CC16 levels into adult life are associated with nitrogen dioxide exposure at birth

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At a Glance:

Scientific Knowledge of the Subject: Lower levels of circulating club cell secretory protein in childhood are associated with reduced lung function in adulthood. Increased exposure to nitrogen dioxide is also associated with reduced lung function growth in children. Evidence from *in vitro* and *in vivo* studies suggest that nitrogen dioxide exposure may induce epithelial damage in the lungs and alter club cell growth, yet no previous publication has assessed the effects of nitrogen dioxide exposure in children on subsequent circulating club cell secretory protein levels.

What this Study adds to the Field: We utilized data from a longitudinal birth cohort study to determine the associations between nitrogen dioxide exposures in early life and circulating club cell secretory protein from age 6 through age 32 years. We determined that higher exposures to nitrogen dioxide at the birth address are associated with persistently lower levels of circulating club cell secretory protein from childhood (age 6) into adult life (age 32). There was no

association between nitrogen dioxide exposures at age 6 with circulating club cell secretory protein levels.

One Sentence Summary: We determined that higher exposures to nitrogen dioxide at the birth address, but not age 6, are associated with persistently lower levels of circulating club cell secretory protein from childhood (age 6) into adult life (age 32).

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

Abstract

Rationale: Lung function and growth are adversely associated with nitrogen dioxide (NO_2) exposure. Lower levels of circulating club cell secretory protein (CC16) in childhood are also associated with subsequent decreased lung function. NO_2 exposure may induce epithelial damage in lungs and alter club cell proliferation and morphology.

Objectives: Our objective was to determine if increased ambient NO_2 levels at participants' home addresses in early life were associated with decreased levels of CC16 from age 6-32 years.

Methods: Participants were enrolled at birth in the Tucson Children's Respiratory Study and had circulating CC16 measured at least once between age 6-32. Linear mixed models were used to determine the association between estimated ambient NO_2 exposure at participants' home address at birth or age 6 with CC16 levels from age 6-32.

Measurements and Main Results: NO_2 exposures at birth or age 6 were available for 777 children with ≥1 CC16 measurement. We found a negative association between NO_2 exposure and CC16 levels, with a 4.7% (95% CI -8.6, -0.7) decrease in CC16 levels from age 6-32 per interquartile (IQR) increase in NO_2 exposure (6.0 ppb) at the participants' birth address. We observed modification by race (p interaction = 0.04), with stronger associations among participants with at least one Black parent (-29.6% [95% CI -42.9%, -13.2%] per IQR). NO_2 at participant's age 6 address was not significantly associated with CC16 levels (-1.9%, 95% CI -6.3, 2.6).

Conclusions: Higher exposure to NO_2 at birth is associated with persistently low levels of CC16 from 6-32 years.

Abstract Word Count: 250

Key Words: air pollution, club cell, biomarker

Introduction

Nitrogen dioxide (NO_2) is a highly reactive oxidizing gas that arises from the reaction of nitric oxide (NO) and oxygen in the atmosphere (1). Both NO_2 and NO are by-products of fossil fuel combustion and are among the most common pollutants measured in both outdoor and indoor air (2). Primary outdoor sources include diesel vehicles and power plants, while common indoor sources are the pilot light for gas cook stoves and hot water heaters. NO_2 may be the best proxy pollutant to assess spatial exposure to the urban air pollution mixture (3), and non-Whites in the United States (US) are significantly more likely to live in areas with increased NO_2 exposure (4).

Deficits in children's lung function and growth are associated with exposure to NO_2 in both cross-sectional and prospective studies (5-7). A 20 year-long study found that long term reductions in levels of NO_2 were associated with significant improvements in children's lung function growth (8). Multiple studies document that inhaled NO_2, due to its reactive nature, can induce epithelial injury in the lungs (1, 9, 10). Of particular concern is evidence that during periods of lung development, epithelial cells may be uniquely susceptible to NO_2-induced death (1). Furthermore, NO_2 exposure is associated with altered club (formerly Clara) cell proliferation and morphology in animal studies (9).

These club cells release a secretory protein, referred to as CC16, which is a homodimeric pneumoprotein with anti-inflammatory properties that may protect the lungs from oxidative stress (11-13). Because CC16 measured in serum has been correlated with levels in bronchoalveolar lavage fluid (BALF) and club cell density, serum CC16 is proposed as a novel biomarker for early respiratory impairment from both acute and/or chronic lung injury (11, 14).

Decreased levels of circulating CC16 are prospectively associated with lung cancer mortality, COPD development and lung function deficits in both children and adults (15-17).

