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Urinary CC16, a potential indicator of lung integrity and inflammation, increases in children after short-term exposure to $PM_{2.5}$/PM_{10} and is driven by the CC16 38GG genotype

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ABSTRACT

Particular matter (PM) exposure is a big hazard for public health, especially for children. Serum CC16 is a well-known biomarker of respiratory health. Urinary CC16 (U-CC16) can be a noninvasive alternative, albeit requiring adequate adjustment for renal handling. Moreover, the SNP *CC16* G38A influences CC16 levels. This study aimed to monitor the effect of short-term PM exposure on CC16 levels, measured noninvasively in schoolchildren, using an integrative approach. We used a selection of urine and buccal DNA samples from 86 children stored in an existing biobank. Using a multiple reaction monitoring method, we measured U-CC16, as well as RBP4 (retinol binding protein 4) and β2M (beta-2-microglobulin), required for adjustment. Buccal DNA samples were used for *CC16* G38A genotyping. Linear mixed-effects models were used to find relevant associations between U-CC16 and previously obtained data from recent daily PM ≤ 2.5 or 10 μm exposure ($PM_{2.5}$, PM_{10}) modeled at the child's residence. Our study showed that exposure to low PM at the child's residence (median levels 18.9 $\mu g/m^3$ ($PM_{2.5}$) and 23.6 $\mu g/m^3$ (PM_{10})) one day before sampling had an effect on the covariates-adjusted U-CC16 levels. This effect was dependent on the *CC16* G38A genotype, due to its strong interaction with the association between PM levels and covariates-adjusted U-CC16 ($P = 0.024$ ($PM_{2.5}$); $P = 0.061$ (PM_{10})). Only children carrying the 38GG genotype showed an increase of covariates-adjusted U-CC16, measured 24h after exposure, with increasing $PM_{2.5}$ and PM_{10} ($\beta = 0.332$; 95% CI: 0.110 to 0.554 and $\beta = 0.372$; 95% CI: 0.101 to 0.643, respectively). To the best of our knowledge, this is the first study using an integrative approach to investigate short-term PM exposure of children, using urine to detect early signs of pulmonary damage, and taking into account important determinants such as the genetic background and adequate adjustment of the measured biomarker in urine.

Abbreviations: β2M, β-2-microglobulin; BMI, body mass index; CC16, club cell protein; COGNAC, COGNition and Air pollution in Children; COPD, chronic obstructive pulmonary disease; IQR, interquartile range; LME, linear mixed effect; LMWP, low-molecular-weight proteins; LRT, likelihood ratio test; ML, maximum likelihood; MRM, Multiple reaction monitoring; PM, particulate matter; $PM_{2.5}$, particular matter with an aerodynamic diameter smaller than 2.5 μm; PM_{10}, particular matter with an aerodynamic diameter smaller than 10 μm; RBP4, retinol binding protein 4; REML, restricted maximum likelihood estimates; SD, Standard Deviation; SNP, single nucleotide polymorphism; U-CC16, urinary CC16; U-β2M, urinary β2M; U-RBP4, urinary RBP4; WHO, World Health Organization; WT, wild-type.

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Ethics approval and consent to participate

The study was approved by the medical ethics committee of Hasselt University and the Eastern-Limburg Hospital (Belgium) ((11/065U). The parents of the children gave informed consent to participate in the COGNAC study.

1. Introduction

Ambient air pollution consists of a complex mixture with multiple substances, damaging for the public health, including the respiratory health. Amongst them is particular matter (PM), with an aerodynamic diameter smaller than 2.5 μm ($PM_{2.5}$) and with an aerodynamic diameter smaller than 10 μm (PM_{10}), which have been shown to cause severe effects, since they can transport a broad range of toxic substances to the respiratory tract (Mannucci et al., 2015). In the European Union, 97% of the urban population is exposed to levels of $PM_{2.5}$ above the most recent guideline levels set by the World Health Organization (WHO) (World Health Organization, 2021). These WHO recommendations are generally stricter than the comparable politically agreed EU standards (European Union, 2008). Some epidemiological studies have demonstrated adverse respiratory health effects of air pollution around or even below these current standards (Adam et al., 2015; Guo et al., 2019). These standards are set for the general population, with no specific values available for children, although they are known to be especially vulnerable for air pollution exposure (Schwartz, 2004). Studies have shown that short-term as well as long-term exposure impact their respiratory health (GBD, 2016 Risk Factors Collaborators, 2017; Guo et al., 2019; Jassal, 2015; Landrigan et al., 2018; Zhu et al., 2017) but only a few of them investigated the short-term effect with specific biomarkers reflecting the respiratory health at the level of the lung. Of interest is the serum club cell protein (hereafter referred to as CC16), a small pneumoprotein (16 kDa), which has a protective role in the respiratory tract due to its anti-inflammatory and immunomodulatory properties (Hermans and Bernard, 1999; Hung et al., 2004; Levin et al., 1986). It is mainly secreted in the epithelial lining fluid of the respiratory tract, where it diffuses across the airway epithelium into blood, followed by rapid clearance by glomerular filtration and tubular catabolism, finding its way into urine (Bernard et al., 1993; Hermans and Bernard, 1999). Several studies have suggested that CC16 may be a sensitive biomarker of lung inflammation, integrity and permeability as well as future lung diseases (Almuntashiri et al., 2020; Arsalane et al., 1999; Bernard et al., 2005; Broeckaert et al., 2000; St Helen et al., 2013; Wang et al., 2017a). Lower serum CC16 levels are associated with increased risks of a number of respiratory diseases and decreased lung function (Guerra et al., 2015; Rong et al., 2020; Shijubo et al., 1999; Zhai et al., 2019). The relationship between serum CC16 and environmental stressors is complex. Chronic exposure to cigarette smoke and other air pollutants, damages the CC16 producing cells and leads to lower serum CC16 levels (Beamer et al., 2019; Bernard et al., 1993; Heldal et al., 2013; Hermans and Bernard, 1999). This is in contrast with short-term environmental exposures where, perhaps due to epithelial damage in the lung, elevated serum CC16 levels were observed (Arsalane et al., 2000; Blomberg et al., 2003; Wang et al., 2017a). Importantly, circulating CC16 levels in serum are also influenced by a genetic factor, i.e. the *CC16* G38A polymorphism (rs3741240), where the mutant A-allele of this single nucleotide polymorphism (SNP) leads to lower CC16 expression and predisposes to respiratory diseases, such as asthma (Chen et al., 2012; Ku et al., 2011; Laing et al., 2000; Taniguchi et al., 2013).

Serum CC16 could therefore be used as a biomarker in biomonitoring studies for investigating the impact of short-term air pollution on the lungs. However, in the context of large-scale biomonitoring studies with children, collecting serum is challenging due to ethical and practical reasons. The use of noninvasive samples is needed to overcome this hurdle and to increase the participation (Mirowsky and Gordon, 2015). A few studies already used urine for CC16 protein investigation and confirmed the association between the levels of serum CC16 and urinary CC16 (U-CC16) (Andersson et al., 2007; Stockfelt et al., 2012; Tufvesson et al., 2013) although conflicting results were observed (Barregard et al., 2008; Egron et al., 2020; Eklund et al., 2021; Stenberg et al., 2018). Furthermore, saliva and buccal cells were shown to be excellent alternatives to blood for genotyping experiments (McMichael et al., 2009; Nauwelaerts et al., 2020b) in which the SNP G38A was successfully investigated (Nauwelaerts et al., 2020b). Several studies have already investigated U-CC16 as biomarker of lung epithelial integrity and permeability following short-term air pollution exposure (Arsalane et al., 1999; Jacquemin et al., 2009; Timonen, 2004) but none of them were conducted in children and none of them took into account the *CC16* genotype. One of the challenges when measuring U-CC16 is the need to adjust its urinary levels for renal handling, including dilution and tubular reabsorption, before it can be used as a valid biomarker. Recently, retinol binding protein 4 (RBP4) and beta-2-microglobulin (β2M) were successfully used as adjuster (Egron et al., 2020; Nauwelaerts et al., 2020a) and could even be quantified simultaneously with U-CC16 by using a high throughput mass spectrometric method based on the multiple reaction monitoring (MRM) technology (Nauwelaerts et al., 2021).

This study aimed to investigate the effect of short-term PM exposure on children in an integrative approach, combining the investigation of biomarkers on different levels, as they might be correlated, including proper adjustment for the source from where they are measured and for the genotype. We used available noninvasive samples of an existing biobank, i.e. urine, created in the context of a previously conducted study (i.e. the COGNition and Air pollution in Children (COGNAC) study (Saenen et al., 2017, 2019; Vriens et al., 2016)), to measure the adequately adjusted U-CC16 by applying a high throughput MRM technology (Nauwelaerts et al., 2021). The *CC16* SNP G38A was examined in the available buccal DNA samples from the same collection. The association with the available PM exposure data of children, collected during the COGNAC study, was investigated.

