{-6} M also suppressed phagocytosis of PM_{10} by rat AM in vitro ($p<0.01$ vs control).

Conclusions Phagocytosis of inhaled carbonaceous PM by AMs is impaired in severe asthma. PGE_2 may contribute to impaired AM phagocytic function in severe asthma.

Key messages

What is the key question?

- Is there evidence of impaired phagocytosis of inhaled carbonaceous particulate matter (PM) in children with severe asthma, and is there a role for prostaglandin (PG) E_2 and D_2 in mediating suppression of phagocytosis?

What is the bottom line?

- The amount of carbon in airway macrophages (AM) from children with severe asthma is 51% lower than healthy controls and mild asthmatics, urinary markers of PGE_2 and PGD_2 are increased in severe asthma, and PGE_2 suppresses phagocytosis of PM by human macrophages in vitro.

Why read on?

- AM carbon is lower in severe asthma, and PGE_2 suppresses AM phagocytosis of PM, results that lead to testable questions; what are the effects of inhaled PM that evades normal AM clearance, and does blocking PGE_2 release improve AM phagocytic capacity?

INTRODUCTION

Studies in children and adults suggest that the capacity of airway macrophages (AMs) to phagocytose inhaled material is impaired in severe asthma. First, Fitzpatrick *et al*[1] reported a 50% reduction of phagocytosis of *Staphylococcus aureus* by AM from children with poorly controlled asthma. Second, Alexis *et al*[2] reported that phagocytosis of opsonised yeast by AM is impaired in adults with eosinophilic asthma compared with healthy controls. Third, Huynh *et al*[3] reported impaired phagocytosis of apoptotic human T cells by AM from adults with severe asthma compared with either healthy controls or adults with less-severe asthma. To date, the mechanism for impaired AM phagocytosis in asthma is unclear, but severe asthma is associated with increased airway levels of eicosanoids that suppress macrophage phagocytosis in vitro. For example, prostaglandin E_2 (PGE_2) is increased in induced sputum from adults with eosinophilic asthma[4 5] and inhibits the phagocytosis of opsonised *Escherichia coli* and sheep red blood cells by rat AM in vitro.[6] Furthermore, PGE_2 inhibits the phagocytosis of sheep red blood cells by murine AM in vitro,[7] and PGE_2, and to a lesser extent PGD_2, inhibits phagocytosis of apoptotic neutrophils by human macrophages in vitro.[8] To date, it is unknown whether PGE_2 and PGD_2 suppress macrophage phagocytosis of fossil fuel-derived particulate matter (PM) in vitro.

Carbonaceous PM is phagocytosed in a dose-dependent manner by AM.[9 10] Studies in animals and humans suggest that the amount of PM in AM is a valid marker of chronic exposure to

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carbonaceous PM from fossil fuel combustion. For example, in rats, the amount of carbon in AM reflects exposure to diesel PM over a 13-week period,[11] and in human adults the amount of carbon in AM is most strongly associated with the modelled mean exposure to inhalable PM (PM <10 microns in aerodynamic diameter; PM_{10}) over the previous 6 months.[12] We, and other groups, have therefore used the area of carbon in AM (AM carbon) to assess the health effects of chronic exposure to PM in children and adults.[13–16] The utility of AM carbon as a marker of personal exposure to PM is based on the assumption that the phagocytic capacity of AM is broadly similar between individuals. However, in a previous small pilot study, we unexpectedly found low to absent AM carbon in a small number of children with severe eosinophilic asthma and speculated that phagocytosis of AM carbon in vivo is impaired in severe asthma.[14] In the present study, we sought to establish definitive evidence for (i) lower AM carbon in children with severe asthma compared with children with either mild asthma or healthy controls, and (ii) a role for PGE_2 and PGD_2 in suppressing phagocytosis.

METHODS

Participants

Asthmatic children were recruited from the Royal London Children's Hospital, the University Hospitals of Leicester, the Royal Brompton Hospital (London) and schools in east London. The major source of carbonaceous PM exposure in children in these urban areas is fossil fuel-derived PM. Current asthma control was established from a review of the clinical notes or a parent-completed International Study of Asthma and Allergies in Childhood questionnaire.[17] Asthma severity was categorised by British Thoracic Society/Scottish Intercollegiate Guidelines Network (BTS/SIGN) criteria. Children with asthma at BTS steps 1–2 were classified as 'mild', and BTS steps 3–5 classified as 'moderate-to-severe'.[18] Healthy children were recruited from east London schools and from siblings of children attending asthma clinics. A subgroup of asthmatic children recruited at the Royal London Children's Hospital also had a urine sample obtained and exposure of their home to PM modelled using (i) the London Air Quality Toolkit (LAQT) and (ii) the home to main road distance. The LAQT is an established emissions dispersion model capable of producing annual mean pollutant concentrations at a resolution of 20 m×20 m.[19] The LAQT models the mean exposure of children's home addresses to PM with mean aerodynamic diameter <2.5 µm ($PM_{2.5}$), for annual, 7-day and 24 h $PM_{2.5}$ prior to sputum induction (see online supplement). Children living in homes within 50 m of a main road were considered to have increased exposure to traffic-derived PM. Written informed assent and consent was obtained from children and parents (Research Ethics Committees approval reference; 08/H0704/139, 11/LO/1732 and 09/H0403/92).

Spirometry and induced sputum

Spirometry was performed according to standard criteria[20] using a Microlab spirometer (Care Fusion, Kent, UK). Post bronchodilator lung function was measured 15 min after 400 µg inhaled salbutamol via a metered dose inhaler and large volume spacer. SD scores (z scores) for FEV_1 and FVC were calculated using Global Lung Initiative software.[21] Children with asthma underwent sputum induction during a clinician-assessed period of stability. Induced sputum was not done within 1 month of an asthma exacerbation requiring oral corticosteroids. Sputum induction and processing was done as previously described[14] using nebulised 4.5% saline via a Multisonic Profi nebuliser (Schill, Germany) (see online supplement).

AM carbon

The area of carbon in AM was determined as previously reported.[14] Briefly, digital images of 50 randomly selected AM from each child were obtained at ×100 magnification, and the mean area (μm^2) of carbon determined. Analysis was blinded to asthma status. Additional details are given in the online supplement.

