belspo

BRAIN-be
Belgian Research Action through Interdisciplinary Networks

PIONEER PROJECTS

STRATEGY TO EVALUATE HEALTH RISKS OF AIR POLLUTION EPISODES IN VULNERABLE INDIVIDUALS

CONTRACT - BR/165/PI/PMOLLUGENIX-V2

FINAL REPORT

15/12/2016-15/12/2019

Promotors

1. Nancy Roosens (Transversal activities in Applied Genomics (TAG), Sciensano, Rue Juliette Wytsmanstraat 14 1050 Brussels)
2. Sigrid De Keersmaecker (Transversal activities in Applied Genomics (TAG), Sciensano, Rue Juliette Wytsmanstraat 14 1050 Brussels)
3. Koen De Cremer (Platform chromatography and mass spectrometry, Sciensano)
4. Natalia Bustos Sierra and Katrien Tersago (Epidemiology of infectious diseases, Sciensano)
5. Alfred Bernard (Laboratory of Toxicology and Applied Pharmacology (LTAP), UCLouvain)
6. Tim Nawrot (Centre of Environmental Sciences, Molecular & environmental epidemiology, UHasselt)

Authors

Sarah J. D. Nauwelaerts (Sciensano)
Koen De Cremer (Sciensano)
Natalia Bustos Sierra (Sciensano)
Alfred Bernard (UCL)
Tim Nawrot (UHasselt)
Nancy H.C. Roosens (Sciensano)
Sigrid C.J. De Keersmaecker (Sciensano)

sciensano
UCLouvain
UHASSELT

.be

Published in 2020 by the Belgian Science Policy Office
WTCIII
Simon Bolivarlaan 30 Boulevard Simon Bolivar
B-1000 Brussels
Belgium
Tel: +32 (0)2 238 34 11
http://www.belspo.be

Contact person: Georges Jamart
+32 (0)2 238 36 90

Nauwelaerts SJD, De Cremer K, Bustos Sierra N, Bernard A, Nawrot T, Roosens NHC, De Keersmaecker S C. ***Strategy to evaluate health risks of air pollution episodes in vulnerable individuals***. Final Report. Brussels : Belgian Science Policy Office 2020 – 43 p. (BRAIN-be (Belgian Research Action through Interdisciplinary Networks))

TABLE OF CONTENTS

SUMMARY

Context

Reduced air quality, in particular with high levels of air pollutants in urban and rural areas could lead to exacerbation of respiratory disorders. Therefore, the increase of air pollution represents a major threat to public health and especially for the most vulnerable population strata, including children. Biomarkers, used as measurable indicators of exposure, effect and susceptibility, may help to assess the impact of air pollution and monitor children's respiratory health. At the present time, most of the studies have included protein biomarkers in blood to assess the effect of environmental exposure on health. Some studies have associated genetic polymorphisms to the development of hypersensitivity response or sickness. Few prospective studies have investigated how epigenetics affected by environmental exposure influences the health response. Promising and future large scale studies should integrate all proteinaceous, genetic and epigenetic aspects in order to optimize the use of potential health biomarkers and consequently to reduce the potential risk and impact on population and substrata of the population. However, the design of such kind of experiments is relatively manpower-, time- and cost-demanding and collecting blood, especially in children, remains difficult, hampering the study on a large scale population necessary to have a powerful epidemiological analysis. Therefore, there is a need of designing a study set-up with the collection of noninvasive samples, using cost-effective and high throughput technologies to investigate biomarkers on an integrated level.This strategy should allow the set-up of the most efficient large scale epidemiological study in order to investigate the adverse effect of air pollution on vulnerable populations, like children.

Objectives

The main objective of the PMOlluGENix-V2 Pioneer project, was to design a future large scale study for measuring the effects of air pollution exposure on the cardiorespiratory system of children, by measuring sensitive indicators of airway damage or inflammation at protein of genetic and epigenetic level in noninvasive samples like saliva and urine from an existing biobank and on collected samples from a conducted field study. The final aim was to evaluate the feasibility of the developed strategy for the preparation, implementation and evaluation of federal policies/strategies for conducting future large scale epidemiological studies about the efficient biomonitoring of the respiratory health in children impacted by the air pollution.

Conclusions

Based on this study the design of a workflow to investigate the effect of air pollution on the respiratory health of children in future large-scale settings was proposed. This design included the advantages of the use of high throughput methods and non-invasive samples for the biomarker measurements. Saliva was found to be the perfect surrogate of blood for genotyping and for epigenetic investigation within this context. Urine was used for the quantification of multiple protein biomarkers using the cost-effective high throughput MRM method. These non-invasive samples were complementary and valuable alternatives in the context of large scale epidemiological studies, where blood collection, especially in children, is generally not accepted. The integrated investigation (at proteinaceous, genetic and epigenetic level) optimized the use of potential health biomarkers by giving an insight on inter-related or complementary relationships and identifying potential confounding effects. In parallel, this study showed that even levels of PM2.5 below the WHO guidelines can impact the respiratory health of children. These evidence-based data need to be used and to be taken into account when policy makers enact the law establishing the goals for air quality, targeting lower thresholds than applicable now. Finally, a few modifications of the selected strategy were proposed to expand this study for a higher number of children, examined over several time points and at different locations. Auto sampling and the use of performant portable air pollution monitors were suggested in order to reduce the required manpower.

