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# Noninvasive integrative approach applied to children in the context of recent air pollution exposure demonstrates association between fractional exhaled nitric oxide (FeNO) and urinary CC16

Sarah J.D. Nauwelaerts [a,b,1], Nina Van Goethem [c,1], Koen De Cremer [d], Natalia Bustos Sierra [c], Jordy Vercauteren [e], Christophe Stroobants [f], Alfred Bernard [b], Tim Nawrot [f,g], Nancy H.C. Roosens [a], Sigrid C.J. De Keersmaecker [a,*]

[a] Transversal Activities in Applied Genomics, Sciensano, Brussels, Belgium
[b] Centre for Toxicology and Applied Pharmacology, University Catholique de Louvain, Brussels, Belgium
[c] Department of Epidemiology and Public Health, Sciensano, Brussels, Belgium
[d] Platform Chromatography and Mass Spectrometry, Sciensano, Brussels, Belgium
[e] Unit Air, Vlaamse Milieumaatschappij, Antwerpen, Belgium
[f] Centre for Environmental Sciences, Hasselt University, Agoralaan Building D, 3590, Diepenbeek, Belgium
[g] Department of Public Health and Primary Care, KU Leuven, Leuven, Belgium

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ABSTRACT

Exposure to the air pollutant particulate matter (PM) is associated with increased risks of respiratory diseases and enhancement of airway inflammation in children. In the context of large scale air pollution studies, it can be challenging to measure fractional exhaled nitric oxide (FeNO) as indicator of lung inflammation. Urinary CC16 (U-CC16) is a potential biomarker of increased lung permeability and toxicity, increasing following short-term $PM_{2.5}$ exposure. The single nucleotide polymorphism (SNP) *CC16* G38A (rs3741240) affects CC16 levels and respiratory health. Our study aimed at assessing the use of U-CC16 (incl. *CC16* G38A from saliva) as potential alternative for FeNO by investigating their mutual correlation in children exposed to PM. Samples from a small-scale study conducted in 42 children from urban (n = 19) and rural (n = 23) schools examined at two time points, were analysed. When considering recent (lag1) low level exposure to $PM_{2.5}$ as air pollution measurement, we found that U-CC16 was positively associated with FeNO (β = 0.23; 95% CI [-0.01; 0.47]; p = 0.06) in an adjusted analysis using a linear mixed effects model. Further, we observed a positive association between $PM_{2.5}$ and FeNO (β = 0.56; 95% CI [0.02; 1.09]; p = 0.04) and higher FeNO in urban school children as compared to rural school children (β = 0.72; 95% CI [0.12; 1.31]; p = 0.02). Although more investigations are needed, our results suggest that inflammatory responses evidenced by increased FeNO are accompanied by potential increased lung epithelium permeability and injury, evidenced by increased U-CC16. In future large scale studies, where FeNO measurement is less feasible, the integrated analysis of U-CC16 and *CC16* G38A, using noninvasive samples, might be a suitable alternative to assess the impact of air pollution exposure on the respiratory health of children, which is critical for policy development at population level.

*Abbreviations:* β2M, beta-2-microglobulin; CC16, club cell secretory protein; CLB, pupil guidance center; FeNO, fractional exhaled nitric oxide; MRM, multiple reaction monitoring; PM, particulate matter; $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5μm; $PM_{10}$, particulate matter with an aerodynamic diameter less than 10μm; RBP4, retinol-binding protein 4; SNP, single nucleotide polymorphism; U-β2M, urinary beta-2-microglobulin; U-CC16, urinary club cell secretory protein; U-RBP4, urinary retinol binding protein 4; WHO, World Health Organization.

* Corresponding author. Rue Juliette Wytsmanstraat 14, 1050, Brussels, Belgium.
*E-mail address:* Sigrid.dekeersmaecker@sciensano.be (S.C.J. De Keersmaecker).

[1] Equally contributed.

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## 1. Introduction

Air pollution is a major threat for public health (Boogaard et al., 2019; WHO Regional Office for Europe, 2013). Elevated levels of particulate matter (PM) with an aerodynamic diameter smaller than 10 μm ($PM_{10}$) but especially with an aerodynamic diameter smaller than 2.5 μm ($PM_{2.5}$) are the most problematic air pollutants in terms of health effects in Belgium (IRCEL-CELINE, 2019), even with levels commonly encountered. They can even cause adverse health effects when their levels are around or below the levels defined by the recently adapted World Health Organization (WHO) guidelines (World Health Organization, 2021). These guidelines aim for an 24-h average exposure that doesn't exceed 45 $\mu g/m^3$ and 15 $\mu g/m^3$ more than 3–4 times per year for $PM_{10}$ and $PM_{2.5}$, respectively. Children are particularly vulnerable for the effects of air pollution with increased risks of respiratory diseases such as asthma, lower track infections, impairment of their lung function (Guarnieri and Balmes, 2014; Guo et al., 2019; Orellano et al., 2017; Zhai et al., 2019) and enhancement of airway inflammation (Barraza-Villarreal et al., 2008; Renzetti et al., 2009). The noninvasive measurement of fractional exhaled nitric oxide (FeNO) can help to assess the adverse effects of air pollution in the airways as an indicator of allergic and eosinophilic airway inflammation (Malmberg et al., 2005). Measuring FeNO is a sensitive test for diagnosing asthma in children (Flamant-Hulin et al., 2009; Guo et al., 2016; Karrasch et al., 2017; Malmberg et al., 2005). FeNO has also been shown to increase following short-term and long-term air pollution exposure (Berhane et al., 2011; Flamant-Hulin et al., 2009; Mohd Isa et al., 2022). The measurement of FeNO is cheap, once the required instrument has been acquired, and easy to perform in children in a small scale study setting with limited children, locations and time points. However, cautious interpretation is needed as there are a number of confounding factors to take into account, such as exercise, exposure to smoke, viral infections or certain food intakes (Bjermer et al., 2014). Moreover, in the context of large scale studies, conducted in multiple locations and at several time points, it can be time consuming and logistically challenging to measure FeNO, as it necessitates trained staff and multiple instruments, increasing the costs (Nauwelaerts et al., 2022a). Furthermore, in the case a set-up with self-sampling is envisaged to facilitate larger-scale studies, the measurement of FeNO is not feasible anymore and other alternatives for monitoring the respiratory health are needed (Nauwelaerts et al., 2022b).