Urinary PGE_2 and PGD_2 metabolites

Urine was obtained from children at sputum induction, transported on ice and stored at −80°C within 1 h of collection. Analysis was by high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS).[22] Negative ionisation was used to detect 13,14-dihydro-15-keto-tetranor-PGE_2 (-tetranor-PGE_2) and 13,14-dihydro-15-keto-tetranor-PGD_2 (-tetranor-PGD_2). Metabolite concentrations were expressed in proportion to urinary creatinine (see online supplement).

Phagocytosis of PM_{10} in vitro

The effect of PGE_2 and PGD_2 on the phagocytosis of urban PM_{10} by human monocyte-derived macrophages and rat AM was assessed using a modification of the in vitro assay described by Aronoff *et al*[6] (see online supplement). The aim was to first assess the effect of PGE_2 and PGD_2 on human macrophages at the optimal suppressive concentration (10^{-6} M) described by Aronoff *et al*,[6] then confirm any positive findings using rat AM. Briefly, either human monocyte-derived macrophages from healthy adult donors or rat AM were adhered to bovine serum albumin-coated coverslips and cultured with PM_{10} (10 µg/mL) collected from urban filters in Leicester city centre with or without PGE_2/PGD_2 (Sigma-Aldrich, St Louis, Missouri, USA) for 1 h.[6] Phagocytosis of PM_{10} was determined by image analysis of 50 cells per well by an operator blinded to exposure status (described above). Experiments were done on at least six separate occasions.

Statistical analysis

Data are presented as median (IQR). Correlations were done by Spearman's rank correlation (rs) and proportions compared by χ^2 test. Data between two groups were compared by either Mann–Whitney test (unpaired) or Wilcoxon matched-pairs signed-rank test (paired). Comparisons between more than two groups were done by Kruskal–Wallis test and Dunn's multiple comparison test. Analyses were performed using Prism 5.00 for Windows (GraphPad Software, California, USA). Results were considered significant at $p<0.05$.

RESULTS

AM carbon and induced sputum eosinophil differential count (figure 1) were determined in 47 healthy controls, 13 children with asthma at BTS steps 1–2 and 36 children with asthma at BTS steps 3–5 (figure 2, table 1). Subject numbers were determined by the study duration (October 2011–December 2012, see online supplementary figure S1). For asthmatic and healthy children recruited at the Royal London Children's Hospital and in east London schools, 195 children were approached, 129 consented to undergo a sputum induction and a technically acceptable induced sputum (IS) sample was obtained from 72 (56%). One asthmatic child with sufficient number of cells for AM carbon had insufficient leucocytes for an induced sputum differential count. All 33 asthmatic children recruited at the

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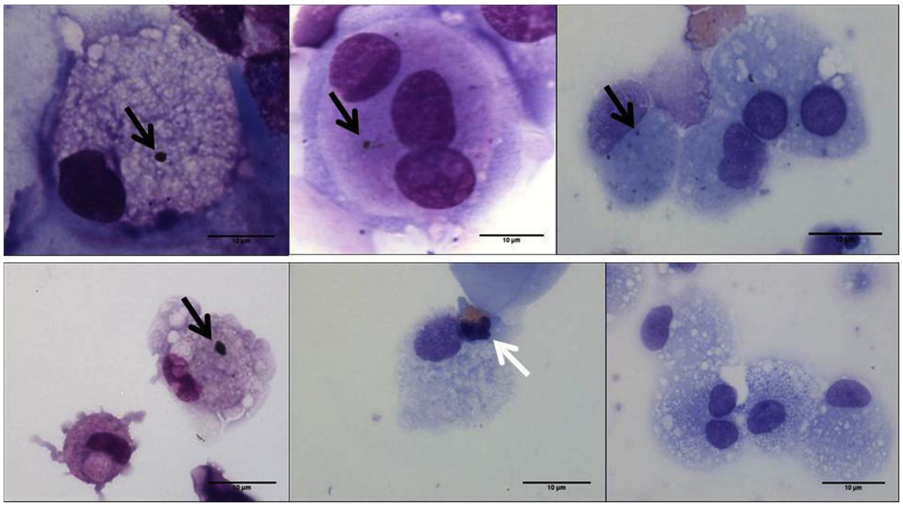

Figure 1 Induced sputum airway macrophages (AM) from children showing phagocytosed inhaled carbon. The black arrows show carbon in AM from healthy children (top row) and from children with moderate-to-severe asthma (bottom row). The white arrow indicates an eosinophil. Images at ×100, bar=10 μm.

Royal London Children's Hospital provided a urine sample for PG analysis. At the University Hospitals of Leicester, 20 children with asthma and 58 healthy controls were approached for IS, 20 asthmatic children and 17 controls agreed to undergo IS. Ten randomly sampled slides from each group were assessed for AM carbon, and AM carbon determined in eight asthmatics and eight controls (see online supplementary figure S1). At the Royal Brompton Hospital, 65 children with asthma were approached for IS, 55 agreed to undergo IS. Eight randomly sampled slides were assessed and AM carbon determined in 8/8 (see online supplementary figure S1). For analysis, data from all three sites were pooled.

Compared with healthy controls, children with moderate-to-severe asthma (BTS steps 3–5), but not those with mild asthma (BTS steps 1–2), had lower postbronchodilator FEV_1 z score and increased induced sputum eosinophils (table 1). In healthy controls, there was no association between AM carbon and age, and no difference in AM carbon between males and females. There was also no significant difference in AM carbon when stratified by site of recruitment in either control or asthmatic groups.