Keywords biomarkers, epidemiological studies, noninvasive, children, feasibility, air pollution

SAMENVATTING

Context

Verminderde luchtkwaliteit, met name hoge waarden van luchtvervuilende stoffen in stedelijke en landelijke gebieden, kan leiden tot verergering van ademhalingsstoornissen. Daarom vormt de toename van luchtvervuiling een grote bedreiging voor de volksgezondheid en vooral voor de meest kwetsbare bevolkingslagen, waaronder kinderen. Biomerkers die gebruikt worden als meetbare indicatoren voor blootstelling, effect en gevoeligheid, kunnen helpen om de impact van luchtvervuiling te evalueren en de ademhalingsgezondheid van kinderen op te volgen. De meeste onderzoeken bestuderen op dit moment eiwitbiomerkers in bloed om het effect van blootstelling aan de omgeving op de gezondheid te evalueren. Sommige studies hebben genetische polymorfismen in verband gebracht met de ontwikkeling van overgevoeligheidsreacties of andere aandoeningen. Weinig prospectieve studies hebben onderzocht hoe epigenetica, die wordt beïnvloed door blootstelling aan de omgeving, de gezondheidsrespons beïnvloedt. Veelbelovende en toekomstige grootschalige studies zouden alle eiwit-, genetische en epigenetische aspecten moeten integreren om het gebruik van potentiële biomerkers te optimaliseren en bijgevolg het potentiële risico en de impact op de (substrata van de) bevolking te verminderen. Het ontwerp van dergelijke studies vereist echter relatief veel mankracht, tijd en budget. Daarbij is de staalname van bloed, vooral bij kinderen, moeilijk, wat zo'n studie op grote schaal, nodig voor een significante epidemiologische analyse belemmert. Bijgevolg is er behoefte aan het opzetten van een onderzoeksstrategie, waarbij niet-invasieve stalen worden verzameld en waarbij, met behulp van kostenefficiënte en high-throughput-technologieën, biomerkers op een geïntegreerd niveau kunnen worden onderzocht. Deze strategie zou de opzet van het meest efficiënte grootschalig epidemiologisch onderzoek moeten toelaten om zo de nadelige effecten van luchtvervuiling op kwetsbare bevolkingsgroepen, zoals kinderen, na te gaan.

Doelstellingen

Het hoofddoel van het PMOlluGENix-V2 pioniersproject was het ontwerpen van een toekomstig grootschalig onderzoek voor het meten van de effecten van blootstelling aan luchtvervuiling op het cardiorespiratoire systeem van kinderen, en dit door het meten van gevoelige indicatoren van luchtweginflammatie of –letsels op het eiwit-, genetisch en epigenetisch niveau in niet-invasieve stalen zoals speeksel en urine van een bestaande biobank en op de verzamelde stalen van een uitgevoerde field study. Het uiteindelijke doel was om de haalbaarheid van de ontwikkelde strategie te evalueren voor de voorbereiding, de implementatie en de evaluatie van federale beleidslijnen/strategieën voor het uitvoeren van toekomstige grootschalige epidemiologische studies over de efficiënte biomonitoring van de gezondheid van de luchtwegen bij kinderen die worden blootgesteld aan luchtvervuiling.

Besluiten

Op basis van deze studie werd het ontwerp van een workflow voorgesteld om het effect van luchtvervuiling op de gezondheid van de ademhaling van kinderen in toekomstige grootschalige studies te onderzoeken. Dit ontwerp omvatte de voordelen van het gebruik van high throughput methoden en niet-invasieve stalen voor de meting van biomerkers. Speeksel bleek het perfecte surrogaat van bloed te zijn voor genotypering en voor epigenetisch onderzoek in deze context. Urine werd gebruikt voor de kwantificering van meerdere eiwitbiomerkers met behulp van de high throughput en kostenefficiënte MRM-methode. In de context van grootschalige epidemiologische studies waren deze niet-invasieve stalen complementaire en waardevolle alternatieven, aangezien bloedafname, vooral bij kinderen, over het algemeen niet wordt geaccepteerd. Het geïntegreerde onderzoek (op eiwit, genetisch en epigenetisch niveau) optimaliseerde het gebruik van potentiële gezondheidsbiomerkers door inzicht te geven in onderling gerelateerde of complementaire relaties en door mogelijke samenhangende effecten te identificeren. Tegelijkertijd toonde deze studie aan dat zelfs PM2.5-waarden onder de WHO-richtlijnen de gezondheid van de luchtwegen van

kinderen beïnvloeden. Deze op bewijs-gebaseerde data zouden gebruikt en in rekening gebracht moeten worden wanneer beleidsmakers de wetgevingsvoorstellen omtrent de luchtkwaliteit opstellen en vastleggen en waarbij wordt gestreefd naar lagere drempels dan nu van toepassing zijn. Ten slotte werden enkele wijzigingen op de geselecteerde strategie voorgesteld om deze studie uit te breiden naar een groter aantal kinderen die op verschillende tijdspunten en locaties worden onderzocht. Zelf de stalen laten afnemen en het gebruik van performante draagbare luchtvervuilingsmonitors werden voorgesteld om de vereiste mankracht te verminderen.

Trefwoorden biomerkers, epidemiologische studies, niet-invasief, kinderen, haalbaarheid, luchtvervuiling

RESUME

Contexte

Une mauvaise qualité de l'air, en particulier avec des niveaux élevés de polluants atmosphériques dans les zones urbaines et rurales, peut entraîner une exacerbation des troubles respiratoires. Par conséquent, l'augmentation de la pollution atmosphérique représente une menace majeure pour la santé publique et en particulier pour les couches de la population les plus vulnérables, y compris les enfants. Les biomarqueurs, utilisés comme indicateurs mesurables de l'exposition, des effets et de la sensibilité, peuvent aider à évaluer l'impact de la pollution atmosphérique et à surveiller la santé respiratoire des enfants. À l'heure actuelle, la plupart des études incluent des biomarqueurs protéiques dans le sang pour évaluer l'effet de l'exposition environnementale sur la santé. Certaines études ont associé polymorphismes génétiques et développement d'une réponse d'hypersensibilité ou d'autres affections. Peu d'études prospectives ont examiné comment l'épigénétique, affectée par l'exposition environnementale, influence la réponse de la santé. Les études prometteuses et futures à grande échelle devraient intégrer tous les aspects protéiques, génétiques et épigénétiques afin d'optimiser l'utilisation de biomarqueurs potentiels pour la santé et, par conséquent, de réduire les risques et l'impact potentiel sur la population (vulnérable). Cependant, la conception de ce type d'expériences est relativement exigeante en main-d'œuvre, en temps et en coûts et la collecte de sang, en particulier chez les enfants, reste difficile, ce qui entrave l'étude sur une population à grande échelle nécessaire pour disposer d'une analyse épidémiologique puissante. Par conséquent, il est nécessaire de concevoir une configuration d'étude incluant une collecte d'échantillons non invasifs, en utilisant des technologies efficaces et à haut débit pour étudier les biomarqueurs à un niveau intégré. Cette stratégie devrait permettre la configuration d'une étude épidémiologique efficace à grande échelle afin d'étudier les effets néfastes de la pollution de l'air sur les populations vulnérables, comme les enfants.