Biomarkers have opened new perspectives with the development of noninvasive tests to monitor the damage and the inflammation in the deep lung. The club cell protein (CC16) is secreted from the nonciliated epithelial cells to the epithelial lining fluid and plays a protective role against lung inflammation and oxidative stress (Broeckaert and Bernard, 2000). CC16 has been proposed as a biomarker of respiratory health and air pollution exposure with a dual character (Broeckaert and Bernard, 2000; Lakind et al., 2007). Chronic exposure to air toxicants damages the CC16-producing club cells, leading to decreased serum CC16 (S-CC16) levels (Bernard et al., 1994; Broeckaert et al., 1999; Heldal et al., 2013; Rava et al., 2013), which were found to be associated with the development of respiratory diseases, and lung function decline in adults and children (Guerra et al., 2015, 2013; Pilette et al., 2001). On the other hand, short-term exposure to irritants such as chemicals, dust and air pollution will lead to transient increases of S-CC16, most likely due to an increased permeability of the bronchoalveolar/blood barrier, explaining the use of CC16 as biomarker of early signs of acute airway injury and increased lung permeability (Arsalane et al., 1999; Blomberg et al., 2003; Provost et al., 2014; I.-J. Wang et al., 2017a, b). Only few studies have investigated the early effects of acute air pollution in children. If this increase of CC16 could be detected and could reflect the sudden increased lung permeability and injury following short-term exposure, quick interventions might help to prevent the transition to the further degradation of the club cells and reduction of produced CC16, in case of chronic exposure.

However, in large-scale studies, involving children, sampling of urine might be more appropriate than serum, as it is more readily accepted and less costly than blood sampling. Urinary CC16 (U-CC16) has gained more interest, as a potential alternative to S-CC16 and as biomarker of lung inflammation, airway toxicity and increased lung permeability (Andersson et al., 2007; St Helen et al., 2013). A few studies have investigated U-CC16 in children and its association with respiratory disease susceptibility (Egron et al., 2020; Ma et al., 2015b; Rosas-Salazar et al., 2015) and with different toxicant exposures (Beamer et al., 2016; Ma et al., 2015a; H. Wang et al., 2017a, b). Similarly to S-CC16, increasing U-CC16 levels have been observed following short-term exposure to air pollution such as ozone in rats (Arsalane et al., 1999) and PM in adults (Jacquemin et al., 2009; Timonen, 2004). Recently, this short-term effect of $PM_{2.5}$ was also demonstrated in children, with increased levels of CC16 in urine, 24 h after exposure (lag 1) (Nauwelaerts et al., 2022b). These findings also emphasized the importance of taking into account two parameters when using U-CC16 as a biomarker. Firstly, the single nucleotide polymorphism (SNP) *CC16* G38A (rs3741240) is an important genetic determinant of the circulating CC16 levels, reflecting the amount of CC16 secreted in the deep lung. Besides being associated with respiratory disease susceptibility (Chen et al., 2012; Zhao et al., 2013), associations were also found between the A-allele and lower circulating S-CC16 levels (Chen et al., 2012; Ku et al., 2011; Laing et al., 2000; Taniguchi et al., 2013) and recently with lower U-CC16 levels (Nauwelaerts et al., 2020a, 2022b). This SNP can easily be measured in buccal DNA obtained from saliva (Nauwelaerts et al., 2020b). Secondly, beta-2-microglobulin (β2M) has been proposed as a potentially more precise alternative to the commonly used creatinine to adjust the measured U-CC16 levels for renal handling and diuresis (Egron et al., 2020; Nauwelaerts et al., 2021, 2022b, 2020a). This protein can be measured using a mass spectrometry based multiple reaction monitoring (MRM) method, allowing the simultaneous measurement of this protein as well as other potentially interesting protein biomarkers, including U-CC16 (Nauwelaerts et al., 2021).

While the measurement of FeNO in larger scale studies might be challenging or impossible, the collection of urine and saliva as source of biomarkers is straightforward, and even feasible in self-sampling modus. The measurement of U-CC16, combined with urinary β2M (U-β2M) and the SNP *CC16* G38A, would allow to detect the early signs of lung damage and increased lung permeability (Nauwelaerts et al., 2022b). Only few studies investigated both parameters, U-CC16 and FeNO, in children following short-term exposure to $PM_{2.5}/PM_{10}$. None investigated their mutual association in that context, neither used an integrated approach by including the genetic background of the child into the analysis. Previously, we conducted a small scale field study, in view of future larger scale studies, that envisaged the collection of samples for the measurement of U-CC16 as well as FeNO at two time points in a small group of school children exposed to air pollution (Nauwelaerts et al., 2022a) at two different locations. Although this was a small scale field study, aimed at providing insight at the logistic and technical level for future large-scale studies (Nauwelaerts et al., 2022a), we used the collected samples and FeNO measurement data in our current integrated study to assess if U-CC16, a potential biomarker of lung epithelial injury, is associated with FeNO in children recently exposed to different levels of air pollution. This would allow us to evaluate if U-CC16 could potentially be used as an alternative for FeNO to assess the impact of air pollution at the level of the lungs. To our knowledge, this has not yet been looked into, while it would facilitate future monitoring studies based on noninvasive (self-) sampling as well as the retrospective investigation of already existing biobanks.