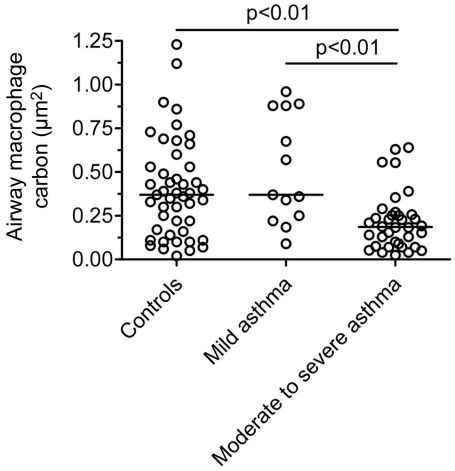

Figure 2 Comparison of airway macrophage (AM) carbon between children with mild asthma (British Thoracic Society (BTS) steps 1–2), moderate-to-severe asthma (BTS steps 3–5) and healthy controls. AM carbon was calculated from 50 AM per child and is expressed as the mean area of carbon per AM (μm^2). Comparison is by Kruskal–Wallis test and Dunn's multiple comparisons test. Bar represents median. Children with moderate-to-severe asthma have lower AM black carbon compared with both healthy controls and mild asthmatics. There is no significant difference between healthy controls and mild asthmatics.

Asthma and AM carbon

Asthmatic children with moderate-to-severe asthma had 51% less AM carbon compared with both healthy controls ($p<0.01$) and mild asthmatics ($p<0.01$, table 1, figure 2). There was no difference in AM carbon between healthy controls and children with mild asthma (table 1, figure 2). In asthmatics (n=49), there was an inverse association between AM carbon and sputum eosinophils (%) (rs=−0.32, $p<0.05$), but not with sputum neutrophils (%) ($p=0.14$). AM carbon was therefore lower in asthmatic children with sputum eosinophilia defined as ≥2.5% (0.16 vs 0.25 μm^2, $p<0.01$, figure 3). AM carbon in asthmatics was also inversely associated with daily inhaled corticosteroid dose (rs=−0.31, $p<0.05$) and age (rs=−0.44, $p<0.01$).

Urinary PG metabolites and AM carbon

In the 33 asthmatic children who had a urine sample taken for PG analysis, exposure to air pollution at the home address was determined by the LAQT air pollution model for 23/33, and the home–road distance assessed in 33/33. There was no difference in modelled annual mean $PM_{2.5}$, 7-day $PM_{2.5}$ or 24 h $PM_{2.5}$ between mild and moderate-to-severe asthmatics (table 2), and no difference in the proportion of homes within 50 m of a main road between the two asthmatic groups (table 2). There was no association between modelled PM exposure and AM carbon in 33 asthmatics who had a urine sample. Urinary PG metabolites (13,14-dihydro-15-keto-tetranor-PGE_2 and 13,14-dihydro-15-keto-tetranor-PGD_2) were highly correlated (rs=0.89, $p<0.001$), and both metabolites were increased in moderate-to-severe asthma ($p<0.05$, $p<0.01$ vs mild asthmatics for -tetranor-PGE_2 and -tetranor-PGD_2, figure 4, table 2). AM carbon was inversely associated with urinary metabolites of PGE_2 (rs=−0.40, $p<0.05$) and PGD_2 (rs=−0.44, $p<0.01$). There was an association between sputum eosinophils and urinary PGE_2 and PGD_2 metabolites (rs=0.35, $p<0.05$, and rs=0.39, $p<0.05$, respectively). Compatible with results from all children, AM carbon in the subgroup with a urine sample was lower in moderate-to-severe asthmatics compared with mild asthmatics ($p<0.01$, table 2, see online supplementary figure S2).

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Table 1 Demographics and airway macrophage carbon in healthy controls and children with asthma

	Healthy controls	Mild asthma (BTS steps 1 to 2)	Moderate-to-severe asthma (BTS steps 3 to 5)
n	47	13	36
Age (year)	9.34 (8.9 to 9.7)	9.41 (9.1 to 9.6)	11.63*** (9.8 to 13.4)
Gender (M/F)	21/26	11/2*	18/18
Postbronchodilator FEV_1 (z-score)	−0.16 (−0.89 to 0.46)	−0.25 (−0.78 to 0.23)	−1.26*** (−1.93 to −0.79)
ICS dose (μg BDP equivalent/day)	Not applicable	0 (0 to 150)	500 (400 to 750)
LABA (n)	Not applicable	0	27
LTRA (n)	Not applicable	0	16
Induced sputum eosinophils (%)	0 (0 to 0)	0 (0 to 0.60)	3.5*** (0.32 to 9.84)
Induced sputum neutrophils (%)	1.75 (0 to 22.5)	1.00 (0.13 to 16.8)	16.00** (6.5 to 39.3)
Induced sputum airway macrophage carbon (μm^2)	0.37 (0.16 to 0.53)	0.37 (0.24 to 0.88)	0.19** (0.08 to 0.26)

Data are described as median (IQR). Comparison between groups by Kruskal–Wallis test and Dunn's multiple comparisons test. There is a higher proportion of males with mild asthma ($p<0.05$ vs controls, χ^2 test). *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus healthy controls.
BDP, beclomethasone dipropionate; BTS, British Thoracic Society; ICS, inhaled corticosteroid; LABA, long acting β_2 agonist; LTRA, leukotriene receptor antagonist.

Phagocytosis of PM_{10} in vitro

Phagocytosis of PM_{10} by human monocyte-derived macrophages was suppressed by PGE_2 10^{-6} M ($p<0.05$ vs control, figure 5A), but not by PGD_2 10^{-6} M (figure 5B). PGE_2 at 10^{-6} M also suppressed phagocytosis of PM_{10} by rat AM ($p<0.05$, figure 6).