2. Materials and methods

2.1. Study population

To perform this study, biobanked samples of 86 children originally participating to a previous study, i.e. the COGNAC study, which was conducted between 2011 and 2014 and with another research question, were included. At that time, these children aged 9–11 years old, were going to three primary schools located in urban areas with substantial amounts of traffic in Flanders (Saenen et al., 2016, 2017, 2019, 2016; Vriens et al., 2016, 2018). The COGNAC study was performed with maximally three examinations per child (described in Table 1). The mean period of time (±SD) between two consecutive examinations was 41 (±23) days and all visits were scheduled at the same day of the week and at the same time point to rule out diurnal variation. The study was approved by the medical ethical committee of Hasselt University and the Eastern-Limburg Hospital (Belgium) in accordance with the Helsinki Declaration (World Medical Association, 2001). Parental informed consent was obtained prior to participation in the study. Parents were asked to complete a questionnaire to provide additional information, such as lifestyle, passive exposure to tobacco, residence, general health and physical activity. The COGNAC study involved the collection and

Table 1
Characteristics and *CC16* G38A genotype of the participants (n = 86), urinary proteins and residential $PM_{2.5}$ and PM_{10} exposure.

General characteristics[a]	
Boys[c]	42 (49%)
Age, years[c]	10.5 [9.8–11.2]
Body mass index (BMI), kg/m^2 [c]	17.2 [15.7–18.5]
Weight, kg[c]	36.1 [33.2–42.0]
Length, m[c]	1.46 [1.41–1.53]
Mother smoked during pregnancy[k d]	6 (7%)
Passive smoking[k e]	10 (12%)
Smoking in family[k]	19 (22%)
Physical activity, hours/week[k e]	3.5 [2.0–4.5]
Medication use [ek]	7 (8%)
Allergy [k d]	16 (19%)
Recent exposure (at residence) [a i, h]	
$PM_{2.5}$ (μg/m^3)	
Lag 0	19.7 [14.6–81.8]
Lag 1	18.9 [10.8–27.2]
Lag 2	15.8 [9.7–22.6]
PM_{10} (μg/m^3)	
Lag 0	26.1 [18.5–37.3]
Lag 1	23.6 [14.1–31.9]
Lag 2	18.2 [13.9–27.3]
Levels of urinary analytes [i b f]	
Relative abundance U-CC16 [j,e]	15.1 [7.4–31.8]
Relative abundance U-RBP4	2.3 [1.6–3.6]
Relative abundance U-β2M [e]	90.1 [60.1–126.5]
SNP *CC16* G38A genotype [g]	
Homozygous WT 38GG	37 (43%)
Heterozygous 38AG	38 (44%)
Homozygous mutant 38AA	11 (13%)

Values are represented as numbers for the categorical variables and as median [IQR] for the continuous variables.
IQR: interquartile range; RBP4: retinol binding protein 4; WT: wild-type; PM: particulate matter with; U-β2M: urinary beta-2-microglobulin; U-CC16: urinary club cell protein; U-RBP4: urinary retinol binding protein 4; aerodynamic diameter smaller than 2.5 μm ($PM_{2.5}$) or smaller than 10 μm (PM_{10}).
[a] Data obtained during COGNAC study and extracted for selection of children (n = 86) used in this study.
[b] Data obtained from analyses conducted in this study.
[c] Parameters obtained/calculated during first examination.
[d] Data missing for 2 children.
[e] Data missing for 1 child.
[f] From a total of 233 measured urine samples collected from a total of 86 children, 189 samples were collected from 63 children examined 3 times, 42 samples from 21 children examined 2 times and 2 urine samples from 2 children examined once.
[g] DNA extracted from buccal samples from 86 children collected during 1 examination.
[h] Average outdoor air pollution at the child's residence over the different periods before sampling and examination. Obtained by spatial temporal interpolation modeling. The different periods are defined as 2 days before (lag 2), one day before (lag 1) or on the day of (lag 0) the examination.
[i] Parameter obtained by calculating the median of the averages of this parameter of each child over the different time points.
[j] 233 samples were analyzed, 11 samples were not determined or below the limit of quantification.
[k] Information obtained from questionnaire.

investigation of samples such as urine and, buccal samples and air pollution measurements, for which details can be found elsewhere (Saenen et al., 2016, 2017, 2019, 2016; Vriens et al., 2016, 2018). In this present study, the available noninvasive samples of the 86 selected children were used for genetic and protein analyses. The selection of the 86 children was based on the availability in the original COGNAC biobank of buccal DNA samples and of other measured data, needed for future analyses (beyond the scope of this study). This resulted in 86 buccal DNA samples and a total of 233 available urine samples, collected from the corresponding 86 children during their examination, maximally at three time points (described in Table 1). Additionally retained from the COGNAC study, were the data from the conducted examinations (such as weight and length), from the questionnaire (such as passive smoking and inquiring for allergies and physical activity) and from air pollution measurements (more details in the next paragraph). As only a proportion of the initial COGNAC samples was included in this study, it was first verified that this selection could be representative for the whole collection. To detect this potential selection bias, comparison of the baseline characteristics between the study population of the COGNAC study (n = 334) and the study population as selected for the current study (n = 86) was done. No selection bias could be observed (see supplementary table S1).

2.2. Air pollution data

The air pollution data were retrieved from the measurements carried out during the earlier COGNAC study (Saenen et al., 2016; Vriens et al., 2016). In the context of the current study, we used modeled data of daily residential exposure levels, based on a spatial temporal interpolation method, taking into account land cover data obtained by satellite images (CORINE land-cover data Set) (Janssen et al., 2008). Estimates of $PM_{2.5}$ and PM_{10} on the day of (lag 0), the day before (lag 1) or two days before (lag 2) the examinations and the collection of samples were used in the analyses. Additionally, PM_{10} and $PM_{2.5}$ exposure levels at the school (inside the class room and on the playground) were available from measurements conducted with portable devices during the COGNAC study (Saenen et al., 2016). However, these measurements were only made on the day of the examinations (lag 0).

2.3. Urinary protein measurements

During each of the examinations (occurring maximally three times) of the COGNAC study, urine samples were collected from the children. Spot urine samples were collected using designated metal and black carbon-free sample jars which were kept at 4 °C for 2–8 h until aliquoting and storage at −80 °C (Saenen et al., 2017). In this study, 233 urine samples from the selected 86 children were analyzed as follows. Tryptic digestion of the urine samples was performed as described in Nauwelaerts et al. (2021) (Nauwelaerts et al., 2021). A validated MRM method was used to measure the relative abundance of U-CC16, which was obtained by comparing the mass spectrometry signal of spiked exogenous isotope-labeled peptides with the signal of the endogenous CC16 peptides, present in the samples (Nauwelaerts et al., 2021). The U-CC16 levels were adequately adjusted with RBP4 and β2M, both quantified using the same MRM method, on the basis of the regression coefficient between the two proteins, obtained by multiple regression analysis. This adjustment corrected for the physiological variations of dilutions as well as the protein tubular reabsorption capacity.

2.4. Buccal DNA analysis

Buccal DNA was obtained during the COGNAC study by collecting the buccal cells using buccal swabs after which the DNA was extracted and stored at −80 °C until further use (Saenen et al., 2019). In this study, aliquots containing DNA originating from buccal cells of the 86 children were analyzed in a genotyping assay for the SNP *CC16* G38A polymorphism (rs3741240) as previously described (Nauwelaerts et al., 2020b).

2.5. Statistical analysis

All continuous variables were described as median with interquartile

range (IQR). A log transformation was used for the normalization of the distribution of PM exposures levels and the protein levels.

As part of the explorative analysis, taking into account the repeated measures of the participants, the repeated measures coefficient was calculated (Bakdash and Marusich, 2017) between U-CC16 and the modeled $PM_{2.5}$ and PM_{10}, respectively. A linear mixed effect (LME) model was used to estimate the effect of $PM_{2.5}$ and PM_{10} on the CC16 levels and how the genotype influences this effect (Wang et al., 2017a). This model takes into account the correlations among the repeated measurements of the participants and includes a random effect for each subject (Baayen et al., 2008). This repeated-measurements design allows each participant to serve as his/her control over time and thereby eliminating within-subject confounding (Verbeke and Molenberghs, 2001).

We calculated the effect estimates for recent modeled $PM_{2.5}$ and PM_{10} exposure. The determinants of U-CC16 were identified through the model selection process recommended by Zuur et al. (2009). This top-down strategy starts by fitting a full model including the following variables (also shown in Table 1) that were defined *a priori*: PM exposure, gender, age, body mass index (BMI), maternal pregnancy smoking, passive smoking, hours of physical activity spent per week, medication use, respiratory allergy, traffic, the time of examination (subdivided in three categories: 9–10.30am (reference), 11am-12.30pm (category 1), 1–2.30pm (category 2)) and the *CC16* G38A genotype. It was decided to keep gender, age and time of examination in the model based on biological plausibility and to use the model selection approach to select for the other variables. The full model and the reduced model (in which one of the fixed effects are dropped) are fitted using maximum likelihood (ML) and subsequently compared using a likelihood ratio test (LRT). The final model is presented using restricted maximum likelihood estimates (REML).

The mixed models analyses were used to investigate the association between the U-CC16 levels and residential PM exposure (with the modeled $PM_{2.5}$ and PM_{10} estimates analyzed in separate models) on the one hand and between the U-CC16 levels and the *CC16* G38A genotype on the other hand, as well as the interaction between both, while controlling for potential covariates identified and retained by the stepwise regression and the biological plausibility. The confounding effect of renal handling and diuresis was compensated by including β2M or RBP4 in two separate models, as adjuster for U-CC16.