We postulated that early life exposure to NO_2 may be associated with lung damage, which is reflected in lower CC16 levels. To test this hypothesis, we used data from the Tucson Children's Respiratory Study (CRS), a longitudinal birth cohort study that enrolled newborns to examine risk factors for acute lower respiratory tract illnesses in early childhood and chronic lung disorders later in life (18, 19). We determined whether ambient NO_2 levels at participants' birth and age 6 addresses were associated with levels of CC16 from age 6-32 years. If early CC16 levels can be shown to reflect lung injury from NO_2 exposure, early-life interventions that target children with low CC16 levels could be developed to prevent potential long-term health effects in high-risk children. Some of these results have been previously reported in the form of an abstract (20).

Methods

Study Population

The Tucson Children's Respiratory Study (CRS) is a longitudinal non-selected birth cohort study. All healthy infants born to mothers using the services of the largest health maintenance organization (HMO) in Tucson, Arizona between 1980 and 1984 were eligible to participate and were contacted by a study nurse. Of the 1,596 eligible families contacted, 1,246 infants (78%) were enrolled. A detailed description of the cohort is published (18, 19). For the purpose of the current analysis, demographic characteristics of the child and parents, as well as home address,

were obtained from a questionnaire administered at enrollment (within two weeks after birth). Blood samples were obtained at age 6 (mean=6.1, sd=0.8), 11(mean=10.8, sd=0.6), 16 (mean=16.6, sd=0.6), 22 (mean=22, sd=0.9), 26 (mean=26.4, sd=0.8) and 32 years of age (31.8, sd=1.0). The study was approved by the University of Arizona Human Subjects Committee. Informed consent was obtained from a parent of a participating child and/or from the participants themselves starting at age 16 years.

CC16 Measurements

Circulating CC16 was measured in stored samples from each follow up period. Serum samples were used for all time points, with the exception of 78 plasma samples from year 6. In sensitivity analyses we excluded the plasma samples. Measurement techniques and the adjustments for plasma samples have been described in detail previously (16). All samples were cryopreserved at -80°C and circulating CC16 was measured using a commercially available enzyme-linked immunosorbent assay kit (BioVendor with branches in Asheville NC and Modrice, Czech Republic; kit range: 1.57-50.0 ng/mL). All samples had detectable values.

Nitrogen Dioxide Exposure Assessment

We estimated NO_2 exposure at each enrollment (1980-1984) and age 6 (1986-1990) address using a land-use regression (LUR) model developed from NO_2 measurements obtained in eastern Pima County (where Tucson is located) between 1987-1991. NO_2 concentrations were measured in residential yards using passive Palmes diffusion tubes (21). Participating homes were from the Pima County Workers Study, who were recruited through county government departments with a wide range of occupations, resulting in a study cohort with varied socio-economic status,

demographic indicators, and geographic distribution. In 1987, the year closest to CRS enrollment, 166 homes were sampled for 1-2 one-week periods, resulting in 311 observations.

We developed and evaluated our NO_2 LUR by adapting protocols for the European Study of Cohorts for Air Pollution Effects (ESCAPE), which has been conducted in 36 study areas in Europe (22). We created 89 geographic predictor variables based on ESCAPE protocols (22). Additional variables important to the Tucson environment were included (e.g., distance to airports, active mining operations, and agricultural land). Following the ESCAPE method, simple regressions were performed with all predictors and the one with the highest R^2 and slope in the predefined direction (e.g., area of nearby natural space is negatively related to air pollution) became the 'seed' model. Other predictors were added one-by-one and kept if: 1) the adjusted R^2 increased by >1%; 2) the slope met the predefined direction; and 3) the slope direction of other predictors did not change. After all predictors were evaluated, variables in the model were removed one-by-one if their p-value was >0.1, beginning with the variable with the largest p-value. Model performance was evaluated using a leave-one-out cross-validation method. Details on the source data and calculations for each variable are provided in supplemental material (Table E1).

Using ArcGIS 10.1 (ESRI, Redlands CA), we geocoded the enrollment and age 6 address for each participant. These coordinates were used as receptor points in the LUR to estimate ambient NO_2 exposure in parts per billion (ppb) at each address as:

$$[NO_2] = 6.18 + (8.48 \times 10^{-6})Pop_{5km} - 0.16\sqrt{Elev} + 247\left(\frac{1}{Dist_{air}}\right) + 67.6\left(\frac{1}{Dist_{rail}}\right)$$

where Pop_{5km} is the population density within 5 km (persons/m^2); *Elev* is the elevation (m); $Dist_{air}$ and $Dist_{rail}$ are the distance to the nearest airport and rail line, respectively (m). To predict exposures corresponding to each participant's birth or age 6 address, these variables were obtained for each address corresponding to the calendar year of enrollment (1980-1984) or age 6 (1984-1992) from the sources listed in Table E1, as in previous ESCAPE studies. Estimates were scaled based on differences between 1987 and the exposure year in NO_2 measurements from the regional monitoring network (7).