DISCUSSION

This study found, compatible with our previous pilot data,[14] that AM carbon in children with moderate-to-severe asthma is approximately half that of mild asthmatics and healthy controls. It is unlikely that lower AM carbon in children with severe asthma reflects lower exposure to PM since no difference was found in either modelled exposure to PM at the home address or in the proportion of homes within 50 m of a main road in the subgroup where this was assessed. Children with more severe asthma were older than mild asthmatics, and as expected received higher doses of inhaled corticosteroids. Although there were inverse associations between AM carbon and both age and daily inhaled therapy in asthmatics, these are unlikely to be causal. First, as reported previously in healthy children,[14] we found no effect of age on AM carbon in healthy controls in the present study. Second, Fitzpatrick *et al*[1] previously reported that phagocytosis of bacteria by AM in vitro is not attenuated in children receiving regular inhaled corticosteroid therapy for non-asthmatic symptoms. Third, AM phagocytic function is not impaired by β_2-adrenoreceptor agonists in vitro.[23]

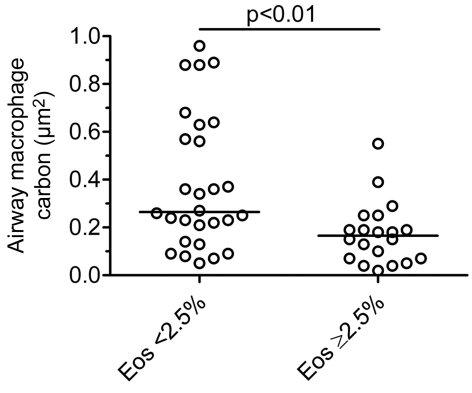

Figure 3 Comparison of airway macrophage (AM) carbon between children with eosinophilic (≥2.5%, n=20) and non-eosinophilic (<2.5%, n=28) asthma. Eosinophil (Eos) differential count is from 400 induced sputum leucocytes per child. AM carbon is lower in children with eosinophilic asthma ($p<0.01$, Mann–Whitney test). Bar represents median.

Lower AM carbon in children with severe asthma is compatible with previous studies of AM using other phagocytic targets. For example, Huynh *et al*,[3] in a study of adults with severe asthma, reported a reduced number of apoptotic bodies in AM (reflecting decreased phagocytosis of apoptotic cells in vivo[24]) compared with healthy controls, and in children with poorly controlled asthma, Fitzpatrick *et al*[1] reported an impaired capacity of AM to phagocytose *S aureus* in vitro. The lack of an effect of mild asthma on the capacity for AM phagocytosis was first suggested by Alexis *et al*,[2] who reported no difference in the capacity of induced sputum AM to phagocytose opsonised yeast in mild asthmatics compared with healthy controls, and second by Lay *et al*,[25] who found that phagocytosis of inhaled particles by AM in vivo was not impaired in mild asthmatics compared with healthy controls. It is unlikely that lower AM carbon in severe asthma is due to less PM inhaled into the lower airway since studies in adults suggest increased lower airway deposition of PM in asthmatics compared with controls.[26 27] An increased number of AM in asthma could also result in lower AM carbon. To date, AM density in children with asthma has not been studied, but no increase in bronchoalveolar lavage AM concentration has been reported in a murine model of asthma.[28]

The mechanism causing impaired AM phagocytosis in severe asthma is unclear. Two studies to date have assessed the association between AM phagocytosis in asthma and airway inflammation. Simpson *et al*[29] reported that uptake of apoptotic bronchial epithelial cells by AM in vitro from adults with non-eosinophilic asthma is impaired compared with adults with eosinophilic asthma. By contrast, Alexis *et al*[2] reported that phagocytosis of opsonised yeast particles by AM in vitro is attenuated in adults with eosinophilic asthma compared with a non-eosinophilic asthmatic phenotype. Compatible with Alexis *et al*,[2] we found that AM carbon was indeed lower in children with eosinophilic asthma when defined either by a cut-off of ≥2.5%[30] or by a cut-off of >5%[2] ($p<0.05$, see online supplementary figure S2).

PGE_2 and PGD_2 are putative mediators of impaired phagocytosis in severe asthma since they both are associated with eosinophilic asthma and directly suppress AM phagocytosis in vitro.[4 31] Profita *et al*[4] reported increased induced sputum PGE_2 in asthmatic adults with bronchodilator reversibility >15% and peak

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Table 2 Prostaglandin E_2 and D_2 metabolites and modelled exposure to air pollution in asthmatic children in whom a urine sample was obtained

	Mild asthma (BTS steps 1–2)	Moderate-to-severe asthma (BTS steps 3–5)
N	13	20
Induced sputum eosinophils (%)	0 (0 to 0)	2** (0 to 10)
Alveolar macrophage carbon (μm^2)	0.37 (0.24 to 0.88)	0.19** (0.11 to 0.26)
Mean annual $PM_{2.5}$ ($\mu g/m^3$)	14.3 (14.1 to 14.6)	14.3 (13.7 to 14.5)
Mean 7-day $PM_{2.5}$ ($\mu g/m^3$)	15.3 (9.3 to 18.0)	12.4 (10.1 to 21.0)
Mean 24 h $PM_{2.5}$ ($\mu g/m^3$)	9.5 (7.9 to 10.1)	11.5 (8.9 to 18.1)
Home address ≤50 m from a main road (n)	2	4
Urinary 13,14-dihydro-15-keto-tetranor-PGE_2 (pg/mg creatinine)	250 (119 to 450)	505* (218 to 937)
Urinary 13,14-dihydro-15-keto-tetranor-PGD_2 (pg/mg creatinine)	225 (170 to 470)	779** (259 to 1149)

$PM_{2.5}$, particulate matter <2.5 μm in aerodynamic diameter modelled at home address for 23/33 children. Sputum eosinophil differential is derived from 400 induced sputum leucocytes per child. AM carbon is derived from 50 AM per child. Urine samples were obtained from all children recruited via the Royal London Hospital. Data are described as median (IQR).
*p<0.05 **p<0.01 versus mild asthmatics by Mann–Whitney test.
AM, airway macrophage; BTS, British Thoracic Society; PG, prostaglandin.

