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^{o}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^{o}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 x 10^5 cells/ml and stimulated with 100ng/ml of GM-CSF (PeproTech) and incubated at 5% CO_2 at 37^{o}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.

References (Online)

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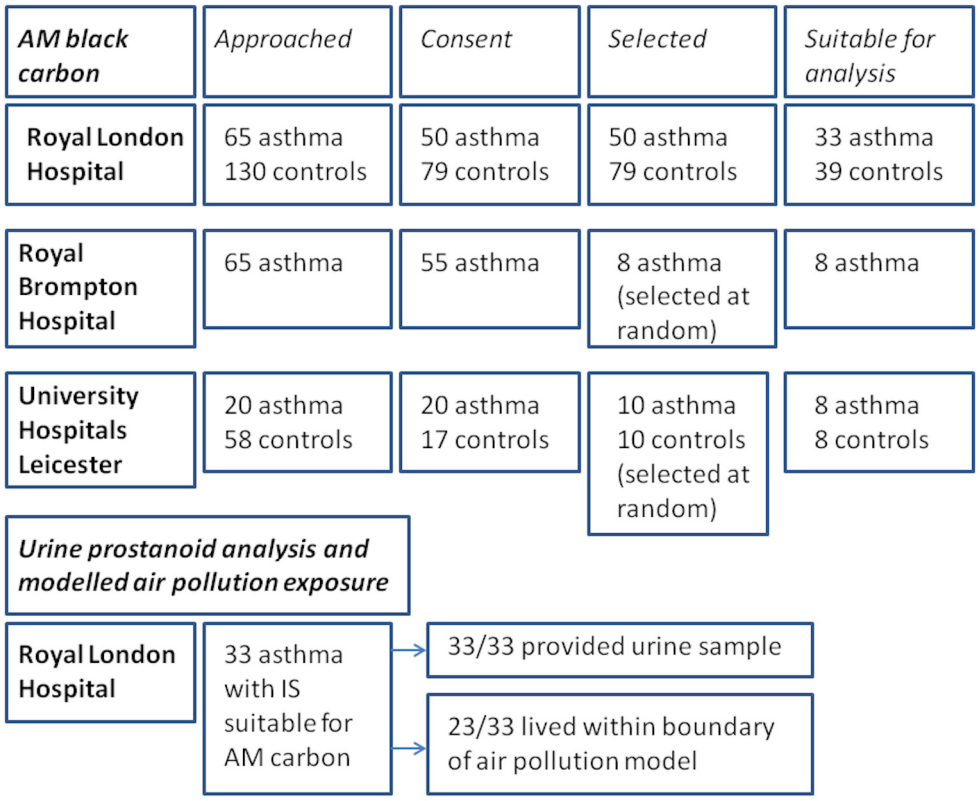
Online figure 1.



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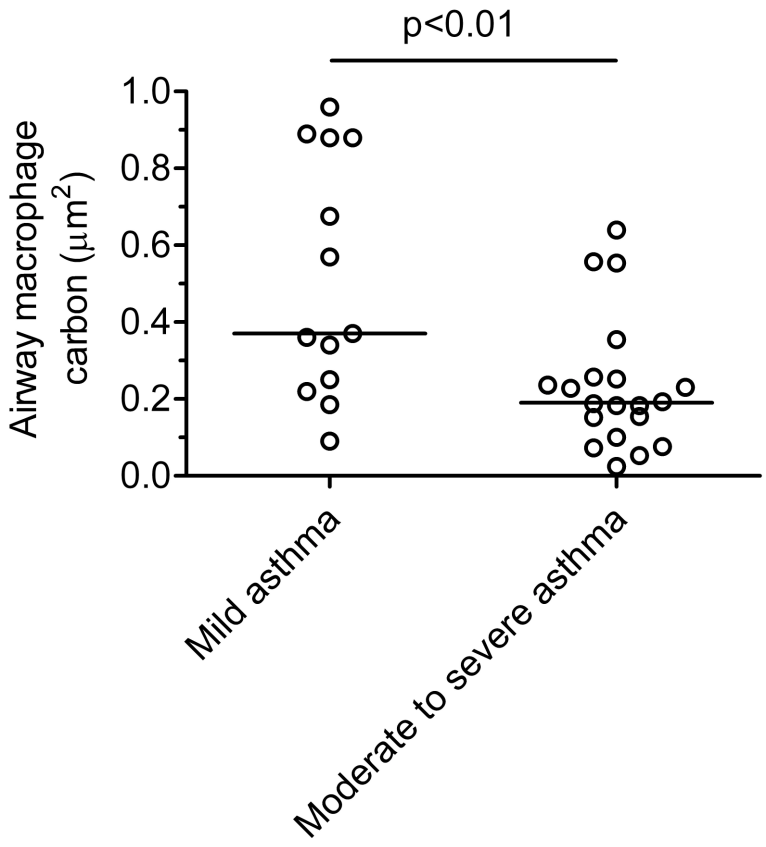

Online figure 3.