Explorative analysis was also done, using the available data of a portable monitor, installed at the schools on the day of the study. The overall correlation between the $PM_{2.5}$ and PM_{10} levels, measured at school (in the class rooms and on the playground) on the day of the study and U-CC16 was investigated.

All analyses were performed using the R software (R version 3.6.0). P-values are 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. Characteristics of study population, biomarkers and air pollution exposure

Baseline characteristics, obtained from the examinations and from the questionnaire, the *CC16* G38A genotype determined from the buccal DNA, measured relative urinary protein abundances (CC16, RBP4 and β2M) and residential exposure to $PM_{2.5}/PM_{10}$ of the current study population are summarized in Table 1. The median age of the children was 10.5 years and approximately the same numbers of boys and girls were included in the study. 12% and 7% of the children were exposed to passive smoking and maternal pregnancy smoking, respectively. The children performed physical activity around 3.5 h per week. Sixteen children (19%) suffered from any type of allergy. Results of the *CC16* G38A genotyping assay indicated that 43% of the children were homozygous WT 38GG, 44% heterozygous 38AG and 13% homozygous mutant 38AA. These observed allele frequencies follow the same tendency as the frequencies in the general population, described by the 1000 Genome Project (The 1000 Genomes Project Consortium, 2010) (45% 38GG, 44% 38AG, 11% 38AA for the *CC16* G38A SNP). This confirmed again that the selected sample population was representative for the whole population and that there is no selection bias.

The modeled estimates of recent residential ambient exposure, characterized by the median concentrations of $PM_{2.5}$ and PM_{10} on the day of (lag 0), the day before (lag 1) or two days (lag 2) before the examinations and the urine sampling, were 19.7 μg/m^3, 18.9 μg/m^3, 15.85 μg/m^3, respectively, for $PM_{2.5}$ and 26.1 μg/m^3, 23.6 μg/m^3,18.2 μg/m^3, respectively, for PM_{10}.

3.2. Effect of recent PM exposure on U-CC16

3.2.1. Selection of the retained potential confounders using mixed model analysis

To investigate the effect of PM exposure on U-CC16, a LME model analysis was used, including all relevant potential confounding factors and parameters as fixed effects and a random effect for each child (i.e. child-specific intercepts, as each child will have its own unique effect added to the overall intercept). This model included β2M (or RBP4 presented in Supplementary data) as adjuster to compensate for the confounding effects of renal handling and diuresis when measuring U-CC16. Additionally, based on the biological plausibility, age, gender and time of examination were retained as variables in the mixed model. The model selection approach was used to select for other potential confounders (listed in Materials & Methods) but did not retain any (results not shown). To investigate the separate effect of the modeled PM estimates on U-CC16, the pollutants $PM_{2.5}$ and PM_{10}, both mutually correlated, were included in two separate models, summarized in Fig. 1A ($PM_{2.5}$) and 1C (PM_{10}). The complete results, showing the associations between air pollution on each day (lag 0, lag 1, lag 2) and U-CC16, with and without adjustment with the retained variables can be found in the supplementary Table S2.

3.2.2. Association between the adjuster (U-β2M) and U-CC16 levels

In the two separate models, the U-CC16 levels were strongly correlated with the levels of U-β2M as well when investigating $PM_{2.5}$ (Fig. 1A, $\beta = 0.688$, 95% CI 0.575 to 0.802; $P < 0.001$) as PM_{10} (Fig. 1C, $\beta = 0.680$, 95% CI 0.565 to 0.794; $P < 0.001$). Similar correlations were found between U-CC16 and U-RBP4 (see supplementary data Figure S3). These results confirm the adequate use of U-β2M as well as U-RBP4 as adjusters of renal handling and diuresis when measuring U-CC16.

3.2.3. Association between modeled $PM_{2.5}/PM_{10}$ (lag 1) and U-CC16

From the calculation of the repeated measures coefficient, a significant linear association was observed between U-CC16 and the child's residential $PM_{2.5}$ and PM_{10} estimates, respectively (see Supplementary Figure S4). From the multivariable analysis investigating the exposure to $PM_{2.5}$ (Fig. 1 A) and PM_{10} (Fig. 1 C), respectively, and its effect on U-CC16 levels (adjusted with U-β2M), the overall average association between U-CC16 levels and the $PM_{2.5}$ exposure levels of the day before (lag 1) was borderline significant ($P = 0.062$). A similar association with PM_{10} (lag 1) exposure ($P = 0.051$) was observed. No association was found between the U-CC16 and lag 0 or lag 2 of the $PM_{2.5}$ and PM_{10} exposure estimates (results not shown). Similar results were found when using U-RBP4 as adjuster for U-CC16 (see supplementary data Figure S3).

Data of a portable monitor, installed in the schools on the day of the study, were also used to potentially gain additional insight on the effect of PM exposure at the school. However, explorative analysis didn't reveal any association between the $PM_{2.5}/PM_{10}$ levels measured and U-CC16 (results not shown).

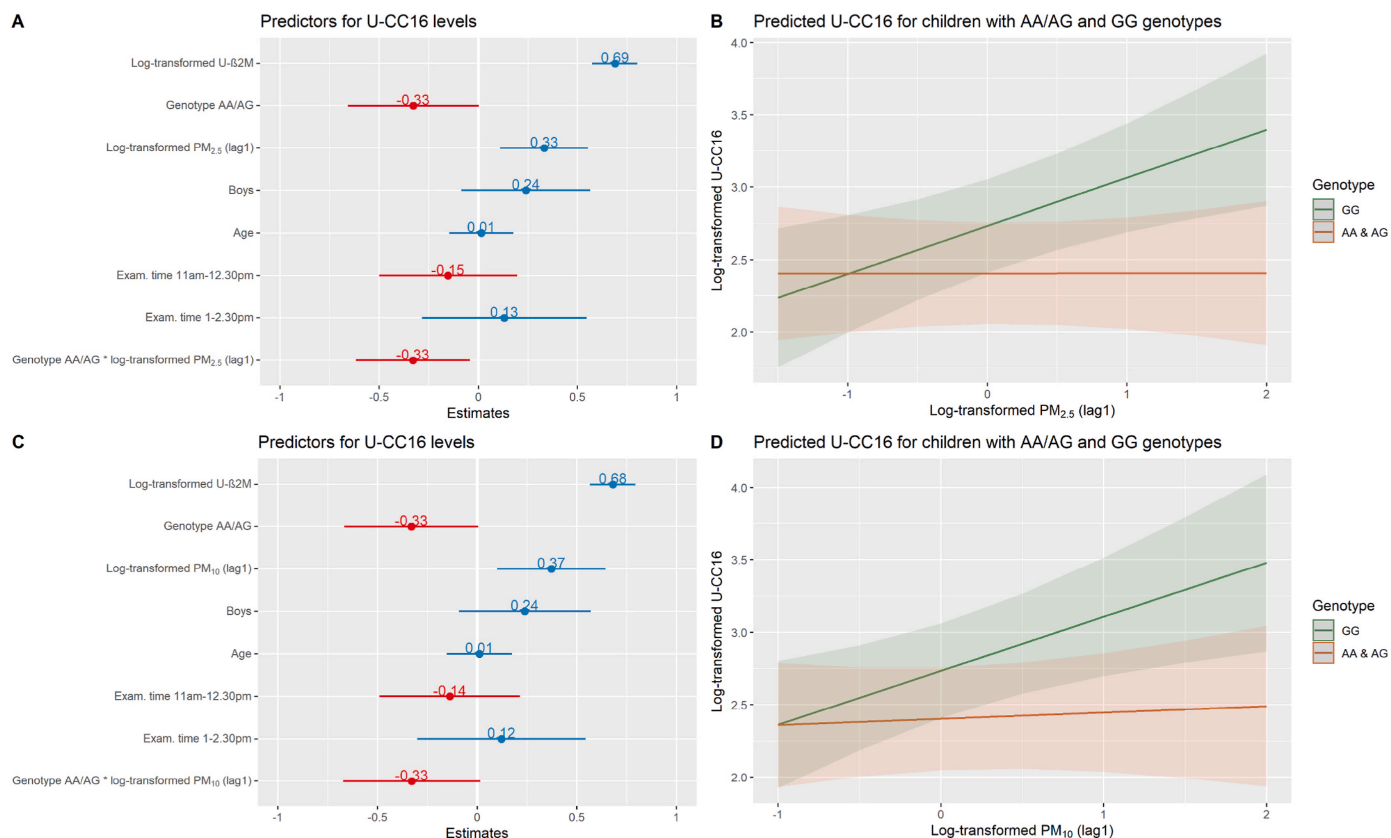

Fig. 1. Multivariable analysis of the association between urinary CC16 (U-CC16) (adjusted with urinary β2M (U-β2M)) and modeled $PM_{2.5}$ (lag 1) or PM_{10} (lag 1) exposure. Panels A ($PM_{2.5}$ (lag 1)) and C (PM_{10} (lag 1)) show the fixed effects estimates and associated 95% confidence intervals of the retained predictors (based on biological plausibility and model selection) in the mixed model with a random intercept for each child assessing the association with U-CC16 levelsin the situation when the genotype is equal to 38 GG. There is a significant association between the air-pollution measurements ($PM_{2.5}$ (lag 1) (panel A) and PM_{10} (lag 1) (panel C) and U-CC16 for children with the 38GG genotype (i.e. when taking into account the interaction effect of the genotype (GG vs. AA/AG) on the association between U-CC16 and $PM_{2.5}$ (lag 1) (panel A) or PM_{10} (lag 1) (panel C)). The predicted values of U-CC16 in function of the $PM_{2.5}$ (lag 1) (panel B) or PM_{10} (lag 1) (panel D) are shown stratified per genotype (GG vs. AA/AG), and adjusted for other variables as included in the models shown in panels A and C, respectively. .