Statistical Analysis

All analyses were performed with R (V3.3.4, R Foundation for Statistical Computing, Vienna, Austria). NO_2 exposures were scaled by dividing by the interquartile range to aid interpretability of the coefficients (i.e., one-unit increase is equal to the interquartile range). Because the distribution of CC16 values was right skewed, and concentrations are theoretically log-normally distributed (23), values were natural log-transformed. We used Pearson's chi-square test to assess if the demographic characteristics of those who were included in the analyses were different from participants who were excluded. Simple linear regression was used to determine if there were associations between NO_2 exposures and participant characteristics.

To estimate longitudinal associations between NO_2 exposure and CC16 levels throughout life, we used linear mixed models and included participants with complete covariate and exposure data, and with CC16 measurements for at least one time point. Results are presented as percent change in CC16 per interquartile range increase in NO_2. Percent change was calculated from the beta coefficients according to methods for log-transformed outcome variables (24). To select covariates for inclusion, we identified covariates *a priori* that are important in the air

pollution-lung function literature, and examined associations between NO_2 exposure and those covariates (maternal education, maternal age, race/ethnicity, delivery method, marital status, maternal smoking, child sex, home heating, socioeconomic status, birth order, and parity) as well as covariates previously associated with CC16 in our cohort (25) (maternal age, gender, parental education, maternal smoking at age 6, CC16 genotype, race/ethnicity). We excluded variables that were strongly associated with the exposure but not with the outcome to avoid inducing instrumental variable bias (26). The remaining variables were included if they resulted in a change-in-estimate of the NO_2 – CC16 relationship by >10% or if they enhanced the standard error of the estimate.

Final model covariates included fixed effects for child race/ethnicity, child sex, maternal smoking at enrollment, and follow-up visit (6-32 years), and random effects for participant ID. Unadjusted models omitted the fixed effects (with the exception of follow-up visit) but adjusted for random effects of participant ID. We examined associations between NO_2 exposure at birth and at age 6 separately, and CC16 levels at age 6, 11, 16, 22, 26, and 32 years. We assessed modification by several variables including race/ethnicity (non-Hispanic White, any Hispanic parent, any Black parent, Other), maternal education (high school or less vs greater than high school), maternal smoking, child gender, and indices of neighborhood socioeconomic status and housing conditions derived from the US census, (27) with an alpha cutoff of 0.05 on the interaction term, using a likelihood ratio test. To assess if later CC16 measurements were associated with active smoking, we conducted a sensitivity analysis with active smoking between age 18-32 years.

Results

Of the original 1,246 children enrolled, 1,137 had enrollment and 806 children had age 6 addresses, for which NO_2 exposure could be estimated and were included in the analyses (Table 1). NO_2 values were estimated from the LUR models as described and reflect estimates at the child's address during each time period. NO_2 levels were slightly higher during the enrollment period than during the first follow up period (geometric means birth vs. age 6, p=0.05). At enrollment, children classified as "Other" race/ethnicity had significantly lower exposures at their home address than children of any other race/ethnicity category (Table E2). There were no other significant differences in NO_2 exposure at enrollment by participant characteristics. However, at age 6, children who had a Hispanic or Black parent had higher NO_2 exposure levels, as did children with younger mothers or those whose mothers had lower educational attainment.

Characteristics and CC16 levels for the study population included in the analysis are presented by follow-up period in Table 2. As previously reported, CC16 levels in this cohort increased with age (25). There were no large differences in demographic characteristics of the participants by follow-up period (Table 2). However, CRS participants included in this analysis were more likely to be non-Hispanic White or Hispanic and have mothers with more education and that were less likely to smoke than those who were not included in the analysis (Table E3).

An interquartile increase in NO_2 exposure at birth was associated with a decrease in CC16 levels from age 6-32 in both unadjusted and adjusted models (Unadjusted -4.8% [95% CI -8.6, -0.7]; Adjusted -4.7% [95% CI -8.6, -0.7]) (Figure 1). Children in the highest quartile of exposure at birth had 8.6% lower [95% CI -15.0, -1.65] average levels of CC16 compared to children in the lowest quartile of exposure at birth. These trends were consistent in models stratified by follow-up period (Figure E1). There was no significant association between

children's NO_2 exposures at age 6 and their CC16 levels from age 6-32 in either unadjusted or adjusted models (Figure 1). Results were consistent when models included only children with reported exposures for both time periods. When models were stratified by follow-up period, there was an increase in CC16 levels at age 6 with concurrent NO_2 exposures at age 6 (Figure E1), and a decrease in CC16 levels at age 22-32. These trends were not significant.