## 2. Material and methods

### 2.1. General health parameters and noninvasive sampling

42 children of two primary schools, aged 9–11 years old, participated to the field study, conducted on two seasonally different time points, i.e. one in the summer (t1, i.e. September 2018) and one in the winter (t2, i.e. January 2019). For each time point, the study took place with three days apart between the two schools. The two primary schools were each located in a distinct urban or rural area in Belgium, aiming at different air pollution exposures. The study was approved by the Ethics committee of the Cliniques universitaires Saint-Luc (Registration number B403201734310). Informed consents were obtained from the parents and the children prior to participation in the study. A questionnaire, addressing the social and medical background of the children and their family as well as their in- and out-of-house environment was completed by the parents.

General health parameters (such as weight and height) were measured and noninvasive samples were collected. Second morning urine samples were collected, as it was logistically more feasible than collecting the first morning urine and as it also minimizes the time of the urine spent in the bladder, where increased proteolysis might occur (Thomas et al., 2010). The urine samples were immediately aliquoted and stored at −80 °C until further downstream processing. On the first time point of the study, a saliva sample was collected to investigate the *CC16* SNP G38A genotype. More details on the complete set-up of the field study, on the measurements and sampling have been described elsewhere, where we have reported on the set-up and logistic bottlenecks for this type of studies (Nauwelaerts et al., 2022a).

### 2.2. FeNO measurement

A FeNO test was performed with the NOBreath monitor (Bedfond, Maidstone, UK), following the protocol established by the American Thoracic Association (American Thoracic Society and European Respiratory American Thoracic SocietyEuropean Respiratory Society, 2005). From the three measurements, the two best ones were retained for further analysis. FeNO was measured on the same day as the collection of the noninvasive samples of the corresponding children.

### 2.3. Air pollution measurement

Information on the air pollution exposure was obtained from measurements conducted in the context of this small scale field study, described into detail elsewhere (Nauwelaerts et al., 2022a). Briefly, air pollutant levels were obtained from air quality measurement campaigns of two stationary measuring stations. One was installed just before the study on the parking lot of the rural school. Due to the lack of space at the urban school, air pollution data were obtained from a measuring station, already present in the vicinity of the urban school. These stations continuously measured several pollutants, including $PM_{2.5}$ and $PM_{10}$, before, and during the day of the examinations and sample collections. The hourly pollutant levels of $PM_{2.5}$ and $PM_{10}$ levels were measured with an aerosol spectrometer (Palas Fidas 200 (Karlsruhe, Germany) without major technical problems. Daily median concentrations of $PM_{2.5}$ and $PM_{10}$ at both school sites are described on the day of (lag 0), the day before (lag 1) or two days before (lag2) the examinations and the collection of the samples.

### 2.4. Sample analysis

#### 2.4.1. Urinary CC16 protein and its adjuster urinary β2M

The urine samples, collected at both time points of the field study, where thawed and underwent a trypsin digest as described previously (Nauwelaerts et al., 2021). Subsequently, using a validated MRM method (Nauwelaerts et al., 2021), relative abundances of the relevant proteins were obtained simultaneously. U-CC16 was measured as potential biomarker of lung injury and permeability, while U-β2M was measured and used to adjust for renal handling and diuresis (Nauwelaerts et al., 2021).

#### 2.4.2. CC16 G38A genotyping

The saliva samples, stored at room temperature since their collection during the first time point (t1) of the field study, were used to extract DNA with a commercial kit (prepIT-L2P, DNA Genotek, Ottawa, Ontario, Canada), which was stored at −20 °C until further analysis. The DNA was genotyped, using a genotyping assay for the SNP *CC16* G38A polymorphism (rs3741240), as described in Nauwelaerts et al. (2020b). The *CC16* genotyping was successfully performed for a total of 19 and 21 children of both the urban and rural school, respectively.

### 2.5. Statistical analysis

All continuous variables were described as median with interquartile range (IQR) or as daily mean (for the PM values). A log transformation was used for the normalization of the distribution of PM exposures levels, the protein levels (U-CC16, U-β2M), and the FeNO measurements.

