**Urinary prostanoids in preschool wheeze**

Jonathan Grigg MD 1, Abigail Whitehouse MBChB 1, Hitesh Pandya MD 3, Stephen Turner MD 4, Christopher J Griffiths MD 1, Tom Vulliamy PhD 1, Robert Walton MD 1, David Price MRCGP 4, Marek Sanak PhD 5, John W Holloway PhD 6, Lee Noimark MD 1, Maia Lesosky7, Rossa Brugha PhD 1, Lee Koh BSc 1, Chinedu Nwokoro MB BChir 1.

1. MRC and Asthma UK Centre in Allergic Mechanisms of Asthma, Blizard Institute, Queen Mary University of London, London, UK

2. Centre for Primary Care and Public Health, Blizard Institute, Queen Mary University of London, London, UK

3. Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, UK

4. University of Aberdeen, Aberdeen, UK.

5. Department of Medicine, Jagiellonian University Medical School, Krakow, Poland

6. Human Development and Health, University of Southampton, Southampton General Hospital, UK

7. Division of Epidemiology and Biostatistics, School of Public Health and Family Medicine, University of Cape Town, Cape Town, South Africa.

*Corresponding author*: Professor Jonathan Grigg, Blizard Institute, Queen Mary University of London, 4 Newark Street, London, E1 2AT, UK.

*Phone* 00 44 207 882 2206

Fax 00 44 207 882 2195

*E-mail*: [j.grigg@qmul.ac.uk](mailto:j.grigg@qmul.ac.uk)

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*Author Contributions*

JG was the chief investigator, planned, and provided overall supervision of the study, wrote the manuscript and vouches for these data; AW did data analysis and contributed to final manuscript; ML did the multiple regression analysis and contributed to the final manuscript, CN supervised the study, and contributed to the final manuscript. HP contributed to study planning, and to drafting the final manuscript; ST contributed to study planning, and to drafting the final manuscript; TV contributed to study planning, and contributed to the final manuscript; JH contributed to study planning, and contributed to the final manuscript. RW contributed to study planning, and to drafting the final manuscript. DP contributed to study planning, and to drafting the final manuscript, MS performed urinary prostanoid analysis and contributed to the final manuscript. LN obtained data from controls and contributed to the final manuscript, RB supervised the study, and contributed to the final manuscript, LK supervised data from controls, collated data, and contributed to the final manuscript, CG contributed to study planning, and to the final manuscript.

**To the Editor**

Acute episodes of wheeze in preschool are frequently triggered by viral upper respiratory tract infections and result in a significant burden to health services (1). However, to date, the inflammatory mechanisms underlying preschool wheeze remain unclear. A class of mediators that have not been studied in preschool wheeze, but are implicated in the pathogenesis of wheeze in adults with asthma, are the pro-inflammatory prostanoid prostaglandin D2 (PGD2) (2) and the anti-inflammatory prostanoid PGE2 (3, 4). In this study, we sought evidence for either increased PGD2 biosynthesis, or reduced PGE2 biosynthesis, or a combination of both in children with preschool wheeze. To achieve this we measured the major metabolites of PGD2 and PGE2 in the urine; 9α-hydrox-11,15-dioxo-2,3,4,5-tetranor prostan-1,20-dioic acid (tetranor-PGDM) and 9,15-dioxo-11α-hydroxy-13,14-dihydro-2,3,4,5-tetranor-prostan-1,20-dioic acid (tetranor-PGEM) respectively (5, 6).

Preschool children with a history suggestive of ongoing wheeze were recruited from the Wheeze And Intermittent Treatment trial (ClinicalTrials.gov, NCT01142505). Urine samples for prostanoid analysis were obtained from children while asymptomatic, after informed parental consent, and before the issue of trial medication (UK National Health Service Multicenter Research Ethics Committee Ref: 09/H1102/110). Children were aged between 10 months and 5 years with a history of 2 or more episodes of wheeze, at least one of which was physician-confirmed, and at least one of which had occurred within the preceding 3 months (7). Urine was randomly obtained from 0900 to 1600 h. Healthy controlswere preschool siblings of children attending the outpatient clinics of the Royal London Hospital (UK), and preschool children with atopic disease were recruited from a paediatric allergy clinic with a clinical diagnosis of food allergy, but with no history of wheeze (atopic disease controls).