Objectifs

Le principal objectif du projet pionnier PMOlluGENix-V2 est de concevoir une étude future à grande échelle pour mesurer les effets de l'exposition à la pollution de l'air sur le système cardiorespiratoire des enfants, en mesurant des indicateurs sensibles de dommages ou d'inflammation des voies respiratoires à niveau protéique, génétique et épigénétique dans des échantillons non invasifs comme la salive et l'urine d'une biobanque existante et sur des échantillons prélevés lors d'une étude de terrain menée. L'objectif final est d'évaluer la faisabilité de la stratégie élaborée pour la préparation, la mise en œuvre et l'évaluation des politiques / stratégies fédérales pour la réalisation de futures études épidémiologiques à grande échelle sur la biosurveillance efficace de la santé respiratoire chez les enfants impactés par la pollution de l'air.

Conclusions

Sur base de cette étude, la conception d'un flux de travail a été proposée pour étudier l'effet de la pollution de l'air sur la santé respiratoire des enfants dans des études futures à grande échelle. Cette conception a inclu les avantages de l'utilisation de méthodes à haut débit et d'échantillons non invasifs pour les mesures de biomarqueurs. La salive s'est avérée être le substitut parfait du sang pour le génotypage et pour les investigations épigénétiques. L'urine a été utilisée pour la quantification de biomarqueurs protéiques multiples en utilisant la méthode MRM qui est rentable et à haut débit. Ces échantillons non invasifs sont des alternatives complémentaires et précieuses dans le contexte d'études épidémiologiques à grande échelle, où la collecte de sang, en particulier chez les enfants, n'est pas acceptée. L'enquête intégrée (au niveau protéique, génétique et épigénétique) a optimisé l'utilisation de biomarqueurs potentiels pour la santé en donnant un aperçu des relations interdépendantes ou complémentaires et en identifiant les effets confondants potentiels. Parallèlement, cette étude a montré que même des niveaux de PM2.5 inférieurs aux directives de l'OMS peuvent avoir un impact sur la santé respiratoire des enfants. Ces données

factuelles doivent être utilisées et prises en compte lorsque les décideurs politiques adoptent la loi fixant les objectifs de qualité de l'air, en ciblant des seuils plus bas que ceux applicables actuellement. Enfin, quelques modifications de la stratégie choisie ont été proposées pour étendre cette étude à un plus grand nombre d'enfants, examinées sur plusieurs points dans le temps et à différents endroits. L'auto-échantillonnage a et l'utilisation de moniteurs de pollution de l'air portables et performants ont été suggérés afin de réduire la main-d'œuvre requise.

Mots-clés biomarqueurs, études épidémiologiques, non invasif, enfants, faisabilité, pollution de l’air

1. INTRODUCTION

Projected climate change could increase respiratory diseases associated with reduced air quality in urban and rural areas. Poor air quality is caused by anthropogenic activities and subsequent climate changes leading to high levels of particulate matter (PM), ozone and nitrogen oxides (NO_x), the latter being a precursor for O_3 (Akhtar and Palagiano 2018). According to the annual report from 2017 concerning the air quality in Belgium (IRCEL-CELINE 2017), the PM and O_3 concentrations are the most problematic air pollutants in terms of health effects in Belgium, when compared with the EU and WHO guidelines. The WHO guideline values are set for the protection of health, and are generally stricter than the comparable politically agreed EU standards, as shown in the summary in Table I. The air pollutants target values are calculated for the entire population. However, special consideration should be given to determining appropriate levels in order to protect vulnerable groups, including healthy children, elderly, pregnant woman and patients with heart or lung diseases (Provost et al. 2014; Schwartz 2004; Annesi-Maesano et al. 2013).

Table I: Air quality standards under the Air Quality Directive, and WHO air quality guidelines

EU Air Quality Directive				WHO Guidelines	
Pollutant	Averaging Period	Objective and legal nature and concentration	Comments	Concentration	Comments
$PM_{2.5}$	Hourly			25 µg/m³	99th percentile (3 days/year)
$PM_{2.5}$	Annual	Limit value, 25 µg/m³		10 µg/m³	
PM_{10}	Hourly	Limit value, 50 µg/m³	Not to be exceeded on more than 35 days per year	50 µg/m³	99th percentile (3 days/year)
PM_{10}	Annual	Limit value, 40 µg/m³		20 µg/m³	
O_3	Maximum daily 8-hour mean	Target value, 120 µg/m³	Not to be exceeded on more than 25 days per year, averaged over three years	100 µg/m³	
NO_2	Hourly	Limit value, 200 µg/m³	Not to be exceeded on more than 18 times a calendar year	200 µg/m³	
NO_2	Annual	Limit value, 40 µg/m³		40 µg/m³	

Source: EU Air Quality Directive (2008/50/EC), WHO, 2006, Air quality guidelines: Global update 2005