We investigated the association between the measured U-CC16 and the measured FeNO, taking into account the SNP *CC16* G38A genotype, the air pollution measurement, and potential confounding factors identified from the questionnaire, using a linear mixed-effects (LME) model. Gender, age, and time of examination (to account for potential diurnal variation) of the FeNO test (categorized as before (reference value) or after first (playtime) or second (lunch period) school break) were kept in the model based on biological plausibility. A stepwise model selection approach was used to select for the other potential confounding variables: body mass index (BMI), exposure to tobacco smoke, occurrence of respiratory allergies (to house dust mite, animal hair, pollen), the use of bleach during cleaning, the occurrence of baby swimming in swimming pool and the use of wood during heating. The confounding effect of renal handling and diuresis was compensated by including β2M as adjuster for U-CC16. Details on the statistical modeling strategy have been described previously (Nauwelaerts et al., 2022b). A sensitivity analysis excluding children with >10% deviation between the two best FeNO measurements was performed to assess how the inclusion of all samples versus only the average of the two samples with less than 10% variation, would influence the robustness of the results. The same modeling strategy was used to assess the association between air pollution measurements and FeNO, and between air pollution measurements and U-CC16. In the latter model, the time of examination (to account for potential diurnal variation) for urine sampling (categorized as before (reference value) or after first (playtime) or second (lunch period) school break) was taken into account. All analyses were performed using the R software (R version 4.0.5). P-values were calculated based on Type II Wald Chi-square tests. All P-values were two-sided with the level of statistical significance set at $p < 0.05$.

## 3. Results

### 3.1. General characteristics, sample analysis and air pollution exposure of the study population

Baseline characteristics obtained from the questionnaire and from the examinations are summarized in Table 1. The median age of the children was 9.6 years and approximately the same number of boys and girls were included in the complete group of children. However, differences per schools were observed. In the urban school, 21% of participating children were boys, whereas this percentage was higher (68%) in the rural school. From the whole study group, three (7%) children were exposed to passive smoking and only one to maternal pregnancy smoking. Thirteen children (31%) suffered from any type of respiratory allergy.

**Table 1**
General characteristics and CC16 G38A genotype of the children at urban and rural school.

| | Urban (N = 19) | | Rural (N = 23) | | Total (N = 42) | |
|---|---|---|---|---|---|---|
| General characteristics | | | | | | |
| Boys[a] | 4 | (21%) | 13 | (68%) | 17 | (41%) |
| Age, years[a] | 9.4 | [9.1–9.9] | 9.5 | [9.2–10.2] | 9.6 | [9.1–10.1] |
| Body Mass Index (BMI), kg/m$^3$[a] | 16.4 | [15.4–18.9] | 16.2 | [15.4–19.0] | 16.3 | [15.3–19.0] |
| Weight, kg[a] | 30.0 | [29.0–38.5] | 33.8 | [28.3–37.1] | 31.1 | [28.6–38.1] |
| Length, cm[a] | 138 | [135–143] | 139 | [138–145] | 139 | [134–144] |
| Passive smoking[b] | 1 | (5%) | 2 | (9%) | 3 | (7%) |
| Mother smoked during pregnacy | 0 | (0%) | 1 | (4%) | 1 | (2%) |
| Respiratory allergy[b] | 8 | (42%) | 5 | (22%) | 13 | (31%) |
| SNP *CC16* G38A genotype[c] | | | | | | |
| Homozygous WT 38 GG | 14 | (74%) | 9 | (43%) | 23 | (58%) |
| Heterozygous 38AG | 3 | (16%) | 7 | (33%) | 10 | (25%) |
| Homozygous mutant 38AA | 2 | (11%) | 5 | (24%) | 7 | (18%) |

Values are represented as numbers for the categorical variables and as median [IQR] for the continuous variables; WT: wild-type; SNP: single nucleotide polymorphism.
[a] Parameters obtained/calculated during first examination (i.e., t1).
[b] Data obtained from the questionnaire.
[c] Data missing for 2 children.

**Table 2**
Urinary proteins (U-CC16 and U-β2M), fractional exhaled nitric oxide (FeNO) and PM ($PM_{2.5}$, $PM_{10}$) exposure of the children at their urban and rural school.

| | | Urban (N = 19) | | | | Rural (N = 23) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | t1 (in summer) | | t2 (in winter) | | t1 (in summer) | | t2 (in winter) | |
| **Relative levels of urinary analytes** | | | | | | | | | |
| U-CC16 | | 0.43 | [0.25–0.90][b] | 1.11 | [0.53–2.84] | 0.32 | [0.20–0.65][a] | 0.41 | [0.21–0.97][c] |
| U-β2M | | 76.65 | [63.0–95.1][b] | 162 | [110–280] | 57.9 | [39.8–88.0][b] | 66.59 | [48.4–76.4] |
| **FeNO of child (ppb)** | | 11.0 | [8.8–31.8] | 29.5 | [14.3–56.8] | 6.5 | [4.0–10.0] | 8.0 | [3.3–10.8] |
| **PM exposure (μg/m$^3$)**[d] | | | | | | | | | |
| lag 0 | $PM_{2.5}$ | 5 | | 19 | | 9 | | 13 | |
| | $PM_{10}$ | 13 | | 29 | | 17 | | 24 | |
| lag 1 | $PM_{2.5}$ | 8 | | 16 | | 11 | | 7 | |
| | $PM_{10}$ | 16 | | 22 | | 21 | | 16 | |
| lag 2 | $PM_{2.5}$ | 7 | | 9 | | 9 | | 9 | |
| | $PM_{10}$ | 13 | | 14 | | 17 | | 17 | |

Values are represented as numbers for the categorical variables and as mean or as median [IQR] for the continuous variables. ppb: parts per billion, PM: particulate matter with aerodynamic diameter smaller than 2.5 μm ($PM_{2.5}$) or smaller than 10 μm ($PM_{10}$); NA: not available; t1: summer time point; t2: winter time point; WT: wild-type.
[a] Data missing for 2 children.
[b] Data missing for one child.
[c] Data missing for 3 children.
[d] Daily mean concentrations (00h00 till 24h00), expressed in μg/m$^3$.