3.2.4. Interaction effect of CC16 G38A genotype on the association between $PM_{2.5}$/PM_{10} (lag 1) and U-CC16

In both models (Fig. 1A and C), an independent association was observed between U-CC16 and the *CC16* G38A genotype (P = 0.044 and P = 0.047, respectively). In addition, there is an interaction effect of the *CC16* G38A genotype on the association between the air pollution (both for $PM_{2.5}$ and PM_{10}) and U-CC16 (95% CI: 0.617 to −0.044; P = 0.024 and 95% CI: 0.673 to 0.016; P = 0.061, respectively). Therefore, the association between $PM_{2.5}$/PM_{10} and U-CC16 should be interpreted with caution and the *CC16* G38A genotype should be taken into account. This means that the overall effect of PM exposure on the CC16 protein is clearly dependent on the *CC16* genotype. Children carrying the 38AA/AG genotype (i.e. heterozygous or homozygous mutant) showed no significant association between U-CC16 and $PM_{2.5}$ and PM_{10} (β = 0.001; 95% CI: 0.186 to 0.188; P = 0.991 and β = 0.043; 95% CI: 0.181 to 0.267; P = 0.706, respectively). However, as presented in Fig. 1A and C, when the children carried the 38GG genotype (i.e. homozygous WT), a strong significant positive association was observed between U-CC16 and $PM_{2.5}$ and PM_{10} (β = 0.332, P = 0.004; 95% CI: 0.110 to 0.554 and β = 0.372, P = 0.008; 95% CI: 0.101 to 0.643, respectively).

The strong interaction effect of the *CC16* G38A genotype on the association between the $PM_{2.5}$/PM_{10} (lag 1) exposure levels and U-CC16 is also illustrated in Fig. 1B and D, which present the predicted values of the U-CC16 in function of the air pollution stratified per genotype and corrected for the other variables as included in the model. When assuming an increase of 50% of $PM_{2.5}$ (lag 1) exposure, there is no increase of U-CC16 in children with the 38AG or 38AA genotype. This is in contrast with the estimated increase of 14.4% U-CC16 in children carrying the 38GG genotype. Similarly, when assuming an increase of 50% of PM_{10} (lag 1) exposure, there is a slight increase of 1.8% of U-CC16 in children with the 38AG or 38AA genotype, compared to an estimated increase of 16.3% U-CC16 in children with the 38GG genotype.

Similar results were found when adjusting U-CC16 with U-RBP4 (supplementary data Figure S3).

4. Discussion

In this study the positive association between $PM_{2.5}$/PM_{10} (lag 1) levels and U-CC16 levels, mainly driven by children carrying the *CC16* 38GG (homozygous WT) genotype, was demonstrated. Most of the investigated biomarkers of air pollution exposure in children are often related to general inflammation (Li et al., 2010, 2017) or oxidative stress (Lin et al., 2015; Van Miert et al., 2012). To the best of our knowledge, this was the first time that a study, focusing on the respiratory health of children, was conducted in an noninvasive and integrative way, and that significant associations between recent air pollution and potential impact on the lung inflammation and integrity of children were observed. This was achieved by selecting the appropriate biomarker, measurable in noninvasive samples. CC16, one of the common biomarkers for evaluating the inflammation, permeability and integrity of the pulmonary epithelium (Arsalane et al., 1999; Broeckaert et al., 2000; St Helen et al., 2013; Wang et al., 2017a), was successfully measured in urine samples, using proper adjustment with RBP4 and β2M. Importantly, by including the genetic background of the children, more

particularly, the effect modifier *CC16* SNP G38A, an integrated approach was used to allow proper analysis.

CC16 is usually measured in serum and several studies have observed increased levels of circulating CC16 in adults following short-term exposure to air toxicants such as ozone (Blomberg et al., 2003; Broeckaert et al., 1999), wood smoke (Stockfelt et al., 2012) and PM (Wang et al., 2017a; Provost et al., 2014). As our study involved young children, obtaining serum was challenging and we therefore used urine as noninvasive source of the CC16 biomarker. Until now, only few studies investigated urinary CC16 in children, generally in relation to respiratory disease susceptibility (Egron et al., 2020; Ma et al., 2015a; Rosas-Salazar et al., 2015), exhaled nitric oxide (Nauwelaerts et al., 2020a) and different toxicant exposures, such as tobacco smoke, cadmium or arsenic (Beamer et al., 2016; Ma et al., 2015b; Wang et al., 2017b), long term outdoor pollution (Zhang et al., 2021), but none with recent outdoor air pollution exposure. Within the context of using U-CC16 as a biomarker in children for recent PM exposure, our manuscript is the first one reporting on this.

However, cautious interpretation is needed when using urinary biomarkers, due to the occurrence of confounding effects of renal handling for small proteins such as CC16 (Bernard et al., 1994). A similar tendency of association between recent air pollution exposure and U-CC16, as described with serum CC16 in previous studies for adults (Blomberg et al., 2003; Broeckaert et al., 2000; Stockfelt et al., 2012; Wang et al., 2017a), was found for children. The significant positive association in our study between U-CC16 and $PM_{2.5}$ and PM_{10} was found by taking into account these confounding effects of tubular reabsorption and diuresis. In most of the published studies investigating U-CC16, adjustment is performed with creatinine, which is mainly correcting for diuresis only. Instead, we used RBP4 and β2M, which are of similar size as CC16, and which follow a similar path as U-CC16 in the kidneys. This alternative adjustment was recently applied with RBP4 (Egron et al., 2020; Nauwelaerts et al., 2020a), measured using immunoassays, in studies investigating U-CC16 in children. On the other hand, β2M is more challenging to measure using classical immunoassays, as this protein degrades in acidic pH. In our study, its measurement was made possible, by using a recently developed cost-effective and high throughput MRM method (Nauwelaerts et al., 2021), which allowed the simultaneous measurement of U-CC16, U-β2M and RBP4, giving an accurate detection of even degraded and fragmented β2M in urinary acidic environment. The simultaneous measurement of CC16 and its adjusters also limits inter-assay variations, potentially occurring in the alternative simplex immunoassay of each individual protein, therefore reflecting more accurately the true abundances of each protein within the samples.

Interestingly, the increase of U-CC16 was only associated with the increase of the modeled $PM_{2.5}/PM_{10}$ exposure, measured 24 h before urine collection. This delayed increase of U-CC16 might be the result of increased inflammation in the airways. Several studies have indeed shown that PM can cause inflammation in airways (Bonvallot et al., 2001; Hirota et al., 2015; Kumar et al., 2015; Li et al., 2019; Shen et al., 2018). The CC16 protein protects the respiratory tract against oxidative stress and is a potent anti-inflammatory agent (Janicova et al., 2019; Pang et al., 2017). The inflammatory reaction occurring in the lungs, leads to an increased production of CC16, resulting in increased levels of serum CC16 (Arsalane et al., 2000; Barregard et al., 2008; Doyen et al., 2016; Seys et al., 2015). The time needed for this increase to occur and to be observed, can vary depending on the source and the levels of the toxicant causing the inflammation. Irritants such as ozone (Alexis et al., 2008; Arsalane et al., 1999; Blomberg et al., 2003; Broeckaert et al., 2000), smoke (Rosenberg and Kalhan, 2012; Stockfelt et al., 2012) and lipopolysaccharides (Doyen et al., 2016; Michel et al., 2005) lead to serum CC16 levels rapidly increasing within a few hours after exposure. Similarly, serum CC16 increased in the first 2 h and lasted up to 24 h after $PM_{2.5}$ exposure (Wang et al., 2017a). These results suggest that exposure to PM levels might have an acute effect on the epithelial integrity and that it increases the epithelial barrier permeability in the lungs of children. As the correlation between serum and urine CC16 is high in subjects with no renal failure, similar tendencies of increased levels after acute exposure can be expected for U-CC16. Arsalane et al. reported a transient increase of serum CC16 in rats, following acute ozone exposure, paralleled by an increase in U-CC16, measured the next day (Arsalane et al., 1999). Stockfelt et al. observed an increase of serum CC16 in adults following wood smoke exposure, correlating with an increase of U-CC16 measured the next morning (Stockfelt et al., 2012). Similar findings to the latter study were found in our study where a positive association between U-CC16 levels 24h after $PM_{2.5}/PM_{10}$ exposure (lag 1) was observed. Of note, explorative analysis did not show any association between PM exposure at the school (measured with portable monitors) and U-CC16. This might have been because portable monitor data were only available on the day of sampling for the biomarker measurements (lag 0), not the day before (lag 1), which would have been interesting as in our study we found associations between U-CC16 and the modeled pollutants on lag 1. Unfortunately, the set-up of additional air pollution measurements before the start of the study with the portable monitor was not planned at that time of the COGNAC study, as it was outside the scope of the initial investigations (Saenen et al., 2016). Therefore, this data was not available for our retrospective study. To improve the understanding of the kinetics of CC16, it would be interesting to increase the period of personal monitoring and to measure U-CC16 more frequently and shortly after the $PM_{2.5}$ exposure in future studies.