flow variability >20%,[4] Aggarwal *et al*[5] reported increased induced sputum PGE_2 in severe, but not in mild asthmatics, and Fajt *et al*[31]reported increased bronchoalveolar lavagefluid PGD_2 in severe asthmatic adults. PGE_2 in vitro inhibits phagocytosis of opsonised sheep red blood cells and Klebsiella *pneumoniae* by rat AM,[6] and PGE_2, and to a lesser extent PGD_2, suppresses phagocytosis of apoptotic neutrophils by human monocyte-derived macrophages.[8] In the present study, the inverse association between urinary metabolites of PGE_2 and PGD_2 and AM carbon provides indirect evidence of a role for both mediators in suppressing the phagocytosis of inhaled fossil fuel-derived carbon. Further support for a role of PG is provided by our in vitro studies that show, for the first time, that PGE_2 10^{-6} M markedly suppresses phagocytosis of PM_{10} by human macrophages and rat AM. The lack of effect of PGD_2 at 10^{-6} M on phagocytosis of PM_{10} is compatible with Rossi *et al*,[8] who reported that while PGE_2 10^{-6} M suppresses by 60% phagocytosis of apoptotic cells by human monocyte-derived macrophages, PGD_2 at a concentration of 10^{-5} M suppresses phagocytosis by only 20%.[8] A limitation of our study is that we did not measure airway PG directly since HPLC-MS is not suitable for small sample volumes obtained using induced sputum, and PGs are rapidly metabolised in the plasma during sampling.[32] However, the urinary metabolites measured in the present study are plausible markers of systemic levels since they result from a common metabolic pathway that includes a reduction of the double bond between C-13 and C-14 and oxidation of the hydroxyl group at C-15, producing 13,14-dihydro-15-keto PGs[33]—metabolites that are excreted unchanged in the urine.[22 34] Although we have shown that PGE_2-mediated suppression of phagocytosis is biologically plausible and have provided indirect evidence for this association in vivo, we cannot exclude a role for 'intrinsic' changes in phagocytic capacity of AM in severe asthma. For example, Fitzpatrick *et al*[35] reported that AM from children with severe asthma are characterised by a distinct molecular phenotype that does not have a clear Th1 or Th2 pattern.

In summary, in children with moderate-to-severe asthma with no evidence of lower exposure to fossil fuel-derived PM, we found lower AM carbon compared with mild asthmatics and healthy controls. Indirect evidence was found of increased systemic concentrations of both PGE_2 and PGD_2—mediators that suppress alveolar macrophage phagocytosis in vitro. Of these, our in vitro studies suggest that PGE_2 is a potent suppressor of AM phagocytosis of PM in vivo. The consequences of impaired removal of inhaled PM by AM are unknown in humans, but in mice inhaled particles that are normally non-toxic to wildtype animals are toxic to animals deficient in the receptor required for efficient AM phagocytosis of PM.[36]

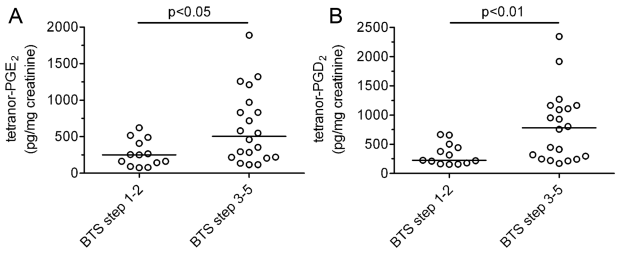

Figure 4 Effect of asthma severity on urinary metabolites of (A) prostaglandin E_2 (PGE_2) and (B) PGD_2 in the subgroup of 33 asthmatic children where a urine sample was obtained. Urinary 13,14-dihydro-15-keto-tetranor-PGE_2 and 13,14-dihydro-15-keto-tetranor-PGD_2 (-tetranor-PGE_2/D_2) is higher in moderate-to-severe asthmatics compared with mild asthmatics. Urinary metabolites of PGE_2 and PGD_2 were measured using high-performance liquid chromatography–tandem mass spectrometry. Comparison by Mann–Whitney test. Bar represents median.

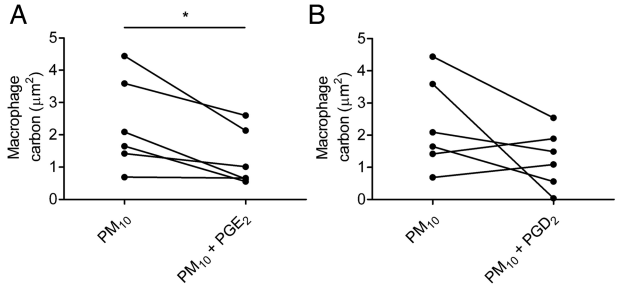

Figure 5 Effect of (A) prostaglandin E_2 (PGE_2) and (B) PGD_2 on the phagocytosis of fossil fuel-derived particulate matter (PM) <10 μm in aerodynamic diameter (PM_{10}) by human monocyte-derived macrophages. Macrophages were adhered onto coverslips, cultured with either PGE_2 10^{-6} M or PGD_2 10^{-6} M for 10 min, then incubated with PM_{10} 10 μg/mL for 1 h. Phagocytosis of carbon was assessed by image analysis of 50 randomly selected macrophages per coverslip by an operator blinded to exposure and expressed as mean area of carbon (μm^2). Preincubation with PGE_2, but not with PGD_2, suppressed phagocytosis of PM_{10} (*p<0.05, vs medium control by Wilcoxon matched-pairs signed-rank test). Data are from six separate experiments done at separate times.

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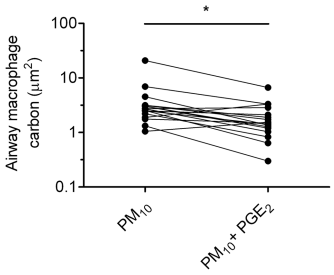

Figure 6 Effect of prostaglandin E_2 (PGE_2) on phagocytosis of urban particulate matter <10 μm in aerodynamic diameter (particulate matter$_{10}$ (PM_{10})) by rat airway macrophages (AMs). AM were obtained by bronchoalveolar lavage and adhered onto coverslips. Rat AM were cultured with PGE_2 10^{-6} M for 10 min, then incubated with PM_{10} (10 μg/mL) for 1 h. Phagocytosis of carbon was assessed using image analysis. Paired data are from AM from the same animal. Preincubation with PGE_2 10^{-6} M suppresses PM_{10} phagocytosis by AM (*p<0.01 by Wilcoxon matched-pairs signed-rank test). Data are from 17 separate experiments in separate animals done at separate times.