The protein abundances of U-CC16 and U-β2M were successfully measured for almost all children and are summarized in Table 2. From the *CC16* G38A genotyping, determined with the salivary DNA, 58% of the children were found to be homozygous WT 38 GG, 25% heterozygous 38AG and 7% homozygous mutant 38AA for the SNP *CC16* G38A (Table 1). Moreover, we observed a difference in genotype frequencies between the urban and the rural school children. This might be due to the limited sample number or to the differences in ethnic background that are represented in both schools. This can lead to deviations from the minor allele frequency and the expected genotype frequencies (The 1000 Genomes Project Consortium, 2010). However, because of to the limited sample size this is difficult to confirm.

No peak values, as defined by the latest WHO thresholds of 2021 (World Health Organization, 2021) of the $PM_{2.5}$ and $PM_{10}$ were reached, except for the urban school during the winter time point of the study, where slightly higher, though still low levels of $PM_{2.5}$ (lag 0) (19 μg/m$^3$) and $PM_{2.5}$ (lag1) (16 μg/m$^3$) were measured (Table 2). As expected, PM values were usually found to be higher in the winter than in the summer in both locations, especially in the urban school area. However, the expected tendency of higher PM levels in the urban school area, compared to the rural school, was less clear, especially in the summer.

### 3.2. U-CC16 is positively associated with FeNO

The following covariates were retained as fixed effects (based on the model selection and biological plausibility) in the final LME model to assess the association between U-CC16 and FeNO: age, gender, CC16 genotype, time of examination, respiratory allergy, air pollution, and U-β2M. A total of 78 observations from 40 children where available for the analysis. When using $PM_{2.5}$ (Fig. 1A) and when considering $PM_{10}$ (Fig. 1B), both measured 24 h before (i.e., at lag1), as air pollution measurement in the model, we found a (borderline) statistically significant positive association between U-CC16 and FeNO ($\beta = 0.23$; 95% CI [−0.01; 0.47]; $p = 0.06$, and $\beta = 0.24$; 95% CI [−0.01; 0.48]; $p = 0.06$, respectively). As expected, a statistically significant association was found between FeNO and respiratory allergy ($\beta = 0.88$; 95% CI [0.34; 1.43]; $p < 0.01$, and $\beta = 0.90$; 95% CI [0.35; 1.46]; $p < 0.01$, respectively).

Fig. 2 presents the predicted values (i.e., marginal effect) of FeNO in function of the U-CC16 protein levels, adjusted for the other covariates as included in the model that is presented in Fig. 1A. We expect a 2% increase in FeNO when U-CC16 increases with 10%. The association between U-CC16 and FeNO did not depend on the SNP *CC16* G38A genotype, nor on the occurrence of respiratory allergy (no significant interaction effects detected).

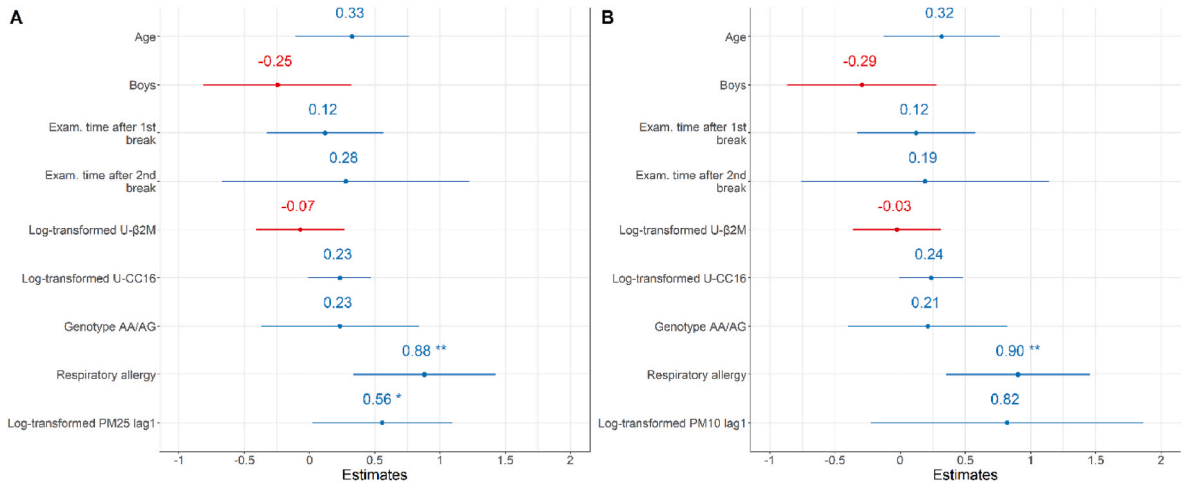


**Fig. 1. Multivariable analysis of the association between U-CC16 and FeNO using particulate matter (PM) as air pollution exposure parameter in the model.** Panels A (using $PM_{2.5}$ measured at lag1) and B (using $PM_{10}$ measured at lag1) present the fixed effects estimates and the associated 95% confidence intervals of the retained covariates (based on biological plausibility and model selection) in the linear mixed effects model assessing the association with FeNO. Red: negative association; blue: positive association. *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