The major parameter that had to be taken into account in this study was the genetic background of the children, using DNA derived from noninvasive buccal samples. The *CC16* G38A polymorphism is the main genetic determinant of serum CC16 and previous studies have reported that children with the 38AA mutant genotype show decreased plasma levels compared to the 38GG genotype (Chen et al., 2012; Ku et al., 2011) as well as associations with asthma (Laing et al., 2000; Taniguchi et al., 2013; Zhao et al., 2013). Our study showed that this effect modifier resulted in a similar tendency of lower U-CC16 levels in children carrying the 38AA genotype. The lower U-CC16 levels, excreted from lower serum levels, were probably due to the presence of the lower intrapulmonary pool of this protein, provoked by this mutation. This type of association between the *CC16* G38A genotype and the U-CC16 has only recently been observed in a previous study, involving the investigation of fractional exhaled nitric oxide in children (Nauwelaerts et al., 2020a) but has never been reported when investigating air pollution exposure. Moreover, our current study showed that an important interaction was observed between the genotype and the air pollution exposure. Children carrying the 38GG genotype strengthened the significant association between the $PM_{2.5}/PM_{10}$ (lag 1) levels and the increased U-CC16 levels. This was in contrast with the children carrying the AA/AG genotype, where almost no increase in U-CC16 was observed with increasing $PM_{2.5}$ and PM_{10} exposure (lag 1). This finding shows the importance of investigating the genetic background of the subjects when measuring protein biomarkers. Without this knowledge on the genotype, our study only showed a borderline significant association between the U-CC16 and the $PM_{2.5}$ and PM_{10} (lag 1) levels. By including the genotype in the analysis, the outcome was finetuned and a distinction was made between the 38GG (wild-type) carrying population that is more reactive to PM exposure, by producing more CC16, and the AA/AG carrying group that is not. Due to their lower production of anti-inflammatory CC16 levels, the children carrying the CC16 38A-allele may therefore be less protected against irritants such as PM. Therefore, this genetic background is crucial information when investigating U-CC16 in children and should be taken into account in future studies.

Until now, most of the studies, that have investigated U-CC16, did not take the aspects of the SNP G38A and the adjustment for renal handling, as mentioned above, into account. Some of these studies described contradictory or lacking associations of U-CC16 as well in adults with wood smoke (Barregard et al., 2008), recent $PM_{2.5}$ exposure

(Bräuner et al., 2009), physical exercise (Eklund et al., 2021) and asthma (Stenberg et al., 2017) as in children with childhood asthma (Ma et al., 2015a). These studies might have suffered from the missing genotype information that was not investigated or the lack of adjusting potentially more sensitively with RBP4 and β2M for the renal handling of U-CC16.

This study presents a few limitations. Firstly, our results are based on a limited sample size. Nevertheless, strong significant associations are observed, which can unlikely be explained by chance only. Secondly, the quantification of the protein abundances, using the MRM method, was relative and not absolute. However, this relative quantification sufficed to observe significant protein level changes due to changes in pollutant exposure. Thirdly, potential socioeconomic confounders were not taken into account, but should be in future large scale studies as some of them they might influence CC16 levels (Zhai et al., 2018). Finally, it was not possible to measure CC16 levels in serum of the participating children in this study. However, this study, as well as a previous study (Nauwelaerts et al., 2020a), confirmed a similar association between serum CC16 and the SNP G38A observed in several studies (Chen et al., 2012; Ku et al., 2011; Laing et al., 2000) by using U-CC16, adequately adjusted. Therefore, we believe that U-CC16 might be a good surrogate noninvasive alternative biomarker of serum CC16 (when not available) for monitoring the adverse effect of air pollution on the lung permeability and integrity of children. However, U-CC16 should not be investigated without the knowledge of the CC16 G38A background, adequate adjustment and potential confounders.

5. Conclusions

Our study showed that the short-term exposure to low $PM_{2.5}$ and PM_{10} levels impacts the urinary CC16 levels in school children. Urinary CC16 is proposed as a proxy of serum CC16 and could be a useful biomarker, especially in young children in a large scale study setting, where blood collection is challenging. However, several important factors had to be taken into account for a proper interpretation. Firstly, our study showed that the association between U-CC16 and PM levels is highly dependent on the *CC16* G38A, as the association was mainly driven by the *CC16* 38GG genotype. Secondly, the adjustment with β2M or RBP4, instead of creatinine, leads to more accurate results of U-CC16 measurement. These different proteins – and eventually additional potential biomarkers could be measured simultaneously in numerous samples, avoiding inter-assay variation, using our validated a high-throughput MRM method. To the best of our knowledge, this is the first study demonstrating the short-term impact of PM on children, using urine samples to detect early signs of pulmonary damage, and taking into account important determinants such as the genetic background and adequate adjustment of the measured biomarker in urine. Our results illustrate the importance of conducting high throughput integrative studies, using noninvasive samples, where proteomics are combined with genetic information, which should be considered when performing large scale epidemiological studies in children. The observed levels of $PM_{2.5}$ and PM_{10} were just around of the EU and the most recent WHO guidelines, which are usually calculated for the entire population. However, in order to protect the more vulnerable strata, such as healthy children, and incited by this study, it could be proposed to define specific thresholds of pollutant levels per strata of the population.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2022.113272.

References

Adam, M., Schikowski, T., Carsin, A.E., Cai, Y., Jacquemin, B., Sanchez, M., Vierkötter, A., Marcon, A., Keidel, D., Sugiri, D., Al Kanani, Z., Nadif, R., Siroux, V., Hardy, R., Kuh, D., Rochat, T., Bridevaux, P.-O., Eeftens, M., Tsai, M.-Y., Villani, S., Phuleria, H.C., Birk, M., Cyrys, J., Cirach, M., de Nazelle, A., Nieuwenhuijsen, M.J., Forsberg, B., de Hoogh, K., Declerq, C., Bono, R., Piccioni, P., Quass, U., Heinrich, J., Jarvis, D., Pin, I., Beelen, R., Hoek, G., Brunekreef, B., Schindler, C., Sunyer, J., Krämer, U., Kauffmann, F., Hansell, A.L., Künzli, N., Probst-Hensch, N., 2015. Adult lung function and long-term air pollution exposure. ESCAPE: a multicentre cohort study and meta-analysis. Eur. Respir. J. 45, 38–50. https://doi.org/10.1183/09031936.00130014.

Alexis, N.E., Lay, J.C., Haczku, A., Gong, H., Linn, W., Hazucha, M.J., Harris, B., Tal-Singer, R., Peden, D.B., 2008. Fluticasone propionate protects against ozone-induced airway inflammation and modified immune cell activation markers in healthy volunteers. Environ. Health Perspect. 116, 799–805. https://doi.org/10.1289/ehp.10981.

Almuntashiri, S., Zhu, Y., Han, Y., Wang, X., Somanath, P.R., Zhang, D., 2020. Club cell secreted protein CC16: potential applications in prognosis and therapy for pulmonary diseases. JCM 9, 4039. https://doi.org/10.3390/jcm9124039.

Andersson, L., Lundberg, P.-A., Barregard, L., 2007. Methodological aspects on measurement of Clara cell protein in urine as a biomarker for airway toxicity, compared with serum levels. J. Appl. Toxicol. 27, 60–66.

Arsalane, K., Broeckaert, F., Knoops, B., Buchet, J.-P., Bernard, A., 1999. Increased serum and urinary concentrations of lung clara cell protein in rats acutely exposed to ozone. Toxicol. Appl. Pharmacol. 159, 169–174. https://doi.org/10.1006/taap.1999.8738.

Arsalane, K., Broeckaert, F., Knoops, B., Wiedig, M., Toubeau, G., Bernard, A., 2000. Clara cell specific protein (CC16) expression after acute lung inflammation induced by intratracheal lipopolysaccharide administration. Am. J. Respir. Crit. Care Med. 161, 1624–1630. https://doi.org/10.1164/ajrccm.161.5.9812157.

Baayen, R.H., Davidson, D.J., Bates, D.M., 2008. Mixed-effects modeling with crossed random effects for subjects and items. J. Mem. Lang. 59, 390–412. https://doi.org/10.1016/j.jml.2007.12.005.

Bakdash, J.Z., Marusich, L.R., 2017. Repeated measures correlation. Front. Psychol. 8, 456. https://doi.org/10.3389/fpsyg.2017.00456.

Barregard, L., Sallsten, G., Andersson, L., Almstrand, A.-C., Gustafson, P., Andersson, M., Olin, A.-C., 2008. Experimental exposure to wood smoke: effects on airway inflammation and oxidative stress. Occup. Environ. Med. 65, 319–324. https://doi.org/10.1136/oem.2006.032458.