The increase of air pollution related to climate change represents a major threat to public health (WHO Regional Office for Europe 2004). Long term ambient air pollution exposure is reported to increase premature (Silva et al. 2013) and all-cause mortality and morbidity (Samek 2016; Bustos Sierra and Aikainen 2017). In Belgium, in 2016, ozone and PM were responsible for 1900 and 75,800 Years of Life Lost (YLL) and for 180 and 7600 premature deaths, respectively (European Environment Agency 2019). Numerous epidemiological studies have demonstrated the link between the ambient air pollution and various health disorders. Elevated levels have been associated with neurological diseases, increased blood pressure (Sughis et al. 2012; Choi et al. 2019) and increased risks of cardiovascular disorders (Collart et al. 2018; WHO Regional Office for Europe 2004). Air pollution is an increasing factor of many respiratory diseases, such as asthma (Orellano et al. 2017; Guarnieri and Balmes 2014), COPD (Li et al. 2016; Jiang, Mei, and Feng 2016) and lung cancer (Puett et al. 2014). It has been associated with the decrements of the lung function (Johnson and Theurer 2014; Barraza-Villarreal et al. 2008) and can lead to enhancement of allergic reaction and airway inflammation (Renzetti et al. 2009; Barraza-Villarreal et al. 2008).

Assessment of potential risks and impact of air pollution exposure on the respiratory health through monitoring is critical to better develop policy or measures with adequate targeted exposure levels of these pollutants at population level. Molecular epidemiology is a powerful tool for this monitoring. At the present time, most of the studies have included protein biomarkers to assess the effect of environmental exposure on respiratory health. Multiple proteins have been studied in the aim to assess the integrity of the deep lung epithelium which reflects the respiratory health and diseases and most of them were measured in serum using immunological assays. Immunological assays

are expensive and time-demanding especially when samples are numerous. Alternative cost-effective and high-throughput technologies should be developed. In addition, serum or other invasive sampling is the preferred sample for serological, genetic and epigenetic studies. However, the necessity of venous blood collection in a healthy population for monitoring studies especially in infants, small children and elderly is hindered by the invasive origin of the test and such type of collection may be difficult in field conditions outside the hospital. Therefore, the use of noninvasive samples, such as urine and buccal samples, for the measurement of biomarkers could be a suitable alternative. So far, no study has used urine protein biomarkers in young children to assess the integrity of the deep lung epithelium because no valuable protein measuring the confounding effect is known for adjustment of variations in diuresis and renal handling of proteins.
Several scientific studies have already demonstrated that the individual genetic background modulates the response to air pollutants and that it directly affects disease risk phenotypes (J. Hong and Khurana Hershey 2012; Favé et al. 2018). Some studies have associated genetic polymorphisms to the development of hyper sensibility response or respiratory diseases (Akhabir and Sandford 2011; Chen, Wong, and Li 2016; Yang et al. 2009).
In the last years, the epigenome has been shown to be an important target of environment-induced modifications such as air pollutants that can cause epigenetic variations contributing to increased disease susceptibility (Vrijens, Bollati, and Nawrot 2015; Silveyra and Floros 2013; J. Hong and Khurana Hershey 2012). Few prospective studies have investigated how epigenetics affected by environmental exposure influences the health response, showing differentially methylated genes or differentially expressed miRNAs after exposure to air pollution or respiratory health (Langie et al. 2016; Vriens et al. 2016; Acevedo et al. 2015).
To measure the respiratory health and the related lung function and lung inflammation, well known tests have been described. A high level of fractional exhaled nitric oxide (FeNO) has been proposed as a non-invasive biomarker of airway inflammation and has been correlated with air pollution (Eckel et al. 2016; Berhane et al. 2011). The feNO can also be used as a tool to help with the diagnosis of asthma (Bastain et al. 2011). The spirometry, on the other hands, gives information about the lung capacity and if lungs are obstructed or not. A reduced lung capacity has been associated with air pollution but the spirometer is also used to give an indication about COPD and/or asthma conditions (Johnson and Theurer 2014).
Not many studies have investigated the combination of different types of biomarkers in the context of respiratory health and air pollution. Promising and future directions should integrate all proteinaceous, genetic and epigenetic aspects in order to optimize the use of potential health biomarkers and consequently to reduce the potential risk and impact on population and substrata of the population. However, the design of such kind of experiments is relatively manpower-, time- and cost-demanding, hampering the study on a large scale population necessary to have a powerful epidemiological analysis.

The goal of this PMOlluGENix-V2 Pioneer project was to develop efficient, generic, standardized, policy supporting tools and methods that allow evaluating in a future follow-up larger scale epidemiological study, the risks of acute respiratory effects during air pollution episodes. In order to achieve this, a design of a study set-up is needed including the non-invasive biomarker measurements at different levels. These should be linked with general and respiratory health assessment obtained through examinations and questionnaire data. By the measurement of sensitive indicators of airway damage or inflammation (protein biomarkers) and of genetic and epigenetic variations in children in the summer and the winter, an integrated approach needs to be proposed for the monitoring of exposure, effect and susceptibility in children’s cohorts. This may help to understand the complex relationship between cause and effect and is also of critical importance for health care management purposes, public health decision making, and primary prevention activities.

2. METHODOLOGY AND RESULTS

Preamble: design of the scientific strategy and work packages (WP)

As mentioned above, the main objective of this project was to design a workflow that could be used for a future large scale study for measuring the effects of "critical" levels of air pollution on the cardiorespiratory system of children. A scientific strategy was designed in five work packages (WP) described below. First, the design of a small scale field study was elaborated and evaluated by the Ethics committee (WP1). This small scale field study was organized in order to go through all steps from start to end of the design. It included the selection of the target group and target location. Furthermore, the detailed study protocol, a questionnaire for the parents and the consent documents were established. Once accepted, this small scale field study (42 children) was conducted, resulting in a number of collected noninvasive samples and the measurements of the respiratory health of the children and of air pollution parameters (WP2). Finally, the potential biomarker candidates had to be measured at several levels. Genetic and protein biomarkers (WP3) were measured but also interesting epigenetic biomarkers were investigated (WP4). In order to increase the statistical significance of the biomarker measurements, samples from an additional large scale biobank were analyzed as well. This biobank included urine samples and buccal samples from 334 children (9-11 years old), from a previous study that was conducted and for which air pollution data was already available. The combination of both the small scale field study and the existing biobank allowed us to investigate practical and technical bottlenecks and to evaluate the feasibility of such a study at large scale (WP5). The type of study design, the use of noninvasive samples and cost-effective methods were assessed. The most efficient approach giving a maximal result with a minimal effort (cost, time, manpower) was proposed as part of the optimal design of future large scale epidemiological studies.