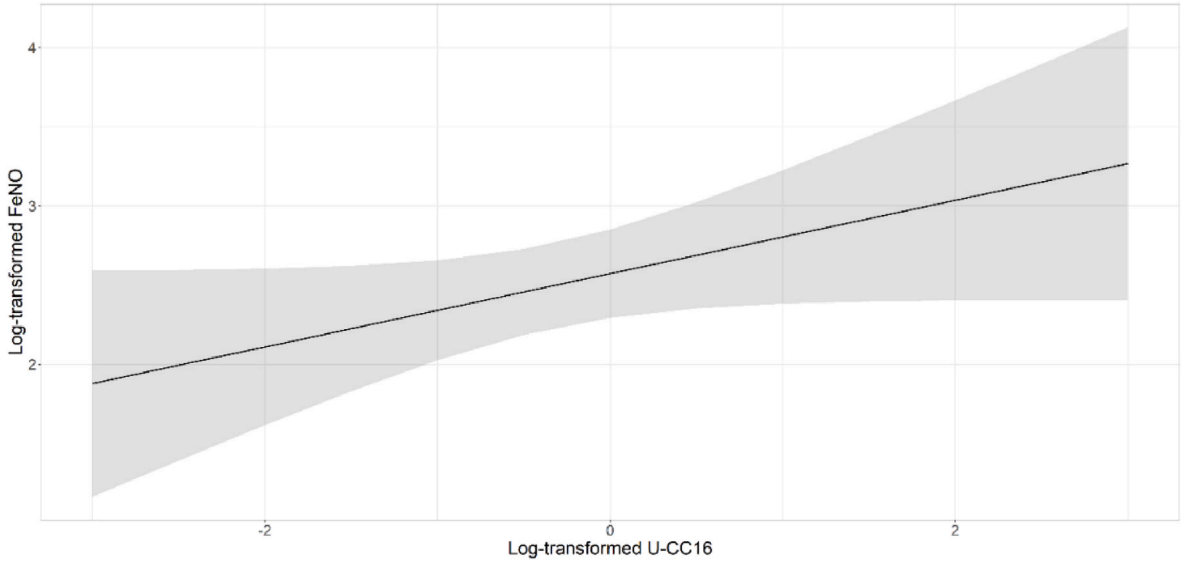


**Fig. 2. Adjusted predicted log-transformed values of FeNO for the log-transformed values of U-CC16**. The marginal effect is based on a mixed effects model with a random effect for each child and fixed effects for age, gender, CC16 genotype, time of examination, respiratory allergy, U-β2M and $PM_{2.5}$. Means are used to fix continuous variables and proportions are used to fix categorical variables.

As an additional analysis, we included the time point (occurring in the summer (t1) or winter (t2)) and location (rural or urban school) instead of the PM measurements in the multivariable linear mixed-effects model (Supplementary Fig. S1). Again, we observed a positive association between U-CC16 and FeNO (β = 0.24; 95% CI [0.01; 0.48]; p = 0.05).

Finally, a sensitivity analysis, excluding children with >10% deviation between the two best FeNO measurements, leaving a total of 51 observations from 37 children, also yielded a positive association between U-CC16 and FeNO in the model considering location and time point as an alternative for air pollution (Supplementary Fig. S2) but no significant association when using the model considering PM measurements (Supplementary Fig. S3).

### 3.3. FeNO is affected by $PM_{2.5}$ and location

Fig. 1 also illustrates that both $PM_{2.5}$ and $PM_{10}$ are positively associated with FeNO values, but only the association between $PM_{2.5}$ and FeNO was found to be statistically significant in the current model (β = 0.56; 95% CI [0.02; 1.09]; p = 0.04). In the additional analysis, where the time point (occurring in the summer (t1) or winter (t2)) and location (rural or urban school) were included instead of the PM measurements in the multivariable model (Supplementary Fig. S1), living in an urban location was statistically significantly associated with increased FeNO levels (β = 0.72; 95% CI [0.12; 0.31]; p = 0.02).

Assuming that the CC16 protein level can potentially play a role in the pathway (i.e., acts as an intermediate factor) between air pollution and FeNO, we assessed the association between PM measurements and FeNO in a separate model without adjustment for U-CC16 (Supplementary Fig. S4). Here, both $PM_{2.5}$ and $PM_{10}$ showed a statistically

significant positive association with FeNO (β = 0.63; 95% CI [0.17; 1.08]; p = 0.01, and β = 1.02; 95% CI [0.11; 1.93]; p = 0.03, respectively).

As a sensitivity analysis, we excluded the children with >10% deviation between the two best FeNO measurements leaving a total of 52 observations from 37 children. Again, a positive association was found between $PM_{2.5}/PM_{10}$ measurements and FeNO (Supplementary Fig. S5).

### 3.4. Non-statistically significant positive association between U-CC16 and $PM_{2.5}$

Lastly, the association between U-CC16 and air pollution was investigated. Although a positive trend was observed, the association between U-CC16 and the PM exposure was not statistically significant (shown for $PM_{2.5}/PM_{10}$ (lag1) in the Supplementary Fig. S6), neither with location and time point of the study (Supplementary Fig. S7). In all models (Supplementary Figs. S6 and S7), a significant association was observed between the *CC16* G38A genotype and U-CC16. However, no significant interaction effects were detected between air pollution and the *CC16* G38A genotype.

## 4. Discussion

The current study assessed if a noninvasive biomarker and test of respiratory health, i.e. U-CC16 and FeNO, are associated in the context $PM_{2.5}/PM_{10}$ exposure and how these parameters were mutually correlated, with the aim to investigate if U-CC16 might serve as an alternative for FeNO in certain cases. To our knowledge this is the first study conducted in children, in the context of short-term exposure to PM, where FeNO and U-CC16 were measured, where their mutual association was investigated, and where specific consideration was taken for the genetic background and renal handling adjustment when using U-CC16 as a biomarker. Although our study involved only a limited number of participants, as it was initially conceived as a feasibility study (Nauwelaerts et al., 2022a), relevant and statistically significant associations were obtained.