We observed a significant interaction between race/ethnicity and NO_2 exposure at birth on CC16 levels (p for interaction = 0.04). The strongest NO_2/CC16 association was among Blacks, though this estimate was quite imprecise (- 29.6%, 95% CI -42.9%, -13.2%, Figure 2). Our cohort had a relatively small Black population (n participants=26, n observations=73), and thus these exploratory results should be interpreted with caution. Given that their exposures at birth were not significantly higher (Table S2), this may indicate an increase in susceptibility to NO_2 exposure. Although we hypothesized that race may be a proxy for socioeconomic status in our cohort, we did not observe modification by maternal age, maternal education, or smoking. To ensure that this was not an effect of living in a neighborhood with lower socioeconomic status or poorer housing conditions, we also assessed effect modification by indices we previously derived from US Census variables (27). There was no effect modification by these variables either. In our cohort, participants with any Black parent were not more likely than other participants to have younger mothers, mothers who smoked, mothers with lower education, or live in neighborhoods with lower socioeconomic status or poorer housing. In a sensitivity analysis without Black children, each interquartile increase in NO_2 exposure at birth was associated with a decrease in CC16 levels from age 6-32 years (Adjusted -3.5% [95% CI -7.5, 0.7]). While not a significant association, these results are still suggestive of a negative association between NO_2 exposure and CC16 levels among children of other racial or ethnic heritage.

Discussion

To the best of our knowledge, this is the first study to prospectively document an association between early-life NO_2 exposures and CC16 levels in humans. We demonstrated that ambient NO_2 exposures at birth address were associated with decreased circulating CC16 levels from age 6-32 years. However, there was no association between ambient NO_2 exposures at age 6 home address with CC16 levels. There was effect modification by race/ethnicity. Participants with at least one non-Hispanic Black parent had significantly larger reductions in CC16 levels per interquartile range of NO_2 exposure compared to participants from other racial/ethnic backgrounds. CC16 may be a sensitive biomarker of early-life damage to the respiratory tract following exposure to oxidizing air pollutants like NO_2.

Our study confirms findings from the only other study we identified that assessed the relationship between NO_2 exposure and CC16 levels in humans. Workers chronically exposed to nitrogen oxides at a nitric acid production factory were significantly more likely to have decreased levels of CC16, compared to unexposed workers (10). In our study, we demonstrated that increased NO_2 exposure levels at birth were associated with decreased CC16 levels through age 32 years. Of the participants included in our analyses with measured CC16 at enrollment and age 6, 56% had moved residences between birth and age 6. However, exposures at age 6 were not significantly associated with CC16 levels from age 6-32 years. This supports conclusions from animal studies that there may be critical periods of lung development where epithelial cells may be uniquely susceptible to NO_2-induced death (1). Thus, children with increased NO_2

exposures during this critical window may be predisposed to having fewer club cells and reduced potential to produce CC16.

One of the complexities of CC16 as a biomarker of lung damage is the finding from *in vitro* and *in vivo* studies that show that acute or recent exposures to NO_2 may temporarily increase bronchial epithelial permeability, thereby allowing more CC16 into circulation, resulting in temporarily elevated levels (1, 9, 10). Although not significant, increased NO_2 exposures at age 6 were associated with increased CC16 levels at age 6 in our study (Figure E1). However, similar to exposures at birth, increased NO_2 exposures at age 6 years were associated with decreased levels of CC16 in adulthood (age 22-32 years). These findings indicate that the critical window of exposure may include more of early childhood.

Although there have not been any studies examining associations between ambient NO_2 exposure and CC16 levels in humans during critical periods of lung growth, a few studies in adults document that daily exposures to ambient particulate matter (PM) are associated with increased CC16 concentrations (28, 29). These associations are stronger with PM attributed to combustion sources (30), which are also more likely to be correlated with NO_2 in most ambient environments. Timonen *et al*. and Jacquemin *et al*. hypothesize that increased CC16 levels associated with ambient PM exposures on the same day may indicate that acute PM exposure is also associated with increased epithelial permeability (29, 30). In one study, concurrent increase in CC16 levels with PM exposures are associated with temporary deficits in lung function (28), indicating that the immediate decrease in lung function following exposure to PM may be attributed to loss of pulmonary epithelium integrity (28). They demonstrated in a mediation model that increased levels of CC16 accounted for 4-36% of the lung function deficits associated with PM exposure. Thus, it is plausible that the positive association we observed in our cohort

for concurrent NO_2 exposure and CC16 levels at age 6 may be explained by a similar mechanism (28), as NO_2 is often correlated with PM in ambient environments.