Contributors REB recruited participants, supervised sampling, analysed sputum samples and performed phagocytosis assays, and wrote the first draft of the manuscript. NM processed sputum samples, supervised phagocytosis assays and contributed to the manuscript. TR recruited participants, supervised sampling and contributed to the manuscript. DHG recruited participants and supervised sampling. ID recruited participants, and supervised sampling and spirometry. EG and LJF provided samples for validation and contributed to the manuscript. LK differentiated monocytes, conducted phagocytosis assays on human cells and contributed to the manuscript. DJL performed geocoding and contributed to the manuscript. MS performed analysis of samples and contributed to the manuscript. HEW recruited participants and supervised sampling and contributed to the manuscript. BB provided pollution exposure estimate calculations and contributed to the manuscript. ISM participated in subject recruitment and sample collection and contributed to the manuscript. FJK and CJG supervised recruitment of participants and sampling. JG devised the study, supervised recruitment of participants, supervised sampling, data analyses, and phagocytosis assays and wrote the first draft of the manuscript with REB.

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Competing interests None.

Patient consent Obtained.

Ethics approval London—Camberwell St Giles Research Ethics Committee.

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Data sharing statement Additional anonymised data for analysis of carbon in individual macrophages, including stored images, as well as HPLC-MS results from urine and air pollution monitoring data are available upon request to the corresponding author.

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Online supplement

Inhaled fossil fuel-derived particulate matter in airway macrophages from children with asthma

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Exposure to PM at the home address

The London Air Quality Toolkit (LAQT) is an established emissions dispersion model capable of producing annual mean pollutant concentrations across Greater London at a resolution of 20 m x 20 m. Development of the LAQT is described in Kelly *et al* [1]. The LAQT provided an estimate of the annual mean $PM_{2.5}$ concentration (for the year 2010) at each home postcode, representing chronic exposure to black carbon pollution. As children underwent sputum induction over a wide time period, it was important to also consider acute exposure in the period immediately prior to the extraction. This was achieved by scaling the annual mean concentration according to a factor (f), defined as the ratio between $PM_{2.5}$ concentrations measured by a local subset of continuous air pollution monitoring sites (L) in the prior period (t), and the annual mean (a) measured by the same sites. Thus, the acute exposure concentration estimate [P] at time t for point (x,y) was calculated as:

$$[P]_t^{(x,y)} = f.[P]_a^{(x,y)} \quad where\ f = \frac{[P]_t^L}{[P]_a^L}$$

Sensitivity tests were made using 24 h and seven day prior periods. Factors (f) ranged from 0.5 to 2.6 for the 24 h prior period and 0.6 to 2.2 for the seven day prior period, i.e., acute exposures to $PM_{2.5}$ concentrations were between 50% and 260% of the annual mean chronic exposure. To identify homes within 50 m of main road, postcodes were geocoded using the National Statistics Postcode Directory via the geoconvert web-service (http:// geoconvert.mimas.ac.uk). Each postcode was assigned an easting and northing coordinate pair relating to the centroid of the given postcode, using the British national grid projected coordinate system. Main roads with high traffic density ("A" roads using the UK definition) were extracted from the integrated transport network layer of the Ordnance Survey MasterMap product (Ordnance Survey © Crown

copyright 2013). Using Esri ArcGIS ® 10 software (ESRI, CA, USA), the Euclidean (i.e. straight-line/crow fly) distance (m) from the home postcode to the nearest A Road was computed.

Spirometry, sputum induction and sample processing

Spirometry was performed according to ATS/ERS criteria using a Microlab spirometer (Care Fusion, Kent, UK). Children were given 400 micrograms of inhaled salbutamol (albuterol) via a multidose inhaler and large volume spacer (Volumatic, Allen and Hanbury, UK). Post-bronchodilator lung function was measured after 15 min. Sputum induction was performed using nebulised 4.5% hypertonic saline via a Multisonic Profi nebuliser (Schill, Germany) for a maximum of 20 minutes following ERS guidelines for sputum induction in children [2]. FEV_1 and FVC were measured at 5-minute intervals to monitor for the risk of bronchospasm. Standard deviation scores (z-scores) were calculated using GrowingLungs software [3,4]. Samples were placed on ice for transport until processing within 1 h. Sputum was processed by selecting for cell-rich plugs of airway mucus as per the method of Pizzichini *et al* [5]. Whole-sputum samples were diluted in phosphate buffered saline (PBS) and agitated. Airway plugs were resuspended in PBS and then selected on visual inspection, aspirated via a Pasteur pipette into a Bijou. 0.1% DTT (Dithiothreitol, Sigma-Aldrich, St Louis, MO, USA) was added and the samples were vortexed, then gently agitated on ice for 15 min to facilitate mucolysis. Samples were then passed through a gauze filter and centrifuged at 10000 rpm for 10 min. Following centrifugation, the cell pellets were re-suspended in PBS. An aliquot was then transferred into cytospin funnels and spun at 1500 rpm for 3 min. Microscope slides were stained with Hemacolour (Merck,

Darmstadt, Germany), allowed to dry and mounted with glass coverslips using Vectamount fluid, for imaging under oil.

Airway macrophage black carbon

Analysis of the area of carbon in induced sputum AM was based on our previously reported method [6]. Digital photographs of 50 AM per child were obtained using the x100 objective under oil. Digital images were transferred into Photoshop 12.0 (Adobe, San Jose, CA, USA) to generate an individual image for each macrophage. Image files were then imported into ImageJ 1.44p (National Institutes of Health, USA) and converted into 32-bit black and white images. In conjunction with the original image, the image threshold is adjusted to identify darkly stained areas of the cell; typically this includes the nucleus, phagocytosed inorganic carbon particulates (which are black) and adherent bacteria (which are typically Gram positive and stain purple). In direct comparison with the original image, areas of carbon deposited within the macrophage are selected using the freehand tool and the software generates a number of pixels which is converted to an area in micrometers squared (by comparing a known distance on a graticule to a set number of pixels in the image: for our analysis 1473 pixels correspond to 100 micrometers when imaging at x100 magnification). AM carbon is generated as mean area of carbon (μm^2). The operator was blind to subject or exposure status. This methodology was used to assess macrophage phagocytosis of PM *in vitro* (below)

