CHEST Online Supplement

Airway Hyperresponsiveness in Children With Sickle Cell Anemia

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e-Appendix 1.

Institutional review board approval for participating sites

Washington University School of Medicine, St. Louis, MO: approved by Human Research Protection Office at Washington University, federal wide assurance (FWA) #00000163.

Case Western Reserve, Cleveland, OH: approved by Office of Institutional Review, FWA #00004428.

Institute of Child Health University College, London, UK: approved by Northwick Park Ethics Centre and Brent Research Ethics Committee, FWA #00001221.

Pulmonary function testing

Spirometry was performed by SAC-certified pulmonary function technicians using a pneumotachograph-type spirometer interfaced with a personal computer system (Jaeger MasterScope, VIASYS, Hoechberg, Germany). Spirometry was performed at least 4 hours after the use of a short-acting bronchodilator.[1] American Thoracic Society (ATS) standards for the performance of spirometry were adapted for children.[1,2] Appropriate prediction equations for FEV_1 and FVC were used taking into account height, age, gender and ethnicity.[3] To measure response to bronchodilator, technicians administered 4 inhalations of albuterol from a metered dose inhaler (90 mcg/puff) using an AeroChamber (Forest Pharmaceuticals, New York, NY) to participants. Spirometry was repeated 15 minutes post-albuterol. Baseline and post-bronchodilator FEV_1 were compared, with percent response to albuterol

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defined as post-bronchodilator FEV_1 minus pre-bronchodilator FEV_1 divided by pre-bronchodilator FEV_1 times 100. An increase of $\geq$ 12% in FEV_1 following albuterol was considered a positive bronchodilator response.[4]

Methacholine Airway Challenge

Methacholine testing and calibration of nebulizers (Airlife Sidestream High-Efficiency Nebulizer, Cardinal Health, Dublin, OH, with a particle size of 1-5 microns, calibrated to deliver 0.13 ml/min) were carried out according to ATS procedures.[5] The two-minute tidal breathing method used by the Childhood Asthma Management Program[6,7] and the Childhood Asthma Research and Education Network was used.[8] FEV_1 was measured following administration of doubling concentrations of methacholine (Provocholine, Methapharm, Coral Springs, FL) starting at 0.195 mg/ml, reaching a maximum dose of 25 mg/ml, or when there was a 20% decrease in FEV_1 post methacholine administration. The procedure was not performed within 1 month after hospitalization for pain or ACS, a course of systemic corticosteroids or symptoms consistent with a respiratory infection, and was performed at least 4 hours after use of a short-acting bronchodilator, 12 hours after the last use of a long-acting bronchodilator and 24 hours after the use of theophylline. A physical examination to preclude any acute exacerbations was undertaken prior to testing. Children with a baseline FEV_1 <70% predicted based on ethnic-specific reference equations[3] did not undergo methacholine challenge. All females who had started menses or were at least 10 years of age had a negative pregnancy test documented within 2 weeks of the testing or at the time of the visit.

Method for measurement of methacholine concentration

To determine if there was a difference in concentration of methacholine at the three participating clinical sites, sample methacholine concentrations were compared from each site. Methacholine concentrations were measured

by LC tandem mass spectrometry on an API 3200 system (Applied Biosystems, Foster City, CA) using acetylcholine as an internal standard. Samples were prepared in mobile phase containing 50% acetonitrile, 0.15 mM ammonium acetate, and 0.15 mM ammonium formate (pH 4.0) and injected at a flow rate of 100 μL/min. Measurements were made using an electrospray ion source operated at 250°C and 5500 V with curtain gas at 20 psi, nebulizer gas at 30 psi, turbo gas at 5 psi, and collision gas set to medium. Methacholine was monitored using a Q1→Q3 transition of m/z 160→101 and acetylcholine was monitored using an m/z transition of 146→87. Quantitation was performed using a linear 6 point standard curve over the range of 0.02 to 2 mg/mL with internal standard present at 0.2 mg/mL.

The difference between calculated methacholine concentrations and known sample concentrations were compared. The mean differences in methacholine concentration between calculated and known samples were 0.13 ± 0.41 mg/ml at site 1, 0.78 ± 1.21 mg/ml at site 2 and 0.31 ± 0.45 mg/ml at site 3. Using an ANOVA analysis to compare the mean difference in methacholine concentration between calculated and known values, there was no difference between the three sites (p=0.186).