Previously, we reported that diminished CC16 levels (age 6-32 years) in our cohort were associated with socioeconomic factors (i.e., Hispanic ethnicity, younger maternal age, maternal smoking, lower parental education attainment) (25). While children with lower socioeconomic status are likely to have higher NO_2 exposures, in our study increased NO_2 exposure at birth was only associated with maternal smoking and none of the other factors. Interestingly, increased NO_2 exposure at age 6 was associated with all of these factors, yet there was not a significant association between NO_2 exposures at age 6 and longitudinal CC16 levels. In our cohort, socioeconomic status is not likely the explanation for the association of NO_2 exposure with CC16 levels (25). Future work is needed to determine if there are other factors that could explain these associations, such as additional environmental exposures, nutritional status, or access to health care.

In our analysis, children with any non-Hispanic Black parent had significantly larger deficits in CC16 levels per interquartile increase in NO_2 exposures at birth compared to children from any other racial/ethnic background (Figure S2). As only 3.6% of our study population had any Black parent, which is representative of Tucson, AZ, further analyses in cohorts with a larger proportion of Black children are needed to confirm this increased potential susceptibility.

However, there is a strong suggestion from the literature that Black children may be more susceptible to NO_2 exposures. Multiple studies demonstrated significantly stronger associations between NO_2 exposures and hospital admissions or emergency room visits for asthma among Black children compared to other children (31-34). This effect modification remained significant after controlling for insurance status and socioeconomic variables in a cohort of low-income

children. Other studies demonstrated that racial disparities in childhood asthma are not just a function of income disparities (35-37). In a laboratory-controlled study, adult Black males demonstrated significantly larger lung function deficits following exposure to ozone, another oxidizing air pollutant (38). Multiple hypotheses have been proposed, including differences in access to healthcare (39), oxidant defense genes (40, 41), or levels of micronutrients (42). For example, increased Vitamin A levels are associated with increases in CC16 levels, (43) and increased levels of Vitamin E protect the lungs from the effects of NO_2 (1). Non-Hispanic Black children in the US have lower levels of both Vitamin A and E after controlling for income and socioeconomic factors, compared to children from other racial/ethnic backgrounds (44). Further work is needed to better understand why Blacks are more susceptible to oxidizing air pollutant (i.e., NO_2, ozone) exposures and the role that CC16 may have in this effect.

One of the key limitations of our study is that we only had exposure estimates for NO_2 and not for other air pollutants. As air pollutants are often times highly correlated, it is plausible that the effects we observed may be attributed to other air pollutants such as ozone or PM. However, we were not able to assess for or control for those other exposures. While there is biological evidence of the effect of NO_2 on CC16 levels, club cells, and epithelial integrity, future studies should investigate exposures to mixtures of ambient air pollutants to determine their independent and joint effects on longitudinal CC16 levels.

In summary, in our non-selected birth cohort with 32 years of follow-up, we found that increased NO_2 exposure at birth but not at age 6 was associated with a decrease in CC16 levels through adult life (age 6-32 years). Although the numbers were small, participants with at least one non-Hispanic Black parent had significantly larger deficits in association with NO_2 exposure at their birth. Thus, CC16 may be a sensitive biomarker of early-life lung damage from the

oxidizing air pollutant NO_2 during critical windows of lung development, particularly among Black children. Confirmation of these findings in other cohorts with a larger proportion of Black participants could support the utility of CC16 as a means of identifying those children most susceptible to the effects of air pollution.

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Figure Legends

Figure 1. Percent change in CC16 levels from ages 6-32 years in association with an interquartile range increase in NO_2 exposure at birth and age 6 addresses, respectively. Associations are also shown per quartile. All models include random effects for subject and fixed effects for visit. Adjusted models include fixed effects for child's race/ethnicity, child's gender, and maternal smoking at enrollment. Participant counts include those with complete covariate, exposure and outcome data. Percent change was calculated from the beta coefficients according to methods for log-transformed outcome variables (24).

Figure 2. Percent change in CC16 levels from ages 6-32 years in association with an interquartile range increase in NO_2 exposure at birth, by race/ethnicity. All models include random effects for subject, fixed effects for visit, child's gender and maternal smoking at enrollment, and an interaction term for child's race/ethnicity and NO_2 exposure at birth. Participant counts include those with complete covariate, exposure and outcome data. Percent change was calculated from the beta coefficients according to methods for log-transformed outcome variables (24).

Table 1. Distribution of ambient NO_2 concentrations (ppb) at reported residence at enrollment and first follow-up (age 6).

