Vitamin D Insufficiency and Severe Asthma Exacerbations in Puerto Rican Children

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SUPPLEMENTAL METHODS

Subject recruitment

From March of 2009 to June of 2010, children in San Juan (SJ) were chosen from randomly selected households, using a scheme similar to that of a prior study (8). In brief, households in the Standard Metropolitan Area of SJ were selected by a multistage probability sample design (8). Primary sampling units (PSUs) were randomly selected neighborhood clusters based on the 2000 U.S. census, and secondary sampling units were randomly selected households within each individual PSU. A household was eligible if ≥1 resident was a child 6 to 14 years old. In households with more than one eligible child, a maximum of five children were randomly selected. Within each housing unit selected, children were enumerated and one child per eligible household was selected for screening. In households with multiple eligible children, one child was randomly selected by using Kish tables. On the basis of the sampling design, a total of 7,073 households were selected for inclusion; 6,401 (90.5%) were contacted. Of these 6,401 households, 1,111 had ≥1 child within the age range of the study who met other inclusion criteria (see below). In an effort to reach our target sample size (~700 children), we attempted to enroll a random sample (n=783) of these 1,111 children. Parents of 106 (13.5%) of these 783 eligible households refused to participate or could not be reached, leaving 677 participants. There were no significant differences in age, gender, or area of residence between eligible children who did and did not agree to participate.

The main recruitment tool was a screening questionnaire given to parents of children ages 6 to 14 years to obtain information about the child's respiratory health and PR ancestry. All participants (cases and controls) had to have four PR grandparents and be living in the same household for ≥1 year. We selected as cases children with physician-diagnosed

asthma and wheeze in the prior year, and as controls children with no physician-diagnosed asthma and no wheeze in the prior year.

Study Procedures

Questionnaires

The parents of each participant completed a questionnaire used in the Genetics of Asthma in Costa Rica Study, which was slightly modified from one used in the Collaborative Study of the Genetics of Asthma(E1). Vitamin D intake was estimated using a food frequency questionnaire(E2). Time spent outdoors during weekends and holidays, usual time spent outdoors during daily activities, and sunscreen use were assessed using a validated questionnaire (E3).

Spirometry

All spirometries were conducted with an EasyOne (ndd Medical Technologies, Andover, MA) spirometer. All subjects had to be free of respiratory illnesses for at least 4 weeks before spirometry, and they were also instructed (whenever possible) to avoid use of inhaled short- and long-acting bronchodilators for at least 4 and 12 hours before testing, respectively. Forced expiratory maneuvers were judged to be acceptable if they met or exceeded ATS criteria modified for children(E4). As many as eight forced expiratory flow volume maneuvers from total lung capacity were performed to obtain three acceptable measures.

Dust sample collection

Dust samples were obtained from three areas in the home: the one in which the child sleeps (usually a bedroom), living room/television room, and kitchen. The dust was sifted through a 50-mesh metal sieve, and the fine dust was reweighed, extracted, and aliquoted for analysis of allergens from dust mite (Der p 1) and cockroach (Bla g 2) by two-site monoclonal antibody Multiplex array assays(E5) using the same reagents employed in the

established ELISA(E6). Internal controls were run in each assay to insure inter-assay reproducibility.

Measurements of total and allergen-specific IgE, and vitamin D in serum

Serum levels of total IgE, IgE to dust mite (Der p 1), German cockroach (Bla g 2), mouse (Mus m 1), dog (Can f 1) and cat (Fel d 1) were determined using the UniCAP 100 system (Pharmacia & Upjohn, Kalamazoo, MI). For each allergen, an IgE ≥0.35 IU/ml was considered positive. Plasma vitamin D was measured using the Waters high-performance liquid chromatography system with tandem mass spectrophotometry (Waters Corporation, Milford, MA).