In this study, investigating children recently exposed to low $PM_{2.5}$ levels, a (borderline) significant positive association was found between U-CC16 and FeNO. Following our adjusted analysis, we expect a 2% increase in FeNO when U-CC16 increases with 10%. Very few studies have investigated the direct association between FeNO and CC16 (Li et al., 2020; Wang et al., 2018, 2022) and to our knowledge, none were conducted in children, exposed to short-term PM and using noninvasive samples as source of biomarkers. FeNO is a reliable and easy test of inflammation and oxidative stress in the bronchial epithelium (Kharitonov and Barnes, 2000) and has also been proposed as a test to identify asthmatics. Also in this study, a positive association was found between respiratory allergy and FeNO. FeNO has been used as a noninvasive marker of airway inflammation, which has been shown to be triggered in children and adults during pollution episodes (Flamant-Hulin et al., 2009; Kocot et al., 2020; Van Amsterdam et al., 1999; Wang et al., 2018; Zhang et al., 2016). CC16 has anti-inflammatory and anti-oxidative properties and has been described as peripheral marker of lung permeability and of integrity of the respiratory epithelium following exposure to toxicants (Broeckaert et al., 2003; Broeckaert and Bernard, 2000; Hermans and Bernard, 1996). Until now, no studies investigated this mutual association between FeNO and U-CC16 in short-term toxicant exposure conditions, but a positive association was expected. Indeed, a few studies, investigating both parameters, observed an increase of lung inflammation (FeNO) and an increase in lung injury and permeability, characterized by increased CC16 (serum and/or urine) following short-term exposure to wood smoke (Barregard et al., 2008; Stockfelt et al., 2012) and dust (Andersson et al., 2019) in adults. Although little is known about the underlying mechanisms, the results in this study suggest that the inflammatory response, as evidenced by increased FeNO, seems to be accompanied by increased injury and permeability of the lung epithelium barrier, as evidenced by increased U-CC16. However, more investigation is needed to confirm this.

Our study also confirmed the adverse effect of short-term exposure to ambient $PM_{2.5}$ on FeNO response, as elevated FeNO values were observed with increasing $PM_{2.5}$. Similar results were found in other studies where short-term exposure to traffic related pollutants (Flamant-Hulin et al., 2009; Godri Pollitt et al., 2016; Malmberg et al., 2005; Van Amsterdam et al., 1999; Zhang et al., 2016) led to increased FeNO, reflecting airway inflammation. Additionally, looking at seasonal or location differences gave some additional information on the exposure assessment in our study. When including the time point and location in the multivariable linear mixed-effects model, instead of PM measurements, children living in an urban location had significantly higher FeNO levels, compared to children in rural area. Assessing environmental exposure through the proxy of a certain location has been used in multiple epidemiological studies when pollutants or sources are not explicitly identified, as it is inexpensive and broadly applicable (Huang and Batterman, 2000; Wolfe et al., 2021). However, the choice of working with residence or school location as environmental exposure proxy should be done with caution. The relationship between the origin of the pollutants and the exposed subjects is complex and variable, making the assessment of exposure based only on one location (i.e. school in this study) uncertain.

Although not statistically significant, our study findings suggest a positive association of increasing U-CC16 with increasing PM as well as in the children going to the urban school and examined during the winter. Other studies reported that the acute exposure to toxic pollutants led to an increase of CC16 levels in serum (Barregard et al., 2008; Tufvesson et al., 2013) and to a delayed increase in urine in adults (Barregard et al., 2008; Timonen, 2004; Tufvesson et al., 2013). Recently, children participating to the COGNAC study showed a positive association between recent PM exposure (lag 1) and U-CC16 (Nauwelaerts et al., 2022b). Although a same trend was observed in this study, it was not found to be statistically significant. This can be due to several reasons. Firstly, different methods for air pollution measurement were used in the different studies, thus comparing its impact can be challenging. Indeed, in the COGNAC study (Nauwelaerts et al., 2022b), the used air pollution data were obtained from modeling air pollution exposure at the child's residence. This was not the case in this study, where the focus was more on the exposure at the schools with validated sensitive monitoring stations. Secondly, a limitation of this study was the small number of participants from two schools on two time points, narrowing the window of variation of air pollutant exposure, and therefore also the potential significant effect on the measured U-CC16. Indeed, as U-CC16 can show higher inter-individual variability than S- CC16, it can therefore result in lower power to detect the changes in lung permeability (Andersson et al., 2007; Barregard et al., 2008). This window of variation could be broadened by increasing the number of school locations, which eventually would be the goal of this type of field study set-up (Nauwelaerts et al., 2022a). This was also confirmed in our previous study (Nauwelaerts et al., 2022b), involving a larger number of participating children, and where more variation in exposure levels was observed, which led to the detection of significant changes in U-CC16. We therefore believe that, increasing the number of children and/or locations (and thereby increasing the exposure window) in future large scale studies might strengthen the positive trend that is already observed and could contribute to a significant positive association between U-CC16 and PM. Alternatively, combining modelled residential air pollution exposure data, with school exposure data in future studies might also increase the exposure accuracy and increase the window of exposure differences between the children individually. In the same line, although the association between the SNP G38A and U-CC16 found in previous studies (Nauwelaerts et al., 2020a, 2022b) was again confirmed here, no significant interaction effects of the genotype on the association between PM and U-CC16 was observed in this study. This is in contrast with our previous study (Nauwelaerts et al., 2022b),