	N	GM*	GSD†	Median	IQR‡	Range
Enrollment (1980-1984)	1137	10.96	1.41	11.00	6.00	4.18-27.30
First Follow-Up (1986-1990)	806	10.23	1.48	10.74	6.69	2.73-20.39

*GM: geometric mean, †GSD: geometric standard deviation, ‡IQR: interquartile range

Table 2. Characteristics of participants included in analyses in each follow-up period. Included participants had at least one CC16 measurement between age 6-32 years and their NO_2 exposure at enrollment or age 6 could be determined from their address. Participants were asked about their own smoking status beginning at age 16.

	Enrollment	6 years	11 years	16 years	22 years	26 years	32 years
N	1137*	479†	550†	432†	416†	358†	234†
CC16 (ng/mL) GM (GSD)		7.9 (1.5)	7.5 (1.5)	8.4 (1.5)	11.5 (1.5)	9.5 (1.6)	10.5 (1.5)
NO_2 at Enrollment (ppb) Median (IQR)	11.0 (6.0)	10.8 (6.3)	10.9 (6.2)	10.7 (6.2)	10.8 (6.2)	10.7 (6.0)	10.8 (6.2)
NO_2 at Age 6 (ppb) Median (IQR)		10.5 (6.5)	10.8 (6.5)	10.7 (6.4)	10.5 (6.4)	10.3 (6.4)	10.4 (6.9)
Child Sex % (N)							
Male	48.3 (550)	49.9 (239)	48.7 (268)	48.8 (211)	48.5 (204)	47.2 (169)	43.6 (102)
Female	51.6 (587)	50.1 (240)	51.3 (282)	51.2 (221)	51.5 (217)	52.8 (189)	56.4 (132)
Child Ethnicity Race % (N)							
Non-Hispanic White	59.4 (675)	62.2 (298)	58.6 (322)	60.2 (260)	61.3 (255)	62.0 (222)	60.3 (141)
Any Hispanic	25.5 (290)	27.8 (133)	31.5 (173)	29.6 (128)	29.6 (123)	28.5 (102)	30.3 (71)
Any Black	4.1 (47)	3.6 (17)	3.3 (18)	3.2 (14)	2.2 (9)	2.5 (9)	2.6 (6)
Other	11.0 (125)	6.4 (31)	6.7 (37)	6.9 (30)	7.0 (29)	7.0 (25)	6.8 (16)
Maternal Age at Enrollment % (N)							
<20	5.1 (58)	4.4 (21)	4.5 (25)	3.2 (14)	3.1 (13)	3.3 (12)	2.1 (5)
20-25	32.1 (365)	29.4 (141)	29.6 (163)	29.9 (129)	29.3 (122)	33.5 (120)	33.8 (79)
26-30	38.2 (434)	40.1 (192)	40.2 (221)	43.1 (186)	42.3 (176)	38. 5 (138)	40.2 (94)
>30	24.6 (280)	26.1 (125)	25.6 (141)	23.8 (103)	25.2 (105)	24.6 (88)	23.9 (56)
Maternal Education at Enrollment % (N)							
≤12 years	31.3 (355)	28.2 (135)	28.2 (155)	24.6 (106)	25.0 (104)	25.1 (90)	25.8 (60
>12 years	68.7 (780)	71.8 (344)	71.8 (394)	75.4 (325)	75.0 (312)	74.9 (268)	74.3 (173)
Maternal Smoking at Enrollment % (N)							
Yes	17.2 (195)	15.1 (72)	14.2 (78)	13.7 (59)	13.9 (58)	15.1 (54)	13.7 (32)
No	82.8 (941)	84.9 (406)	85.8 (471)	86.3 (373)	86.1 (358)	84.9 (304)	86.3 (202)
Current Smoking % (N)							
Yes				10.6 (43)	28.9 (120)	26.0 (92)	16.7 (39
No				89.4 (362)	71.1 (296)	74.0 (262)	83.3 (195)

*Number of participants with ≥1 CC16 measurement between age 6-32 years and NO_2 exposure was determined at enrollment address, †Number of participants with a CC16 measurement obtained during that follow-up period and their NO_2 exposure at enrollment or age 6 could be determined from their address.

Table E1. Predictor variables used in land use regression model development. Full references for data sources provided at end of supplemental file.