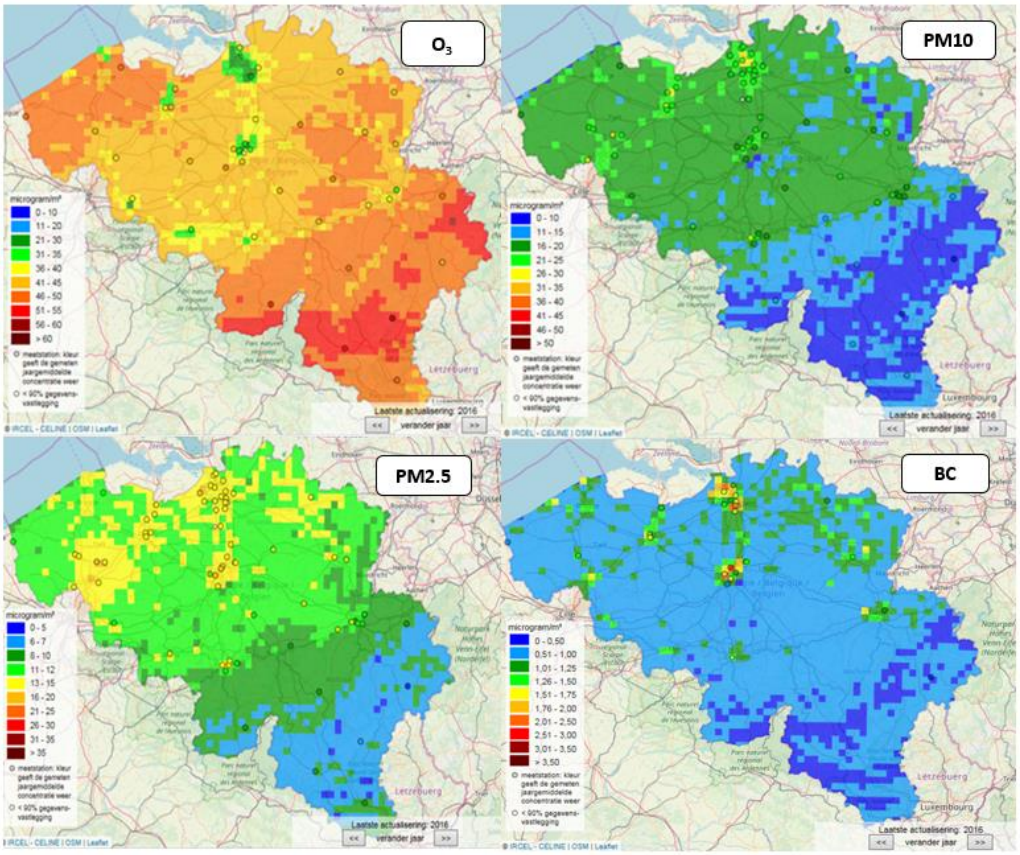
WP1. Selection of vulnerable population strata children

The objective of this WP was to design the optimal set-up for conducting a new small scale study, i.e. to collect the appropriate human samples for biomarker measurements to study the effect of simultaneous exposure to "critical" levels of air pollutants. This included the recruitment of the volunteers, the establishment of a study protocol, a detailed questionnaire and written agreement forms for the parents and children. Additionally, in order to increase the statistical power of the whole study, samples of the existing biobank, involving the study of the effect of air pollution on children's health were also carefully selected and used for analysis.

Task 1.1 Recruitment of volunteers

For the recruitment of healthy children, several locations and activities were investigated, such as schools, summer camps and playground holidays. The initial recruitment strategy was to target, during summer and winter, children living in an urban area and participating to scouts camp/summer camp/playground holidays organized in the city and in the country side. However, opting for this strategy showed multiple logistic and practical hurdles. As those camps start on fixed dated, it was challenging to take into account the short-term character of a pollution peak and mobilizing all the people involved in time. Moreover, finding the medical staff responsible for the sampling is costly. Finally, a follow-up study with the same children in this set-up is very complicated to organize, as the children are required to participate again to the same camp the next winter or summer. Therefore, several reasons led to the option of targeting the children through schools. Firstly, by targeting school classes, the children were automatically grouped by age. Secondly, it was possible to collaborate with the Centrum voor Leerlingenbegeleiding (CLB).This center for student guidance, associated to each school was the perfect candidate to serve as intermediary, ensuring the anonymity of the children. The medical staff of the CLB was able to help on site during the field study by gathering general health parameters of the child. Working together with the CLB, possibly increased the willingness of the parents to allow their child to participate. Thirdly, working with a school allowed to trace back the same children for the next examination. Nevertheless, collaborating with a school also had its inconveniences. The tight school schedule interfered with the planning of the field study. Indeed, the multiple school activities (exams, gym sessions, field trips, (lunch) breaks) and their long closing period during the summer (limiting the chances to aim for very high ozone levels) and during other holidays had to be taken into account.