Beamer, P., Klimecki, W., Loh, M., Van Horne, Y., Sugeng, A., Lothrop, N., Billheimer, D., Guerra, S., Lantz, R., Canales, R., Martinez, F., 2016. Association of children's urinary CC16 levels with arsenic concentrations in multiple environmental media. IJERPH 13, 521. https://doi.org/10.3390/ijerph13050521.

Beamer, P.I., Furlong, M., Lothrop, N., Guerra, S., Billheimer, D., Stern, D.A., Zhai, J., Halonen, M., Wright, A.L., Martinez, F.D., 2019. CC16 levels into adult life are associated with nitrogen dioxide exposure at birth. Am. J. Respir. Crit. Care Med. 200, 600–607. https://doi.org/10.1164/rccm.201808-1488OC.

Bernard, A., Thielemans, N., Lauwerys, R., Vandeleene, B., Lambert, A.E., 1993. The renal handling of protein 1 (clara cell protein): effect of age, sex and renal dysfunction. Contrib. Nephrol. 101, 66–70. https://doi.org/10.1159/000422110.

Bernard, A.M., Thielemans, N.O., Lauwerys, R.R., 1994. Urinary protein 1 or Clara cell protein: a new sensitive marker of proximal tubular dysfunction. Kidney Int. Suppl. 47, S34–S37.

Bernard, A., Carbonnelle, S., Nickmilder, M., de Burbure, C., 2005. Non-invasive biomarkers of pulmonary damage and inflammation: application to children exposed to ozone and trichloramine. Toxicol. Appl. Pharmacol. 206, 185–190. https://doi.org/10.1016/j.taap.2004.10.022.

Blomberg, A., Mudway, I., Svensson, M., Hagenbjork-Gustafsson, A., Thomasson, L., Helleday, R., Dumont, X., Forsberg, B., Nordberg, G., Bernard, A., 2003. Clara cell protein as a biomarker for ozone-induced lung injury in humans. Eur. Respir. J. 22, 883–888. https://doi.org/10.1183/09031936.03.00048203.

Bonvallot, V., Baeza-Squiban, A., Baulig, A., Brulant, S., Boland, S., Muzeau, F., Barouki, R., Marano, F., 2001. Organic compounds from diesel exhaust particles elicit a proinflammatory response in human airway epithelial cells and induce cytochrome p450 1A1 expression. Am. J. Respir. Cell Mol. Biol. 25, 515–521. https://doi.org/10.1165/ajrcmb.25.4.4515.

Bräuner, E.V., Mortensen, J., Møller, P., Bernard, A., Vinzents, P., Wåhlin, P., Glasius, M., Loft, S., 2009. Effects of ambient air particulate exposure on blood–gas barrier permeability and lung function. Inhal. Toxicol. 21, 38–47. https://doi.org/10.1080/08958370802304735.

Broeckaert, F., Arsalane, K., Hermans, C., Bergamaschi, E., Brustolin, A., Mutti, A., Bernard, A., 2000. Serum clara cell protein: a sensitive biomarker of increased lung