Genotyping and data cleaning

Subjects were genotyped using the HumanOmni2.5 BeadChip (Illumina, Inc., San Diego, CA). Each batch included at least one replicate sample. Two channel intensities were brought into Beadstudio workspace, and reclustering was performed using project samples. Subjects with a call rate of < 95% and SNPs with a call frequency < 95% were a priori removed. The remaining markers were then cleaned following Illumina guidelines (http://www.illumina.com/documents/products/technotes/technote_infinium_genotyping_data_analysis.pdf). BeadStudio workspaces were exported to ped file format and further subject and marker cleaning was performed using R (www.r-project.org) scripts in conjunction with PLINK 1.07.(E7) Subject relatedness was estimated using IBD in PLINK. Sex assignment was based on X homozygosity estimate. Samples showing gender discordances when compared with phenotype files were removed. SNPs that were not in Hardy–Weinberg equilibrium ($P<10^{-6}$) in control subjects, had minor allele frequency lower than 1% or a failure rate greater than 2%, or that were in linkage disequilibrium ($r^2 \geq 0.1$) with others were removed from the analysis.

Estimation of Racial Ancestry

After pruning the markers for LD ($r^2 < 0.1$), a total of 85,069 SNPs were used to estimate ancestry using the Local Ancestry in adMixed Populations (LAMP) method (E8). This algorithm uses ancestral proportions from previous studies (in this case from Tang et. al.(E9)) and data from reference panels to estimate ancestral proportions in racially admixed populations. Since Puerto Ricans are an admixture of European, African, and Native Americans populations, we used the CEU (Utah residents with Northern and Western European descent) and Yoruban (West African) reference panels from HapMap(E10), and Native American reference panels (Mayan, Pima, Surui, Karitiana, Colombian) from the Human Genome Diversity Project(E11). As a confirmatory analysis, African ancestry was estimated using STRUCTURE(E12), obtaining nearly identical results as those from LAMP ($r=0.99$, $P < .0001$). The mean estimated ancestral proportions for study participants are shown in **Table E1.**

Table E1 – Prior ancestral proportions used for LAMP analysis, and resulting average ancestral proportion

Cohort	EUROPEAN	AFRICAN	NATIVE AMERICAN
Prior assumption(E9)	*67.0%*	*18.0%*	*15.0%*
Estimated values	63.2%	25.0%	11.8%

Table E2: Multivariate analysis of vitamin D insufficiency and measures of lung function and allergy in controls

Predictors	*Pre-bronchodilator FEV1 (ml)*	*Pre-bronchodilator FEV1/FVC (%)*	*≥1 positive IgE to allergens*
Unadjusted			
Vitamin D level < 30 ng/ml	348(168-528) (<0.001)	2(-1-4) (0.2)	1(0.6-1.7) (0.9)
Multivariate model			
Vitamin D level < 30 ng/ml	-42(-124-39) (0.3)	1(-1-3) (0.4)	1.1(0.6-1.9) (0.7)
Household income < $15,000/year	-88(-171--6) (0.04)	0(-3-2) (0.8)	1(0.6-1.7) (0.9)
BMI (z-score)	2(-35-39) (0.9)		
Each 20% increase in African ancestry	-94(-157--32) (0.003)	-2(-3-0) (0.05)	1.3(0.8-1.9) (0.3)
Virtually always outside	-12(-100-77) (0.8)	0(-2-3) (0.8)	0.9(0.5-1.6) (0.7)
High vitamin D intake (diet or supplements)	-98(-178--19) (0.02)	1(-1-3) (0.5)	1.3(0.8-2.2) (0.4)

[1] *Beta (95% confidence interval (CI)) (P value) for FEV_1 and FEV_1/FVC ratio, and odds ratio (95% CI) (P value) for other outcomes.*

[2] *FEV_1 additionally adjusted for height and height^2*

[3] *All multivariate models additionally adjusted for age and gender.*

FEV_1 = Forced expiratory volume in 1 second. BMI = body mass index.

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