A two-phase-follow-up study (in the summer and winter) was organized in two schools and was preceded by a test phase in third school. The additional test phase was included to evaluate the whole workflow and to test the set-up as described in the designed study protocol, without taking into account the air pollution parameters and measuring all biomarkers. The major practical bottlenecks were identified before starting the following phases of the field study. A school in Boutersem were selected to participate to this test phase. From the 29 children (aged 9 to 11 years old) 20 (69%) finally participated. Following the test phase, a follow-up study was conducted in two schools, specifically located in a rural and urban area and including the measurements of air pollution. The selection of the locations was based on maps representing the yearly averages from 2016 of the different pollutants (Figure 1).



1. Yearly averages of measured air pollutants (2016); source: www.irceline.be

A rural environment in the region south of the Sambre and the Meuse was originally targeted as one of the locations. However, this was not feasible, as the Lames Milieumaatschappij (VMM), the subcontractor in this project, installs the measuring trailers exclusively in Flanders. Finally, the study was conducted in a school located in Diepenbeek (rural area) and another in Molenbeek (urban environment). Meetings were organized with the CLB and the concerned schools and practical arrangements were made. From the selected classes, 36 and 56 children (all aged 9 to 11 years old) were invited to participate in Molenbeek resp. Diepenbeek. Finally, 19 (53%) and 23 (41%) respectively participated. This limited number of participants was sufficient to elaborate a workflow, to identify the bottlenecks and the tools for a future large scale epidemiological study with higher number of samples, which was the main goal of the project.

Task 1.2 Establishment of the study protocol

The whole set-up of the study has been described in a detailed study protocol, including a detailed methodology section, the followed procedures, the examinations and the laboratory investigations. A distinction was made between the procedures followed during the test phase and during the follow-up study. It also included information on safety management issues and data management. A detailed questionnaire and a written agreement for the parents and the children was created (see Task 1.3). All these documents were submitted in November 2017 to the hospital-faculty Ethics Committee of the Cliniques universitaires Saint-Luc. Additional small modifications were made in the agreement document, as was requested in a provisionary opinion from the Ethics Committee. Eventually, the approval was obtained beginning of February 2018 (Registration number B403201734310). Following the test phase in February 2018, bottlenecks were identified and two amendments were submitted, with modifications in the study protocol for the follow-up study: (1) small adaptations in the questionnaire and (2) the establishment of a follow-up letter. This letter was distributed to the parents at the end of the study (see Task 1.3).

Task 1.3 Establishment of the questionnaire & written agreement

A questionnaire was established (in French and Dutch) for the parents, addressing the social and medical background of the children and their family as well as their in- and out-of-house environment. This allowed the identification of potential confounding factors that could influence the measured outcomes. The questionnaire of 8 pages took ~20 minutes to complete (internally tested).

A document of informed consent (DIC), addressed to and to be signed by the parents/legal representative of child, was created (in French and in Dutch). Besides informing that the study was approved by an Ethics Committee, a simplified general description of the study was given as well as of the planned measurements and of what was expected of the parents and their child. It informed them that the participation was voluntary and without compensation, except for an educational gift for the participating school. The DIC also ensured anonymous handling of all samples and questionnaires (by collaborating with the CLB). It also specified that personal results would not be made available. However, general results will be made available at the end of the project in a final report. Contact details were included, in case there was a need for more information.

Taking into account their age and maturity, the assent of the participating children was also requested by the Ethics Committee. A flyer, graphically explaining the goal of the study in a simplified way (Figure 2) was created by a professional illustrator, to be signed by the participating child.

2. Cartoon on the simplified DIC distributed to the children (Dutch version)

Envelopes containing the DIC and questionnaire for the parents and an assent for the children were distributed by the teacher to the parents (via the children) 2-3 weeks prior the of the study. The CLB received the completed documents, made an overview of the participating children and informed the Sciensano team, who prepared the practicalities of the study. The DIC and the first page of the questionnaire, containing the name and a personal anonymized code of the child was kept by the CLB, while the remaining pages of the questionnaire start were transferred to the Sciensano team. These pages were only labelled with the personal code, which was used throughout the whole field study. In addition to the distributed DIC and assent, an info session was organized together with the CLB in the school of Molenbeek, where the parents could attend and ask questions, if necessary.

A follow-up/thank you letter was established (following the submission of an amendment to the Ethics Committee) and distributed to the parents (including those of non-participating children) at the end of the study. It allowed them to give their general feedback and remarks about the field study and to give their reasons for non-participation. It elaborated on which conditions they would agree on to participate (again) to this type of study, including the type of accepted sampling (blood, stool, nasal lavage). Additionally, receiving the personal results or the blood type, was proposed as an option to parents to agree to future similar studies. Finally, 20 (69%), 23 (41%) and 19 (53%) children participated in the study of the schools in resp. Boutersem, Diepenbeek and Molenbeek. 91% of the follow-up letters from the participating children in Diepenbeek and 47% of the follow-up letters from the participating children in Molenbeek were received. In this study, although still in the acceptable range, the participation rate was expected to be higher, as similar rates were described in comparable studies involving more invasive sampling of blood in children (Wennlöf, Yngve, and Sjöström 2003; Michels et al. 2012). This might be due to the limited time (2-3 weeks) offered between the distribution and collection of the forms and the fact that no reminder was sent. Additionally, researchers sometimes have only limited access to parents. These problems can occur more often in urban schools, where parent involvement in the school setting can me more limited, due to language differences and

cultural mismatches (The National Center for Education Statistics 2004; U.S. Department of Health and Human Services 2003). However, in our case, the urban school had a higher participation rate (53%) than the rural school (41%). This was perhaps due to the selection of a motivated and actively involved urban school who already participated in a previous air-pollution related study and to the additional info session that was given there. In future studies, it is advised to plan more time for distributing the documents, to send a reminder and to organize an info session in each participating school. Optionally, parents could immediately agree for the participation of their child and start filling in the requested documents during this info session.