epithelium permeability caused by ambient ozone. Environ. Health Perspect. 108, 533–537. https://doi.org/10.1289/ehp.00108533.
Chen, L.-C., Tseng, H.-M., Wu, Chia-Jen, Kuo, M.-L., Wu, Cheng-Jang, Gao, P.-S., Yeh, K.-W., Yao, T.-C., Lee, W.-I., Ou, L.-S., Huang, J.-L., Huang, S.-K., 2012. Evaluation of a common variant of the gene encoding clara cell 10 kd protein (CC10) as a candidate determinant for asthma severity and steroid responsiveness among Chinese children. J. Asthma 49, 665–672. https://doi.org/10.3109/02770903.2012.697954.
Doyen, V., Pilcer, G., Dinh, P.H.D., Corazza, F., Bernard, A., Bergmann, P., Lefevre, N., Amighi, K., Michel, O., 2016. Inflammation induced by inhaled lipopolysaccharide depends on particle size in healthy volunteers: LPS droplet size predicts bronchial and systemic inflammation. Br. J. Clin. Pharmacol. 82, 1371–1381. https://doi.org/10.1111/bcp.13052.
Egron, C., Labbé, A., Rochette, E., Mulliez, A., Bernard, A., Amat, F., 2020. Urinary club cell protein 16 (CC16): utility of its assay during acute bronchiolitis. Pediatr. Pulmonol. 55, 490–495. https://doi.org/10.1002/ppul.24584.
Eklund, L., Schagatay, F., Tufvesson, E., Sjöström, R., Söderström, L., Hanstock, H.G., Sandström, T., Stenfors, N., 2021. An experimental exposure study revealing composite airway effects of physical exercise in a subzero environment. Int. J. Circumpolar Health 80, 1897213. https://doi.org/10.1080/22423982.2021.1897213.
GBD 2016 Risk Factors Collaborators, 2017. Global, regional, and national comparative risk assessment of 84 behavioural, environmental and occupational, and metabolic risks or clusters of risks, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet 390, 1345–1422. https://doi.org/10.1016/S0140-6736(17)32366-8.
Guerra, S., Halonen, M., Vasquez, M.M., Spangenberg, A., Stern, D.A., Morgan, W.J., Wright, A.L., Lavi, I., Tarès, L., Carsin, A.-E., Dobaño, C., Barreiro, E., Zock, J.-P., Martínez-Moratalla, J., Urrutia, I., Sunyer, J., Keidel, D., Imboden, M., Probst-Hensch, N., Hallberg, J., Melén, E., Wickman, M., Bousquet, J., Belgrave, D.C.M., Simpson, A., Custovic, A., Antó, J.M., Martinez, F.D., 2015. Relation between circulating CC16 concentrations, lung function, and development of chronic obstructive pulmonary disease across the lifespan: a prospective study. Lancet Respir. Med. 3, 613–620. https://doi.org/10.1016/S2213-2600(15)00196-4.
Guo, C., Hoek, G., Chang, L., Bo, Y., Lin, C., Huang, B., Chan, T., Tam, T., Lau, A.K.H., Lao, X.Q., 2019. Long-term exposure to ambient fine particulate matter (PM2.5) and lung function in children, adolescents, and young Adults: a longitudinal cohort study. Environ. Health Perspect. 127, 127008. https://doi.org/10.1289/EHP5220.
Heldal, K.K., Barregard, L., Larsson, P., Ellingsen, D.G., 2013. Pneumoproteins in sewage workers exposed to sewage dust. Int. Arch. Occup. Environ. Health 86, 65–70. https://doi.org/10.1007/s00420-012-0747-7.
Hermans, C., Bernard, A., 1999. Lung epithelium-specific proteins: characteristics and potential applications as markers. Am. J. Respir. Crit. Care Med. 159, 646–678. https://doi.org/10.1164/ajrccm.159.2.9806064.
Hirota, J.A., Gold, M.J., Hiebert, P.R., Parkinson, L.G., Wee, T., Smith, D., Hansbro, P.M., Carlsten, C., VanEeden, S., Sin, D.D., McNagny, K.M., Knight, D.A., 2015. The nucleotide-binding domain, leucine-rich repeat protein 3 inflammasome/IL-1 receptor I axis mediates innate, but not adaptive, immune responses after exposure to particulate matter under 10 μm. Am. J. Respir. Cell Mol. Biol. 52, 96–105. https://doi.org/10.1165/rcmb.2014-0158OC.
Hung, C.-H., Chen, L.-C., Zhang, Z., Chowdhury, B., Lee, W.-L., Plunkett, B., Chen, C.-H., Myers, A.C., Huang, S.-K., 2004. Regulation of TH2 responses by the pulmonary Clara cell secretory 10-kd protein. J. Allergy Clin. Immunol. 114, 664–670. https://doi.org/10.1016/j.jaci.2004.05.042.
Jacquemin, B., Lanki, T., Yli-Tuomi, T., Vallius, M., Hoek, G., Heinrich, J., Timonen, K., Pekkanen, J., 2009. Source category-specific PM2.5 and urinary levels of Clara cell protein CC16. The ULTRA study. Inhal. Toxicol. 21, 1068–1076. https://doi.org/10.3109/08958370902725292.
Janicova, A., Becker, N., Xu, B., Wutzler, S., Vollrath, J.T., Hildebrand, F., Ehnert, S., Marzi, I., Störmann, P., Relja, B., 2019. Endogenous uteroglobin as intrinsic anti-inflammatory signal modulates monocyte and macrophage subsets distribution upon sepsis induced lung injury. Front. Immunol. 10, 2276. https://doi.org/10.3389/fimmu.2019.02276.
Janssen, S., Dumont, G., Fierens, F., Mensink, C., 2008. Spatial interpolation of air pollution measurements using CORINE land cover data. Atmos. Environ. 42, 4884–4903. https://doi.org/10.1016/j.atmosenv.2008.02.043.
Jassal, M.S., 2015. Pediatric asthma and ambient pollutant levels in industrializing nations. Int Health 7, 7–15. https://doi.org/10.1093/inthealth/ihu081.
Ku, M.-S., Sun, H.-L., Lu, K.-H., Sheu, J.-N., Lee, H.-S., Yang, S.-F., Lue, K.-H., 2011. The CC16 A38G polymorphism is associated with the development of asthma in children with allergic rhinitis: CC16 polymorphism and asthma development. Clin. Exp. Allergy 41, 794–800. https://doi.org/10.1111/j.1365-2222.2010.03679.x.
Kumar, R.K., Shadie, A.M., Bucknall, M.P., Rutlidge, H., Garthwaite, L., Herbert, C., Halliburton, B., Parsons, K.S., Wark, P.A.B., 2015. Differential injurious effects of ambient and traffic-derived particulate matter on airway epithelial cells: effect of particles on airway epithelium. Respirology 20, 73–79. https://doi.org/10.1111/resp.12381.
Laing, I.A., Hermans, C., Bernard, A., Burton, P.R., Goldblatt, J., Le Souëf, P.N., 2000. Association between plasma CC16 levels, the A38G polymorphism, and asthma. Am. J. Respir. Crit. Care Med. 161, 124–127. https://doi.org/10.1164/ajrccm.161.1.9904073.
Landrigan, P.J., Fuller, R., Acosta, N.J.R., Adeyi, O., Arnold, R., Basu, N., Nil), Baldé, A. B., Bertollini, R., Bose-O'Reilly, S., Boufford, J.I., Breysse, P.N., Chiles, T., Mahidol, C., Coll-Seck, A.M., Cropper, M.L., Fobil, J., Fuster, V., Greenstone, M., Haines, A., Hanrahan, D., Hunter, D., Khare, M., Krupnick, A., Lanphear, B., Lohani, B., Martin, K., Mathiasen, K.V., McTeer, M.A., Murray, C.J.L., Ndahimananjara, J.D., Perera, F., Potočnik, J., Preker, A.S., Ramesh, J., Rockström, J., Salinas, C., Samson, L.D., Sandilya, K., Sly, P.D., Smith, K.R., Steiner, A., Stewart, R.B., Suk, W.A., van Schayck, O.C.P., Yadama, G.N., Yumkella, K., Zhong, M., 2018. The Lancet Commission on pollution and health. Lancet 391, 462–512. https://doi.org/10.1016/S0140-6736(17)32345-0.
Levin, S.W., Butler, J.DeB., Schumacher, U.K., Wightman, P.D., Mukherjee, A.B., 1986. Uteroglobin inhibits phospholipase A2 activity. Life Sci. 38, 1813–1819. https://doi.org/10.1016/0024-3205(86)90135-9.
Li, N., Harkema, J.R., Lewandowski, R.P., Wang, M., Bramble, L.A., Gookin, G.R., Ning, Z., Kleinman, M.T., Sioutas, C., Nel, A.E., 2010. Ambient ultrafine particles provide a strong adjuvant effect in the secondary immune response: implication for traffic-related asthma flares. Am. J. Physiol. Lung Cell Mol. Physiol. 299, L374–L383. https://doi.org/10.1152/ajplung.00115.2010.
Li, H., Zhou, L., Wang, C., Chen, R., Ma, X., Xu, B., Xiong, L., Ding, Z., Chen, X., Zhou, Y., Xu, Y., Kan, H., 2017. Associations between air quality changes and biomarkers of systemic inflammation during the 2014 Nanjing Youth Olympics: a quasi-experimental study. Am. J. Epidemiol. 185, 1290–1296. https://doi.org/10.1093/aje/kww209.
Li, X., Zhang, X., Zhang, Z., Han, L., Gong, D., Li, J., Wang, T., Wang, Y., Gao, S., Duan, H., Kong, F., 2019. Air pollution exposure and immunological and systemic inflammatory alterations among schoolchildren in China. Sci. Total Environ. 657, 1304–1310. https://doi.org/10.1016/j.scitotenv.2018.12.153.
Lin, W., Zhu, T., Xue, T., Peng, W., Brunekreef, B., Gehring, U., Huang, W., Hu, M., Zhang, Y., Tang, X., 2015. Association between changes in exposure to air pollution and biomarkers of oxidative stress in children before and during the Beijing Olympics. Am. J. Epidemiol. 181, 575–583. https://doi.org/10.1093/aje/kwu327.
Ma, Y.-N., Wang, J., Lee, Y.L., Ren, W.-H., Lv, X.-F., He, Q.-C., Dong, G.-H., 2015a. Association of urine CC16 and lung function and asthma in Chinese children. Allergy Asthma Proc. 36, 59–64. https://doi.org/10.2500/aap.2015.36.3853.
Ma, Y.-N., Qian, Z., Wang, J., Rodemich, E., Lee, Y.L., Lv, X.-F., Liu, Y.-Q., Zhao, Y., Huang, M.-M., Liu, Y., Sun, J., He, Q.-C., Dong, G.-H., 2015b. Environmental tobacco smoke exposure, urine CC-16 levels, and asthma outcomes among Chinese children. Allergy 70, 295–301. https://doi.org/10.1111/all.12559.
Mannucci, P.M., Harari, S., Martinelli, I., Franchini, M., 2015. Effects on health of air pollution: a narrative review. Intern. Emerg. Med. 10, 657–662. https://doi.org/10.1007/s11739-015-1276-7.
McMichael, G.L., Gibson, C.S., O'Callaghan, M.E., Goldwater, P.N., Dekker, G.A., Haan, E.A., MacLennan, A.H., 2009. South Australian cerebral palsy research group. DNA from buccal swabs suitable for high-throughput SNP multiplex analysis J. Biomol. Tech. 20, 232–235.
Michel, O., Murdoch, R., Bernard, A., 2005. Inhaled LPS induces blood release of Clara cell specific protein (CC16) in human beings. J. Allergy Clin. Immunol. 115, 1143–1147. https://doi.org/10.1016/j.jaci.2005.01.067.
Mirowsky, J., Gordon, T., 2015. Noninvasive effects measurements for air pollution human studies: methods, analysis, and implications. J. Expo. Sci. Environ. Epidemiol. 25, 354–380. https://doi.org/10.1038/jes.2014.93.
Nauwelaerts, S.J.D., Roosens, N.H.C., De Cremer, K., Bernard, A., De Keersmaecker, S.C. J., 2020a. Gender-dependent association between exhaled nitric oxide and the CC16 38AA genotype in young school children. Immun. Inflamm. Dis. 1–9. https://doi.org/10.1002/iid3.332.
Nauwelaerts, S.J.D., Van Geel, D., Delvoye, M., De Cremer, K., Bernard, A., Roosens, N.H. C., De Keersmaecker, S.C.J., 2020b. Selection of a noninvasive source of human DNA envisaging genotyping assays in epidemiological studies: urine or saliva? J. Biomol. Tech. jbt 20–3101–004. https://doi.org/10.7171/jbt.20-3101-004.
Nauwelaerts, S.J.D., Roosens, N.H.C., Bernard, A., De Keersmaecker, S.C.J., De Cremer, K., 2021. Development of a multiplex mass spectrometry method for simultaneous quantification of urinary proteins related to respiratory health. Sci. Rep. 11, 1–13. https://doi.org/10.1038/s41598-021-89068-9.
Pang, M., Yuan, Y., Wang, Dong, Li, T., Wang, Dan, Shi, X., Guo, M., Wang, C., Zhang, X., Zheng, G., Yu, B., Wang, H., 2017. Recombinant CC16 protein inhibits the production of pro-inflammatory cytokines via NF-κB and p38 MAPK pathways in LPS-activated RAW264.7 macrophages. Acta Biochim. Biophys. Sin. 49, 435–443. https://doi.org/10.1093/abbs/gmx020.
Provost, E.B., Chaumont, A., Kicinski, M., Cox, B., Fierens, F., Bernard, A., Nawrot, T.S., 2014. Serum levels of club cell secretory protein (Clara) and short- and long-term exposure to particulate air pollution in adolescents. Environ. Int. 68, 66–70. https://doi.org/10.1016/j.envint.2014.03.011.
Rong, B., Fu, T., Gao, W., Li, M., Rong, C., Liu, W., Liu, H., 2020. Reduced serum concentration of CC16 Is associated with severity of chronic obstructive pulmonary disease and contributes to the diagnosis and assessment of the disease. Int. J. Chronic Obstr. Pulm. Dis. 15, 461–470. https://doi.org/10.2147/COPD.S230323.
Rosas-Salazar, C., Gebretsadik, T., Carroll, K.N., Reiss, S., Wickersham, N., Larkin, E.K., James, K.M., Miller, E.K., Anderson, L.J., Hartert, T.V., 2015. Urine club cell 16-kDa secretory protein and childhood wheezing illnesses after lower respiratory tract infections in infancy. Pediatr. Allergy, Immunol. Pulmonol. 28, 158–164. https://doi.org/10.1089/ped.2015.0528.
Rosenberg, S.R., Kalhan, R., 2012. Biomarkers in chronic obstructive pulmonary disease. Transl. Res. 159, 228–237. https://doi.org/10.1016/j.trsl.2012.01.019.
Saenen, N.D., Provost, E.B., Viaene, M.K., Vanpoucke, C., Lefebvre, W., Vrijens, K., Roels, H.A., Nawrot, T.S., 2016. Recent versus chronic exposure to particulate matter air pollution in association with neurobehavioral performance in a panel study of primary schoolchildren. Environ. Int. 95, 112–119. https://doi.org/10.1016/j.envint.2016.07.014.
Saenen, N.D., Bové, H., Steuwe, C., Roeffaers, M.B.J., Provost, E.B., Lefebvre, W., Vanpoucke, C., Ameloot, M., Nawrot, T.S., 2017. Children's urinary environmental carbon load. A novel marker reflecting residential ambient air pollution exposure?