Urinary PGE_2 and PGD_2 metabolites

Urine was obtained immediately following sputum induction and stored on ice until transfer to storage at -80°C (within one hour). Analysis was by high performance liquid chromatography–tandem mass spectrometry (HPLC-MS). Chemically identical deuterated internal standards (1ng all reagents from Cayman Chemical Company, AnnArbor, MI) were added to each urine sample (0.5 mL), acidified with acetic acid (pH 4.5), then extracted twice with methyl tertbutyl-ether and dried under nitrogen. Methanol dissolved aliquots (10 µL, in methanol) were injected onto a reverse phase column (Zorbax Eclipse XDB C-18, Agilent Technologies, Inc. Santa Clara, CA, USA), stabilized thermally at 37 ◦C and a gradient consisting of two mobile phases: A acetonitrile/water/acetic acid (20/80/0.0001) and B acetonitrile/iso-propanol/acetic acid (55/45/0.0001, v/v) was used to elute eicosanoids with the flow rate 0.11 mL/min using HPLC equipped with an autosampler (Shimadzu Sil-2-AC, Shimadzu Scientific Instruments, Inc. Columbia, MD, USA). The mobile phase binary linear gradient was 1 min 8% B, 9.5 min 8–95% B, 0.5 min 95% B, 0.5 min 95–100% B, 2 min 100% B. Analytes were measured using multiple reaction monitoring mode (MRM) tandem mass spectrometry (Qtrap 4000, Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ion source. Negative ionization was used for 13,14-dihydro-15-keto-(tetranor)-PGE_2 and PGD_2 The lowest limit of quantification of the eicosanoids was 1.84 pg/mg creatinine. Urine sample extract was prepared by a two step derivatisation to pentafluorobenzyl and trimethylsilyl esters which modified carboxyl and hydroxyl groups of the compound, and were purified by a thin-layer chromatography. A gas chromatography negative–ion chemical ionization mass–spectrometry was used for quantification (model Engine 5989B series II Helwett Packard, Palo Alto, CA). Detected ions mass to charge ratio and retention times are published elsewhere [7]. All the solvents were of HPLC grade and purchased from Mallincrodt Baker, Inc. Phillipsburg, NJ, USA), while other

chemicals were from Sigma–Aldrich Co. (St. Louis, MO, USA). Ethical approval for urine sampling was limited to children recruited by researchers at the Royal London Hospital.

Phagocytosis assay

Human monocyte-derived macrophages were differentiated from donated human blood (40 ml). This was further diluted at a 2:1 ratio in the medium RPMI 1640 supplemented with 25mM HEPES buffer (Sigma-Aldrich UK). The diluted sample was then layered onto Ficoll-Paque reagent (GH-Healthcare Life Science) in a 50 ml Falcon tube at a 1:1 ratio. Following centrifugation for 20 min at 650g, the peripheral blood mononuclear cell (PBMC) layer was aspirated, diluted further in RPMI, centrifuged for a further 10 min at 2000 rpm, resuspended into minimacs buffer and then washed 3 times (centrifuging for 5 min at 1700 rpm) in minimacs buffer.

Cells were then resuspended in 100µls of minimacs buffer with 40µls of CD14 microbeads (Miltenyi Biotec-Germany), and then incubated at 4°C for 15 min. PBMCs were isolated using the magnetic-activated cell sorting system (MACS) (Miltenyi Biotec, Germany). Following separation, cells were resuspended in RPMI 1640 supplemented with Glutamax and 25mM HEPES (Gibco) along with 1% penicillin-streptomycin (SIGMA) and heat inactivated fetal bovine serum (Lonza) . Cells were plated into 24 well plates at 5×10^5 cells/ml and stimulated with 100ng/ml of GM-CSF (PeproTech) and incubated at 5% CO_2 at 37°C for 7 days. Media and cytokines were refreshed on day 4.

Alveolar macrophages (AM) were obtained from female Wistar rats by bronchoalveolar lavage (BAL). Rats were culled using an intraperitoneal injection of 200mg (in 1mL) phenobarbitone

(Euthatal, Merial Animal Health Ltd, Harlow, UK). Blood was removed from the pulmonary circulation by percutaneous needle aspiration of the heart. The trachea and mediastinum were then exposed. The trachea was cannulated using a 20G Venflon (Becton Dickinson, New Jersey, USA) and 5ml aliquots of sterile PBS were instilled and aspirated via a 3-way tap. Aspirates were collected in a universal container and transported on ice. Prior to the phagocytosis assay, glass coverslips were placed into a 24-well plate and coated with 5% bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO, USA) and left to air dry. 1 $x10^5$ monocyte derived macrophages/rat AM were suspended in 1ml of DMEM (Dulbecco's Modified Eagle's Medium, Lonza) and added to each well. Cells were incubated (37^oC, 5% CO_2) for 2 h to facilitate adherence to the coverslips. PM_{10} on TX40 Teflon-coated glass fibre filter cartridges (Air Monitors Ltd, Tewkesbury, UK) was obtained from an air pollution monitoring station sited in the centre of Leicester (UK). PM was extracted from filters by sonication in PBS (Sigma-Aldrich, Gillingham, UK) and the extracted dose indexed to the optical density of known concentrations of ultrafine carbon black (UF-CB) in phosphate buffered saline, as previously described [8]. PM_{10} was suspended in PBS and sonicated at 10 μm amplitude for 1 min to decrease particle aggregation (Soniprep 150, MSE, London UK).

For the phagocytosis assay, macrophages/rat AM were incubated for 10 min with either PGE_2, PGD_2 (Sigma-Aldrich, St Louis, MO, USA) or medium control, then incubated with PM_{10} 10 μg for 1 h. Glass coverslips were then removed from the wells, air dried, and cells stained with Hemacolor (Merck, Darmstadt, Germany). Following staining, 50 randomly imaged macrophages or rat AM for each exposure condition were imaged and analysed for carbon area as described above.

Figure Legend (Online)

Online figure 1. STROBE flowchart outlining recruitment of children who underwent induced sputum for airway macrophage carbon analysis, modelled air pollution exposure at the home address and provided urine samples.