The process of establishing all the documents for the parents were hampered by several bottlenecks. Firstly, information regarding the ethnicity of the child was not asked in the questionnaire, due to ethical reasons. However, this information appeared to be a relevant parameter, of added value for interpreting the measurement of genetic biomarkers and of the lung parameters. Secondly, missing data was observed for a number of the questions. Thirdly, it is uncertain if some of the questions were answered precisely or truly, potentially leading to misinterpretations. Some of the health-related questions could have been better addressed by including an oral interview with a doctor, yielding a more accurate response. Ideally, a health examination from a doctor present on the day of the study, including the assessment of the respiratory health would result in the most accurate description of the child's health. However, this was not feasible, especially when keeping large scale epidemiological studies in mind.

Task 1.4 Selection of samples from of children exposed to air pollution from an existing biomarker collection

To increase the statistical power, we planned to use the buccal DNA and urine samples that have been bio banked during the COGNAC study conducted by the University of Hasselt (UHasselt). In this study, 334 children aged of 9-11 year old were examined between December 2011 and February 2014. In this study, amongst other analyses, urine and saliva samples were collected, cognitive information was gathered and blood pressure was measured. Additional data was available through a questionnaire completed by the parents. Each examination day, portable devices were used to measure ultrafine particles (UFPs) with a diameter of 10-300nm (Aerasense NanoTracer; Phillips, Eindhoven, The Netherlands) and particulate matter (PM with a diameter <2.5µm (PM2.5) and <10µm (PM10)) (Aerocet 531; MetOne Instruments Inc.; Grants Pass, Oregon, US) in the schools and at the playgrounds. The residential exposure to particulate air pollution was estimated based on the current address, the exposure during pregnancy, during the first 3 years of life, and the average life-time exposure. As exposure indicators, traffic intensity within 100 m of residence and residential proximity to major road using the Geographic Information System was used. In addition to the external ambient residential modelled air pollution using high resolution models, the internal black carbon load was measured, reflecting the passage of nano-sized black carbon from lungs into the system as it is detected in urine (Saenen et al. 2017).

For the present study, 250 buccal DNA samples (of one examination point) and 627 urine samples (collected during max. 3 examinations) were still available from the initial study that was conducted in 334 children. They were transferred from UHasselt to Sciensano and stored under the appropriate conditions. Given the limited time-frame, a selection of the samples was done for further processing. All the DNA samples were used for the genotyping investigation (see WP3, task 3.2). The protein biomarker investigation (WP 3 Task 3.1) was done on 233 urine samples (collected during 1, 2 or 3 examinations), selected to include most available information on the three levels of biomarkers. The urine samples matched with 87 children whose genotyping information was available and for whom their miRNA was measured (from maximally 3 examination points). Additionally, urine samples were added from children whose inclusion of their CC16 A38G genotype to the selection would contribute to obtain a similar distribution of the CC16 SNP A38G genotype as in the 1000 Genome project (The 1000 Genomes Project Consortium 2011). A selection of the buccal DNA samples (n=80) was done in a similar way to investigate the feasibility of measuring a number of potential methylation biomarkers (see WP4, task 4.1). Initially, the urine samples were also intended to be used for investigation of potential miRNA biomarkers.

However, it was decided not to pursue this task (see WP4, task 4.2and WP5 Task 5.1 for more detailed explanation).

Conclusion WP1

The main goal was to set-up and design a small scale field study to collect the appropriate noninvasive samples of children for biomarker measurements to study the effect of simultaneous exposure to "critical" levels of air pollutants. A detailed study protocol was established, describing the set-up of a test phase and a follow-up study. The consent documents for parents and child and a questionnaire including all the relevant questions for this type of study were created. A follow-up/thank you letter was established, to be distributed at the end of the follow-up study to the parents. All the documents were submitted to and accepted by the Ethics Committee. A school in Boutersem was selected for the test phase, where no air pollution was measured and where the workflow was tested and a first set of bottlenecks identified. The follow-up study was conducted in children (9-11year old) of two schools located in distinct urban (Molenbeek) and rural (Diepenbeek) areas, and whose selection was based on expected air pollution trends. Finally, the participation rate was 41% and 53% for respectively rural and urban area. Additionally to the small scale study and in order to increase the statistical power of the biomarker measurements, samples of a larger existing biobank, involving the study of the effect of air pollution on the health of 334 children were also used for analysis. All the available buccal DNA (250) and a selection of the urine samples (233) were used for the measurement of protein biomarkers, genetic biomarkers and epigenetic biomarkers (methylation), leading to evidence-based results (described further in WP3).

WP2. Field work and biological sample handling

Once all administrative and logistical preparations were finalized, the field study was conducted, including the air pollution measurement with a trailer and portable monitors. General health parameters (height, weight, blood pressure) were measured and urine and saliva samples were collected. Urine samples were used for the investigation of protein biomarkers. Together with saliva they were also used for the evaluation of the genetic biomarkers. Epigenetic biomarker investigation was done with the saliva samples. Lung parameters were assessed by spirometry and the fractional exhaled NO (FeNO) test. The final objective of this field work was (1) to evaluate the feasibility of a large scale population study (see WP5), (2) to study the short-term effect of exposure to "critical" levels of O_3 and PM (although not expected to be statistically relevant in this small scale study) on protein, genetic and epigenetic level (WP3 and WP4) and (4) to compare results obtained by different types of human samples.

Task 2.1. Measurements & predictions of local air quality

As mentioned before, no air pollution measurements were done during the test phase of the study. The schools of the follow-up study were selected based on their geographic location in an urban or rural area and where different combinations of critical levels of ozone and PM (peaks) (table II) were expected to be targeted in the summer and winter.