Predictor	**Data Source(s)**	**Specification (Units)**	**Spatial Buffer Sizes (m)**
Distance to nearest road	Pima County GIS (1)	Distance to center of the road (m)	NA
Distance to nearest rail line	Pima County GIS (2)	Distance of object to rail (m)	NA
Distance to nearest bus route	Pima County GIS (3)	Distance of object to bus route (m)	NA
Distance to nearest airport	Pima County GIS (4)	Distance of object to airport (m)	NA

Distance to nearest mine	Pima County GIS (5)	Distance of object to mine (m)	NA
Traffic intensity nearest street	Pima County GIS (1), Pima Association of Governments (6)	Motor vehicles per day (vehicles)	NA
Traffic intensity buffers	Pima County GIS (1), Pima Association of Governments (6)	Motor vehicles per day in buffers (vehicles)	25, 50, 100, 300, 500, 1000
Distance to nearby major road*	Pima County GIS (1), Pima Association of Governments (6)	Distance to center of a major road (m)	NA
Traffic intensity on nearest major road	Pima County GIS (1), Pima Association of Governments (6)	Motor vehicles per day in buffers	NA
Population density	1990 US Decennial Census (7)	Population density in buffers (persons/m^2)	100, 300, 500, 1000, 5000
Household density	1990 US Decennial Census (7)	Household density in buffers (households/m^2)	100, 300, 500, 1000, 5000
Land use	1992 National Land Use Cover Database (8)	Land use in buffers (e.g. residential land, industry, urban green) (m^2)	100, 300, 500, 1000, 5000
Elevation	US Geological Survey	Elevation above sea level (m)	NA

NOTES: *Major road: >5,000 vehicles/day

Table E2. Association between participant characteristics and NO_2 exposure based upon participant's home address at enrollment and at age 6, respectively.

	Enrollment				Age 6			
	Median	Range	β	p-value	Median	Range	β	p-value
Gender								
Male	10.84	4.61-21.80	ref		10.49	2.73-20.12	ref	
Female	10.94	4.18-27.30	0.01	0.87	10.83	2.73-19.18	0.02	0.64
Child Ethnicity/Race								
Non-Hispanic White	11.05	4.37-27.30	ref		10.03	2.78-19.05	ref	
Any Hispanic	10.97	4.82-20.40	-0.01	0.80	11.62	2.73-20.12	0.14	<0.01
Any Non-Hispanic Black	11.88	4.83-18.35	0.07	0.57	11.85	5.12-18.78	0.25	0.06
Other	9.13	4.18-17.60	-0.23	0.01	10.01	4.75-19.18	0.03	0.75
Maternal Age at Enrollment								
<20	11.60	4.61-18.71	ref		13.35	5.94-18.31	ref	
20-25	10.88	5.03-21.80	-0.12	0.23	10.84	2.73-20.12	-0.31	<0.01
26-30	10.98	4.82-27.30	-0.12	0.25	10.23	3.18-18.83	-0.30	<0.01
>30	10.36	4.18-24.75	-0.16	0.13	10.36	2.95-19.18	-0.33	<0.01
Maternal Education at Enrollment								
> 12 years	10.79	4.18-27.30	ref		10.26	2.78-19.18	ref	
≤ 12 years	10.99	4.61-20.83	0.00	0.99	11.22	2.73-20.12	0.12	0.01
Maternal Smoking at Enrollment								
No	10.88	4.18-27.30	ref		10.78	2.73-19.18	ref	
Yes	11.06	4.82-23.58	0.02	0.76	10.46	4.89-20.12	-0.00	0.98

Medians and ranges are reported for the raw NO_2 values. Beta coefficients and p values for differences are per interquartile range of NO_2. Only includes participants with NO_2 and at least one CC16 measurement.

Table E3. Characteristics of participants according to inclusion in the analysis. Participants included had at least one CC16 measurement between age 6-32 years and their NO_2 exposure at enrollment or age 6 could be determined.