Exhaled nitric oxide

Online exhaled nitric oxide (eNO) using the NIOX system (Aerocrine AB, Stockholm, Sweden) was performed according to ATS guidelines.[10] Measurement of eNO used a resistive device that provided a constant low expiratory flow and vellum closure. Participants were required to exhale to residual volume, place their mouth on a mouthpiece connected to a nitric oxide scrubber and then inhale to total lung capacity. Thereafter, the child exhaled for 10 seconds at a constant flow of 0.05 L/second ± 10%. Young children were allowed to exhale for 6 seconds. Following a 30-second relaxation period, the exhalations were repeated until 3 eNO values were obtained that varied less than 10%.

Allergy skin testing

Allergy skin testing was performed by SAC-certified technicians using Multi-test II (Lincoln Diagnostics, Decatur, IL). In St. Louis and Cleveland, ten aeroallergens (Greer Laboratories, Lenoir, NC) were used for skin testing: dust mite (*Dermatophagoides pteronyssinus* and *D garinae*), cockroach (American and German), cat (standardized), dog (mixed breeds), *Alternaria alternans*, *Aspergillus fumigates*, grass (standardized southern mix), tree (eastern 8 tree mix), weed (national mix) and mouse. In London, nine aeroallergens (Alk-Abello, Horsholm, Denmark) were used for skin testing: dust mite (*D. Pteronyssinus*), cockroach (German), cat (*Felix domesticus*), dog (*Canis familiaris*), *Alternaria alternata*, *Aspergillus fumigatus*, grass (6 grass mix), tree (3 tree mix) and Stinging nettle (*Urtica dioica*). All skin tests were administered with histamine (positive) and saline (negative) controls. Wheals present were outlined in ball point pen ink, transferred to tape, and placed on paper as a permanent record. Tests were considered positive when the mean diameter of the wheal was ≥ 3 mm compared to the saline control. Children who were receiving anti-histamines did not undergo allergy skin testing.

Laboratory testing

Lactate dehydrogenase (LDH) (Cobas Integra, Roche Diagnostics, Indianapolis, IN) and IgE (Elecsys 2010, Roche Diagnostics, Indianapolis, IN) testing was performed using stored plasma collected in EDTA tubes and stored at -80ºC. To ensure that freeze-thaw did not influence LDH levels, additional LDH measurements were performed on control samples before and after storage at -80ºC. LDH measurements were performed on 10 consecutive plasma samples collected in EDTA tubes from St. Louis Children's Hospital. Plasma samples were prepared using the same methodology as for the SAC samples. Whole blood was collected in EDTA tubes and platelet poor plasma isolated by centrifuging for 10 minutes at 3,000 rpm. After LDH measurements were performed, the samples were frozen at

80ºC overnight, and then thawed and re-analyzed the next day for LDH level. Spearman rank correlation coefficient between the fresh and frozen/thawed samples was 0.967 ($p<0.001$). Thus, using our methodology to prepare platelet poor plasma, the free-thaw cycle has little influence on LDH values.

Relationship between age and AHR in the Childhood Asthma Management Program Trial

One-thousand thirty-nine children with mild to moderate asthma aged 5-12 years in the Childhood Asthma Management Program Trial underwent methacholine challenge. Tests were performed at randomization when the participants were off all disease modifying medications and free from symptoms of a respiratory viral illness for at least 28 days.[6] There was no linear relationship of PC_{20} (log transformed) and age using linear regression ($\beta=-0.004$, $p=0.82$) (Supplementary table 1).

Sleep Asthma Cohort (SAC) investigative team

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e-Table 1. Association of age with airway responsiveness (PC_{20} mg/ml) among participants in the Childhood Asthma Management Program Trial

Age (years)	N	Median PC_{20}	25^{th} % PC_{20}	75^{th} % PC_{20}
5	96	1.15	0.52	2.33
6	139	1.02	0.52	2.46
7	147	1.08	0.50	2.96
8	162	1.12	0.61	2.65
9	146	1.09	0.42	2.94
10	134	1.04	0.49	3.19
11	123	1.03	0.36	2.87
12	92	1.06	0.48	2.63

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