Urine was collected and stored at -80 oC within 1 h of collection. Urinary tetranor-PGDM and tetranor-PGEM was analysed by high-performance liquid chromatography (HPLC) separation and mass spectrometry (MS) measurements. After thawing on ice, samples were centrifuged for 10 min at 10,000 g at 4°C and 0.5 mL of supernatant was used for extraction and analysis of tetranor-PGDM and tetranor-PGEM. Chemically identical internal deuterated standards were added to each sample: 10 ng tetranor-Prostaglandin E Metabolite-d6 (tetranor-PGEM-d6 or (11R-hydroxy-9,15-dioxo-13,14-dihydro-2,3,4,5-tetranor-prostan-17,17’,18,18’,19,19’-d6-1,20-dioic acid), and 10 ng tetranor-Prostaglandin D Metabolite-d6 (tetranor-PGDM-d6 or (9R-hydroxy-11,15-dioxo-13,14-dihydro-2,3,4,5-tetranor-prostan-17,17’,18,18’,19,19’-d6-1,20-dioic acid) (Cayman Chemical Co. Ann Arbor, MI). Samples were then acidified (pH=3.5) using acetic acid and mixed with 0.5 mL tert-buthyl-ether: methanol (80:20 V/V). The organic phase of the resulting mixture was then separated by a short centrifugation step and then dried under nitrogen at 37 °C. The dried solid extract was re-dissolved in methanol (60 μL). 10 μL of this mixture was used for high-performance liquid chromatography (HPLC) separation and mass spectrometry (MS) measurements; Shimadzu Sil-2-AC, Shimadzu Scientific Instruments, Inc. Columbia, MD, USA equipped with Phenomenex Synergy Fusion RP-100A 100 x 2mm column. The retention times for tetranor-PGEM and tetranor-PGDM retention times were 13.2 and 13.4 min respectively. Analytes were measured using multiple reaction monitoring mode (MRM) tandem mass spectrometry (Qtrap 4000, AB Sciex, Concord, Ontario) equipped with electrospray ion source and operating in negative ionization mode. Both tetranor-PGEM and tetranor-PGDM had the same pseudomolecular ions 327 Mz and monitored ions 309 Mz (333 and 315 Mz for deuterated standards). Quantification was done using a stable isotope dilution method from the area under the peak. Urinary creatinine was assessed using a standard analytical assay and Vitros 350 (Ortho Diagnostics, Raritan, NJ) and prostanoids were indexed to urinary creatinine (pg/mg creatinine). Urinary cotinine was determined using a commercial microplate enzyme immunoassay (Cozart Forensic Microplate, Concateno, Abingdon, UK) and exposure to environmental tobacco smoke was classified as present if the creatinine corrected cotinine concentration was greater than 30 ng/mg (8) (9).

For analysis, we divided children with preschool wheeze into two groups; those recruited at the same site as the healthy controls (group 1), and those recruited at other sites (group 2). Urinary prostanoid concentrations were log10 transformed before analysis. Groups were compared by either ANOVA with *post hoc* Tukey’s multiple comparisons test, or by t test using GraphPad Prism version 6.00 (GraphPad Software, La Jolla, California, USA). Correlations (Pearson correlation coefficient (r)) and multiple regression analyses were done using R v 3.2 (R Core Team, Vienna, Austria). Data are summarised as mean + standard error of the mean (SEM). A P value of <0.05 was considered significant.

We recruited 24 healthy controls, 5 non-wheezy children with atopic disease, 149 children with preschool wheeze recruited at the same site as controls (group 1), and 810 children with preschool wheeze recruited from other sites (group 2). No child had non-steroidal anti-inflammatory therapy in the previous 2 weeks before urine sampling. There was no difference in age between controls and both preschool wheeze groups (healthy controls; 3.0 + 0.27 yr., atopic disease; 2.9 + 0.81 yr., group 1; 3.0 + 0.08 yr., group 2; 2.6 + 0.04 yr.).