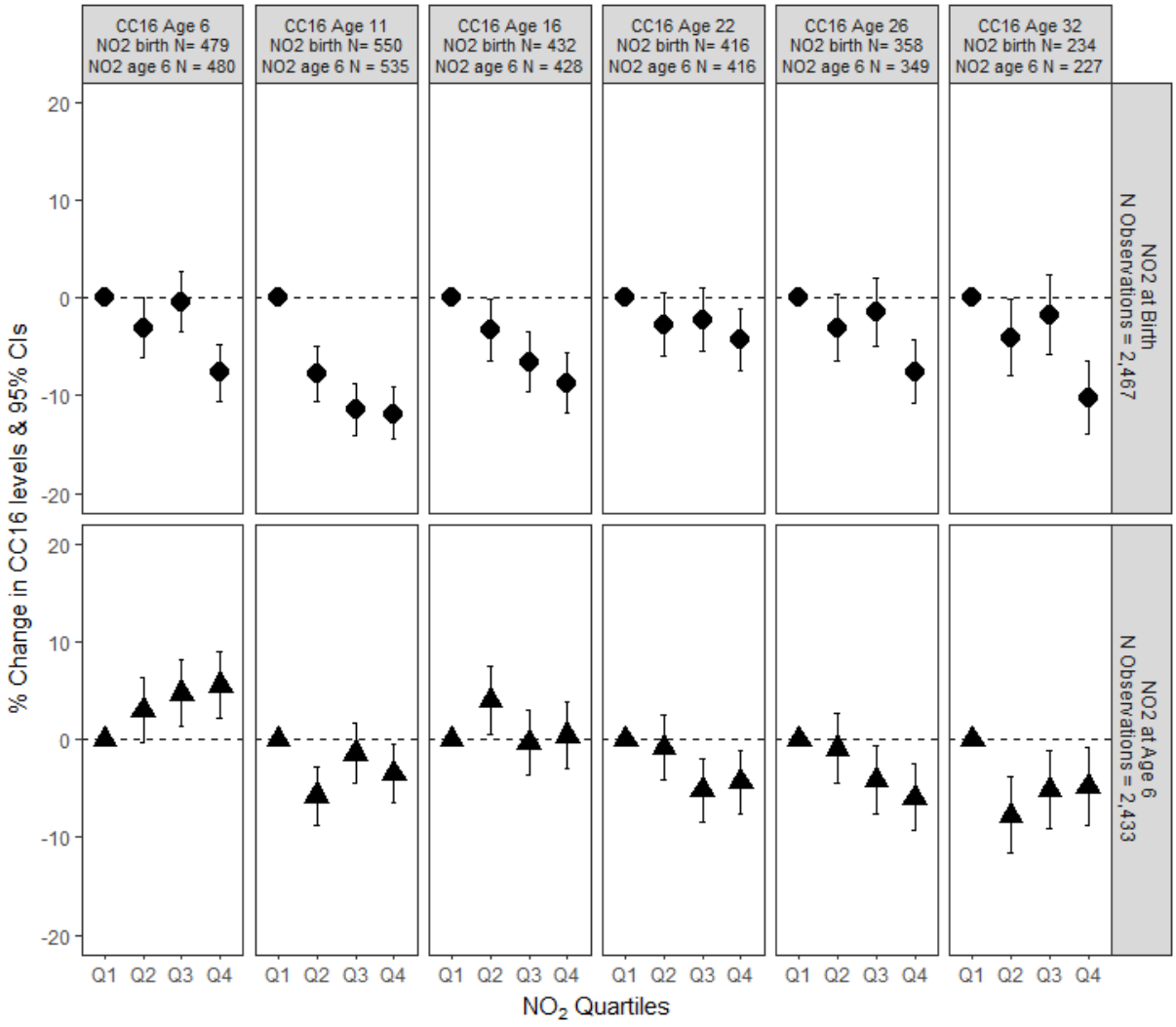
	Not Included		Included		χ^2	p-value
	N=469	%	N=777	%		
NO_2 Enrollment Exposure Quartile*					2.43	0.49
1	97	24.0	187	25.5		
2	97	24.0	187	25.5		
3	112	27.7	172	23.5		
4	99	24.4	186	25.4		
NO_2 Age 6 Exposure Quartile†					2.67	0.45
1	28	24.8	174	25.1		
2	25	22.1	176	25.4		
3	25	22.1	176	25.4		
4	35	31.0	167	24.1		
Gender					0.47	0.49
Male	224	47.8	388	49.9		
Female	245	52.2	389	50.1		
Child Ethnicity/Race					38.4	<0.01
Non-Hispanic White	260	55.4	481	61.9		
Any Hispanic	104	22.2	216	27.8		
Any Non-Hispanic Black	21	4.5	26	3.3		
Other	84	17.9	54	6.9		
Maternal Age‡					9.16	0.03
<20	43	9.2	44	5.7		
20-25	154	32.9	228	29.3		
26-30	169	36.1	303	39.0		
>30	12	21.8	202	26.0		
Maternal Education‡					6.68	<0.01
≤ 12 years	175	37.6	219	28.2		
> 12 years	290	62.4	557	71.8		
Maternal Smoking‡					4.55	0.03
Yes	100	21.4	120	15.5		
No	367	78.6	656	84.5		

*These participants are restricted to those with NO_2 measurements at birth and any CC16 value from age 6-32

†These participants are restricted to those with NO_2 measurements at age 6 and any CC16 value from age 6-32

‡All variables at enrollment

Figure E1. Percent change in club cell secretory protein levels at each age in association with nitrogen dioxide exposure at birth and age 6 addresses, respectively. Associations are shown per quartile. Adjusted models include fixed effects for child's race/ethnicity, child's gender, and maternal smoking at enrollment. Participant counts include those with complete covariate, exposure and outcome data. Percent change was calculated from the b coefficients according to methods for log-transformed outcome variables (10). CC16=club cell secretory protein; CI=confidence interval; NO_2=nitrogen dioxide.



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