conducted on a larger sample size, where the effect of PM exposure on U-CC16 was found to be dependent of the CC16 genotype. Increasing the sample size to assure a sufficient number of participants within each stratum of the CC16 genotype may provide insights on how the association between PM and U-CC16 differs according to the CC16 genotype. Similarly, increasing the sample size may allow to assess the association between PM and FeNO at different levels of allergy occurrence in children, given its importance as risk factor for elevated FeNO levels. Thirdly, the lack of significant increase of U-CC16, in contrast with the increase of FeNO, following short-term exposure, can also suggest that around these low levels of PM, the inflammatory response, evidenced by FeNO, is not yet accompanied by damages at the level of the lung epithelium barrier, characterized by U-CC16 (Bernard et al., 2005), which is in contrast with the effect observed following slightly higher PM exposure in the COGNAC study (Nauwelaerts et al., 2022b).

Our integrated study demonstrated as one of the firsts, a positive association between FeNO and U-CC16 in the context of short-term $PM_{2.5}$ exposure of children. However, more studies are needed to investigate the potential mediating role of U-CC16 in the association between FeNO and short-term PM exposure. This potential mediating role of CC16 was already suggested in chronic exposure situations, and where, due to the dual character of CC16, a negative correlation was found between S-CC16 and FeNO after chronic exposure to metals and diesel exhaust (Li et al., 2020; Wang et al., 2018). Similarly, CC16 might also have a mediating effect in the association between chronic air pollution exposure and subsequent lung function decline (as measured by e.g. spirometry). Indeed, recent studies have demonstrated how chronic air pollution exposure at birth or even prenatally is associated with low S-CC16 later in life (Beamer et al., 2019) and how low S-CC16 during childhood were found to be predictor of future lung function decline and respiratory health impairment (Guerra et al., 2015, Stapleton et al., 2022). In conclusion, more studies are needed to gain insight on how CC16 plays a role in the adverse effects of acute but also chronic air pollution exposure on increased FeNO (i.e. lung inflammation) and lung function. Importantly, these studies should integrate the SNP *CC16* G38A, as it is an important genetic determinant to take into account when measuring U-CC16. This is unfortunately not always the case for previously reported studies. Moreover, with a noninvasive approach as was done in our study, the collection of multiple urine samples, at multiple time points, is facilitated, especially in children. This allows the increase of the exposure window, contributing to obtaining more insight into the complex role of U-CC16 in both acute and chronic air pollution exposure.

Although additional studies are needed to further confirm this positive association between FeNO and U-CC16, and the mediating role of U-CC16 in the association between FeNO and $PM_{2.5}$, we would consider to use U-CC16 as an alternative for FeNO, when the latter cannot be measured, in multiple location field studies involving self-sampling. Using U-CC16 allows to give an insight on the early adverse pulmonary effects, following PM exposure. This small-scale study carried out in only two schools and at two locations, demonstrated the feasibility of using this integrated approach. The ease of repeating this noninvasive sampling allows to follow children individually, at multiple time points, before and after PM exposure, which could also be extended to other air pollutants or exposures. Moreover, the MRM technology allows to include other relevant proteins (needed for adjustment) but also other current and future protein biomarkers, once the method development is done (Nauwelaerts et al., 2021), limiting the inter-assay variation, compared to individual immunological assays. This type of analysis could be applied in future studies but also, retrospectively, in already existing biobanks. Even biobanks, only consisting of urinary samples suffice to measure the U-CC16 and the SNP G38A, although the success rate of genotyping might be a bit lower (Nauwelaerts et al., 2020b), due to the lower quality DNA obtained from urine.

## 5. Conclusion

A positive association was found between FeNO and U-CC16, in children following short-term low level exposure of $PM_{2.5}$. Using existing biobanks where no FeNO data are available or in future studies involving more subjects, time points and especially locations, where it is less or not feasible to measure FeNO, the collection of urine and saliva might be more easily obtained for the subsequent measurement of U-CC16 and the SNP G38A. This would allow in future large-scale studies the noninvasive integrative assessment of the potential impact of air pollution exposure on the respiratory health of children, which is critical for the development of policy or measures for these pollutants at population level.

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## Ethics approval and consent to participate

The study was approved by the Ethics committee of the Cliniques universitaires Saint-Luc (Registration number B403201734310). Both the parents and the children gave their informed consent to participate in the field study.

## Author contributions

**Sarah J. D. Nauwelaerts**: Methodology, Visualization, Investigation, Data curation, Validation, Formal analysis, Writing – original draft; **Nina Van Goethem**: Methodology, Formal analysis, Validation, Visualization, Writing – original draft; **Koen De Cremer:** Methodology, Resources, Writing – Review & Editing; **Natalia Bustos Sierra:** Methodology, Writing – Review & Editing; **Jordy Vercauteren:** Methodology, Writing – Review & Editing; **Christophe Stroobants:** Methodology, Writing – Review & Editing; **Alfred Bernard**: Methodology, Writing – Review & Editing; **Tim S. Nawrot** : Methodology, Resources, Writing – review & editing; **Nancy H. C. Roosens**: Conceptualization, Methodology, Writing – review & editing, project administration, funding acquisition; **Sigrid C. J. De Keersmaecker**: Conceptualization, Methodology, Validation, Supervision, Writing – original draft, writing – review & editing, project administration, funding acquisition.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: All authors reports financial support was provided by Belgian Federal Science Policy Office.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

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