There was no difference in tetranor-PGDM between healthy controls and children with atopic disease and no history of wheeze (3.8 + 0.09, n=24 vs. 3.8 + 0.06, n=5). Tetranor-PGDM was increased in both preschool wheeze groups compared with healthy controls (group 1; 4.3 + 0.04, pg/mg creatinine, group 2; 4.3 + 0.01, healthy controls; 3.8 + 0.09, n=24, P<0.0001, Figure 1A). In a multiple regression for PGDM including age, and preschool wheeze status (group 1 and 2 combined, controls), both age and preschool wheeze status remained statistically significant (R2=0.13, coefficient (standard error) of age: -0.096 (0.01), P < 0.0001; coefficient (SE) of no wheeze (wheeze set as reference level): -0.37 (0.75), P < 0.0001).

In 959 children with preschool wheeze (i.e. group 1 and 2 combined), the correlation between tetranor-PGDM and age was negative (r= -0.30, P<0.0001), and was smaller in those receiving inhaled corticosteroids (ICS, P<0.05). Gender, clinical pattern of wheeze (exclusive viral wheeze vs. multiple trigger wheeze), exposure to environmental tobacco smoke (either parent-reported, or by urinary cotinine), and eczema were not associated with tetranor-PGDM. In a multiple regression analysis limited to children with preschool wheeze, and including age and ICS, only age remained statistically significant (R2=0.11, coefficient (SE) for age: -0.049 (0.005), P < 0.0001; coefficient (SE) for ICS: 0.020 (0.025), P = 0.42).

There was no difference in tetranor-PGEM between healthy controls and children with atopic disease (4.4 + 0.07, n=24 vs. 4.1 + 0.12, n=5). There was no difference in tetranor-PGEM between controls (4.4 + 0.07), and preschool wheeze groups 1 (4.4 + 0.03) and 2 (4 + 0.01, Figure 1B). In 959 children with preschool wheeze, tetranor-PGEM was inversely associated with age (r = -0.33, P<0.0001).

These results suggest that in children with a history of preschool wheeze but with no active wheeze on the day of sampling, PGD2 biosynthesis, but not PGE2 biosynthesis, is increased. The mechanism whereby airway PGD2 contributes to the pathogenesis of preschool wheeze is unclear. One potential mechanism is that increased airway PGD2 rather than directly causing bronchoconstriction, primes the airway for an exaggerated inflammatory response during viral colds - an interaction recently observed in an animal model (10).

There are important limitations to this study. First, although the pattern of urinary prostanoids in preschool wheeze is similar to that reported for adults with mild intermittent wheeze (11), whether increased tetranor-PGDM in the urine reflects either increased levels of PGD2 in the airway, or increased biosynthesis in other organs is unclear. Second, we did not assess a several important potential confounders of PGDM in children with preschool wheeze. For example, atopic status (by either skin prick testing, or specific serum IgE) was not done. Whilst the non significant difference in PGDM between healthy controls and controls with atopic disease is compatible with a lack of effect of atopy, the very small number of children with atopic disease means that a confounding effect of atopy remains possible. We are, however, able to exclude an effect of differences in urine sampling handling since there was no difference in the urinary prostanoid profile between children with preschool wheeze recruited at the same site as controls (group 1) and those recruited at other sites (group 2).

We conclude that PGDM is a marker of potential interest in preschool wheeze, but further studies are required in better defined populations. If airway PGD2 is indeed increased in preschool wheeze, trials of new therapeutic options for this common condition would be suggested, for example of the new oral CHTR2 blockers - which block the action of PGD2 on airway cells (12).

**Legend for Figure**

**Figure; A** Dotplot of urinary tetranor-PGDM (log10) in healthy controls, and children with preschool wheeze recruited at the same site as controls (group 1), and those recruited at other sites (group 2). Urinary tetranor-PGDM is increased in group 1 and group 2 compared with controls (ANOVA and *post hoc* Tukey’s multiple comparisons test). **B**; urinary tetranor PGEM. There is no difference between groups by ANOVA.



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