Am. J. Respir. Crit. Care Med. 196, 873–881. https://doi.org/10.1164/rccm.201704-0797OC.

Saenen, N.D., Provost, E.B., Cuypers, A., Kicinski, M., Pieters, N., Plusquin, M., Vrijens, K., De Boever, P., Nawrot, T.S., 2019. Child's buccal cell mitochondrial DNA content modifies the association between heart rate variability and recent air pollution exposure at school. Environ. Int. 123, 39–49. https://doi.org/10.1016/j.envint.2018.11.028.

Schwartz, J., 2004. Air pollution and children's health. Pediatrics 113, 1037–1043.

Seys, S.F., Hox, V., Van Gerven, L., Dilissen, E., Marijsse, G., Peeters, E., Dekimpe, E., Kasran, A., Aertgeerts, S., Troosters, T., Vanbelle, V., Peers, K., Ceuppens, J.L., Hellings, P.W., Dupont, L.J., Bullens, D.M., 2015. Damage-associated molecular pattern and innate cytokine release in the airways of competitive swimmers. Allergy 70, 187–194. https://doi.org/10.1111/all.12540.

Shen, Y., Zhang, Z.-H., Hu, D., Ke, X., Gu, Z., Zou, Q.-Y., Hu, G.-H., Song, S.-H., Kang, H.-Y., Hong, S.-L., 2018. The airway inflammation induced by nasal inoculation of PM2.5 and the treatment of bacterial lysates in rats. Sci. Rep. 8, 9816. https://doi.org/10.1038/s41598-018-28156-9.

Shijubo, N., Itoh, Y., Yamaguchi, T., Sugaya, F., Hirasawa, M., Yamada, T., Kawai, T., Abe, S., 1999. Serum levels of Clara cell 10-kDa protein are decreased in patients with asthma. Lung 177, 45–52. https://doi.org/10.1007/PL00007626.

St Helen, G., Holland, N.T., Balmes, J.R., Hall, D.B., Bernert, J.T., Vena, J.E., Wang, J.-S., Naeher, L.P., 2013. Utility of urinary Clara cell protein (CC16) to demonstrate increased lung epithelial permeability in non-smokers exposed to outdoor secondhand smoke. J. Expo. Sci. Environ. Epidemiol. 23, 183–189. https://doi.org/10.1038/jes.2012.68.

Stenberg, H., Wadelius, E., Moitra, S., Åberg, I., Ankerst, J., Diamant, Z., Bjermer, L., Tufvesson, E., 2018. Club cell protein (CC16) in plasma, bronchial brushes, BAL and urine following an inhaled allergen challenge in allergic asthmatics. Biomarkers 23, 51–60. https://doi.org/10.1080/1354750X.2017.1375559.

Stockfelt, L., Sallsten, G., Olin, A.-C., Almerud, P., Samuelsson, L., Johannesson, S., Molnar, P., Strandberg, B., Almstrand, A.-C., Bergemalm-Rynell, K., Barregard, L., 2012. Effects on airways of short-term exposure to two kinds of wood smoke in a chamber study of healthy humans. Inhal. Toxicol. 24, 47–59. https://doi.org/10.3109/08958378.2011.633281.

Taniguchi, N., Konno, S., Hattori, T., Isada, A., Shimizu, Kaoruko, Shimizu, Kenichi, Shijubo, N., Huang, S.-K., Hizawa, N., Nishimura, M., 2013. The CC16 A38G polymorphism is associated with asymptomatic airway hyper-responsiveness and development of late-onset asthma. Ann. Allergy Asthma Immunol. 111, 376–381. https://doi.org/10.1016/j.anai.2013.08.005 e1.

The 1000 Genomes Project Consortium, 2010. A map of human genome variation from population-scale sequencing. Nature 467, 1061–1073. https://doi.org/10.1038/nature09534.

Timonen, K.L., 2004. Daily variation in fine and ultrafine particulate air pollution and urinary concentrations of lung Clara cell protein CC16. Occup. Environ. Med. 61, 908–914. https://doi.org/10.1136/oem.2004.012849.

Tufvesson, E., Svensson, H., Ankerst, J., Bjermer, L., 2013. Increase of club cell (Clara) protein (CC16) in plasma and urine after exercise challenge in asthmatics and healthy controls, and correlations to exhaled breath temperature and exhaled nitric oxide. Respir. Med. 107, 1675–1681. https://doi.org/10.1016/j.rmed.2013.08.004.

Union, European, 2008. Directive 2008/50/EC of the European Parliament and of the Council on Ambient Air Quality and Cleaner Air for Europe.

Van Miert, E., Sardella, A., Nickmilder, M., Bernard, A., 2012. Respiratory effects associated with wood fuel use: a cross-sectional biomarker study among adolescents. Pediatr. Pulmonol. 47, 358–366. https://doi.org/10.1002/ppul.21554.

Verbeke, G., Molenberghs, G., 2001. Linear mixed models for longitudinal data, Corr. 2. print. In: Springer Series in Statistics. Springer, New York.

Vriens, A., Nawrot, T.S., Saenen, N.D., Provost, E.B., Kicinski, M., Lefebvre, W., Vanpoucke, C., Van Deun, J., De Wever, O., Vrijens, K., De Boever, P., Plusquin, M., 2016. Recent exposure to ultrafine particles in school children alters miR-222 expression in the extracellular fraction of saliva. Environ. Health 15, 80. https://doi.org/10.1186/s12940-016-0162-8.

Vriens, A., Provost, E.B., Saenen, N.D., De Boever, P., Vrijens, K., De Wever, O., Plusquin, M., Nawrot, T.S., 2018. Children's screen time alters the expression of saliva extracellular miR-222 and miR-146a. Sci. Rep. 8, 8209. https://doi.org/10.1038/s41598-018-26351-2.

Wang, C., Cai, J., Chen, R., Shi, J., Yang, C., Li, H., Lin, Z., Meng, X., Liu, C., Niu, Y., Xia, Y., Zhao, Z., Li, W., Kan, H., 2017a. Personal exposure to fine particulate matter, lung function and serum club cell secretory protein (Clara). Environ. Pollut. 225, 450–455. https://doi.org/10.1016/j.envpol.2017.02.068.

Wang, H., Dumont, X., Haufroid, V., Bernard, A., 2017b. The physiological determinants of low-level urine cadmium: an assessment in a cross-sectional study among schoolchildren. Environ. Health 16, 99. https://doi.org/10.1186/s12940-017-0306-5.

World Health Organization, 2021. WHO Global Air Quality Guidelines: Particulate Matter (PM2.5 and PM10),ozone, 919 Nitrogen Dioxide, Sulfur Dioxide and Carbon Monoxide. World Health Organization.

World Medical Association, 2001. World Medical Association Declaration of Helsinki. Ethical principles for medical research involving human subjects. Bull. World Health Organ. 79, 373–374.

Zhai, J., Stern, D.A., Sherrill, D.L., Spangenberg, A.L., Wright, A.L., Morgan, W.J., Halonen, M., Martinez, F.D., Guerra, S., 2018. Trajectories and early determinants of circulating CC16 from birth to age 32 years. Am. J. Respir. Crit. Care Med. 198, 267–270. https://doi.org/10.1164/rccm.201712-2398LE.

Zhai, J., Insel, M., Addison, K.J., Stern, D.A., Pederson, W., Dy, A., Rojas-Quintero, J., Owen, C.A., Sherrill, D.L., Morgan, W., Wright, A.L., Halonen, M., Martinez, F.D., Kraft, M., Guerra, S., Ledford, J.G., 2019. Club cell secretory protein deficiency leads to altered lung function. Am. J. Respir. Crit. Care Med. 199, 302–312. https://doi.org/10.1164/rccm.201807-1345OC.

Zhang, A.L., Balmes, J.R., Lutzker, L., Mann, J.K., Margolis, H.G., Tyner, T., Holland, N., Noth, E.M., Lurmann, F., Hammond, S.K., Holm, S.M., 2021. Traffic-related air pollution, biomarkers of metabolic dysfunction, oxidative stress, and CC16 in children. J. Expo. Sci. Environ. Epidemiol. https://doi.org/10.1038/s41370-021-00378-6.

Zhao, G., Lin, X., Zhou, M., Zhao, J., 2013. Association between CC10 +38A/G polymorphism and asthma risk: a meta-analysis. Pakistan J. Med. Sci. 29 https://doi.org/10.12669/pjms.296.3724.

Zhu, L., Ge, X., Chen, Y., Zeng, X., Pan, W., Zhang, X., Ben, S., Yuan, Q., Xin, J., Shao, W., Ge, Y., Wu, D., Han, Z., Zhang, Z., Chu, H., Wang, M., 2017. Short-term effects of ambient air pollution and childhood lower respiratory diseases. Sci. Rep. 7, 4414. https://doi.org/10.1038/s41598-017-04310-7.

Zuur, A.F., Ieno, E.N., Walker, N.J., Saveliev, A.A., Smith, G.M., 2009. Mixed Effects Models and Extensions in Ecology with R. Springer, New York, New York, NY.