Online figure 2. Comparison of airway macrophage (AM) black carbon between asthma British Thoracic Society (BTS) step 1-2, and BTS step 3-5 in the subgroup of children in whom a urine sample was obtained for prostaglandin metabolite analysis. Airway macrophage carbon was calculated from 50 AM per child and is expressed as the mean area of carbon (μm^2). Comparison between groups is by Mann Whitney test. Bar represents median. Asthmatic children at BTS step 3-5 have lower AM black carbon compared with both healthy controls and BTS step 1-2.

Online figure 3. Comparison of airway macrophage (AM) black carbon between children with eosinophilic ≥5.0% and non-eosinophilic (< 5.0%) asthma. Airway macrophage black carbon is lower in children with eosinophilic asthma defined using this higher cut-off ($p < 0.05$, Mann Whitney test). Eosinophil (Eos) differential counts are from 400 induced sputum leucocytes per child.

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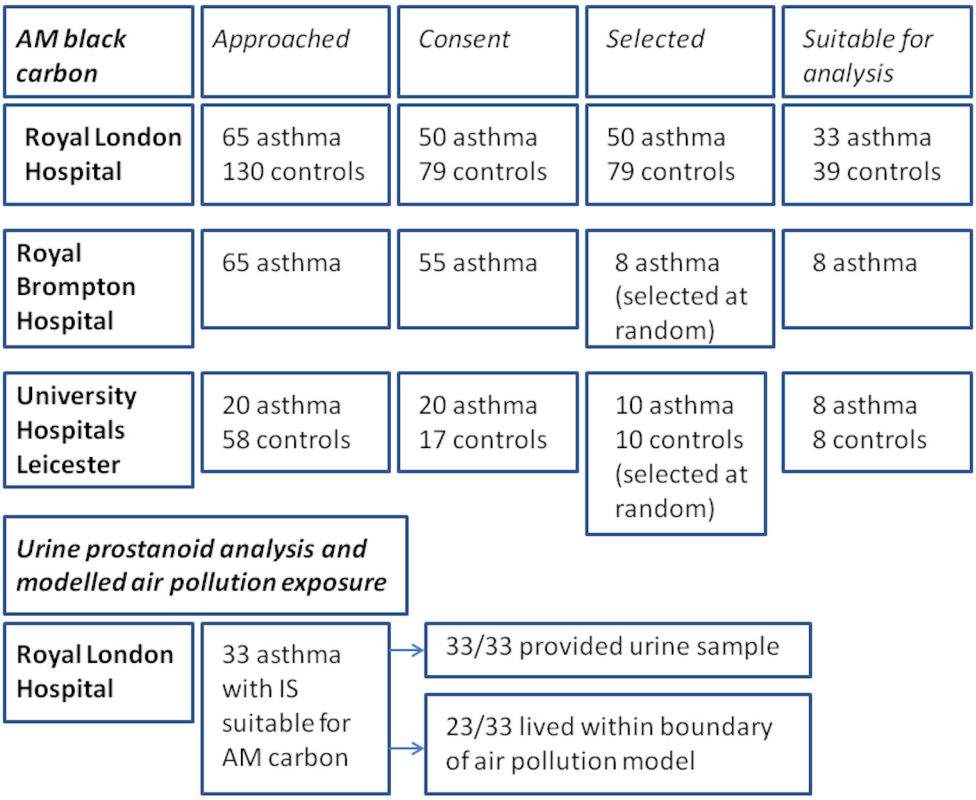

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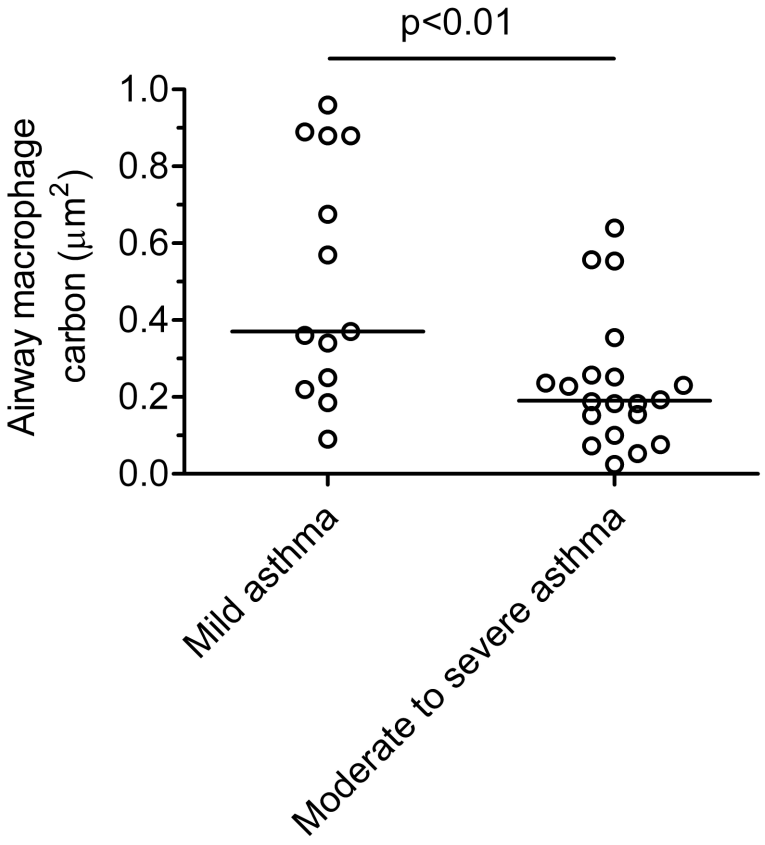
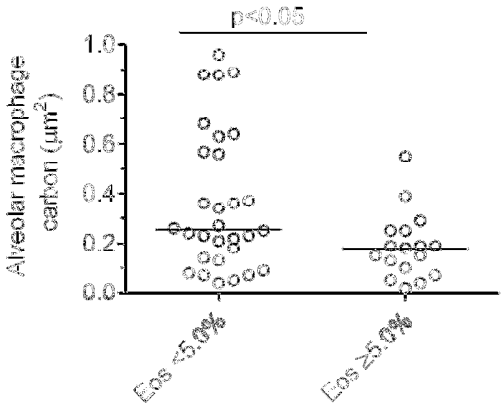
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