Table II: expected PM and Ozone trends for rural and urban areas during winter and summer

	Summer	Winter
Urban	High critical PM High critical O_3	High critical PM Low O_3
Rural	Low PM High critical O_3	Low PM Low O_3

However, targeting the different combinations of PM and ozone peaks was challenging due to several reasons. Firstly, high ozone values in urban areas are not that common: when ozone is accumulating and reaching a high concentration, it quickly reacts with the NO exhausted by traffic to form NO_2 reducing the actual levels of ozone. Secondly, at least one month is needed for the field study preparation and for the school to schedule this study. However, a pollution peak is

maximally forecasted 2 weeks in advance, which is too short notice for the school. Thirdly, the expected trends (in figure 1) are yearly averages that can greatly differ with the measurements made during one study day. Indeed, the quickly changing meteorological conditions (rain, wind,) can have an additional influence on the measured values, leading to differences with the expected air pollution trends in these regions. Moreover, the study in the two schools was conducted on two separate days, on which the meteorological conditions could be different. Fourthly, the schools can be located in micro environments exposed to different air pollutants than depicted on the map (figure 1) showing air pollution trends for larger regions. Indeed, the school in Molenbeek was located in in a street that is flanked by buildings on both sides creating a canyon-like environment (street canyon), modifying the temperature and wind present and consequently the air quality parameters. Similarly, the school in Diepenbeek, although in rural area, was located near a busy road. Therefore **it was decided to shift the investigation from the effect of a (simultaneous) exposure to high values of ozone and PM to the effect of a difference in exposure of several air pollutants, due to location or time point**. Additionally to PM and ozone, the black carbon and NOx were also investigated, as they represent the air pollution mainly provoked by the car industry and thus more present in urban area.

Prior to the start of the follow-up study in the summer (phase 1), a trailer from the VMM was installed on the parking lot of the school in Diepenbeek. Once installed and calibrated, the trailer continuously measured the air quality parameters such as black carbon (BC), nitrogen oxide (NO), nitrogen dioxide (NO_2), ozone, PM2.5 and PM10. This type of trailer was not installed at the school site in Molenbeek. The urban micro environment of the school, characterized by minimal available open space did not allow the installation of this type of large trailer. Therefore, a measuring trailer from Brussels Environment that was already installed 700 meters from the school, near the canal of Brussels was used instead. Averages of each of the pollutants are available for each measuring trailer in Belgium on the website of www.irceline.be. Although not mentioned in the original proposal, and in addition to the measurements done with the trailer, it was decided to test the use of wearable air monitors. The Airbeams (Habitatmap, United States) were installed on both school sites in the class rooms, on the playground, at the street side of the schools. They mapped and graphed the pollution exposure to PM1, PM10 and PM2.5 in real-time via the AirCasting Android app. Additionally, a portable black carbon monitor MicroAeth AE51 (Aethlabs, San Francisco, USA), was placed on the street side of the school in Molenbeek, in order to obtain more precise measurements of the measured black carbon in this street canyon, in contrast to the trailer installed 700 meters further. Table III summarizes the different parameters measured with the trailers near both schools and the Airbeam values of PM2.5 measured during the study.

Table III. Summary of air pollutants measured on the days of the field study

	Molenbeek Urban		Diepenbeek Rural	
Trailer	Summer	Winter	Summer	Winter
O_3	68	34	80	50
BC	0.7	NA	0.9	2.3
NO_2	41	49	29	62
PM10	13	29	17	24
PM2.5	5	19	9	13
Portable monitor				
BC (Street)	1.9	5.3	-	-
Airbeam				
Street side	7.1	34.8	22.3	8.9
Playground	4	30.4	26.2	8.9
Class room	5.2	12.7	14.6	3.9

The trailer is located at 700 m from the school in Molenbeek (urban) and on the school site in Diepenbeek (rural): O_3 (8-hourly maximum), black carbon (BC) (daily average), NO_2 (daily average), PM10 (daily average), PM2.5 (daily average). BC was also measured with a portable monitor on the school site in Molenbeek (average during the 4 hour study). PM2.5 measured by the Airbeam on the street side, on the playground and in the class room. Are all expressed in µg/m³. NA: Not available due to technical issue. "-": not measured.

This table shows that, although no target values (set by EU or WHO) of the pollutants were reached, distinct differences between the two locations and between the two time points were observed. As expected, a higher ozone concentration was measured in the summer, compared to the winter and was higher at the school in Diepenbeek (rural) than the school in Molenbeek (urban). Similarly, expected trends were observed for the BC values, taking into account the most reliable and available values from the portable monitor in Molenbeek and from the trailer in Diepenbeek. They were higher in the winter, compared to the summer and higher in the urban area compared to rural area. As expected, PM values and NO_2 were also higher in the winter than in the summer in both locations.

It should be noted that Airbeam results must be interpreted with caution. For instance, measured PM2.5 values of the Airbeam were not always consistent with the trailer values. This cheap and portable instrument has two main inconveniences: its technology does not detect the smallest (most relevant) and the largest PM particles. Furthermore, the measurement is dependent on the relative humidity. This makes absolute quantification challenging with these instruments and relative quantification should be carefully interpreted. Alternative commercially more powerful portable systems could be investigated in the future.

Eventually, we can conclude that the use of performant portable instruments and stationary trailer, should ideally be combined, as they give complementary air pollution data. Indeed, the calibrated and validated trailer allows the accurate measurement of all relevant parameters at a fixed point. However, organizing a measuring campaign with a trailer requires a significant budget. Additionally, the trailer is voluminous instrument and cannot be installed everywhere. This is in contrast with the cheap and portable air monitor, which can follow the child everywhere, offering another insight on the dynamic exposure to a number of pollutants. The portable Airbeam that was used in this study was an attempt to gather and compare the air quality in and outside the school area. However, due to its limited technology, the results were difficult to interpret. Nevertheless, this instrument might be of use for sensibilizing the population and for doing small comparisons in similar conditions. For example, the Airbeam could be used to give an insight on the air quality before and after lighting up a fire in the fire place.
Another way of collecting information about the air quality is by using a spatial temporal interpolation method (similarly to what was done in the COGNAC study) which models daily residential exposure levels of PM2.5, at each child's home address. This was not done during this study but might be of added value for future large scale studies.

Task 2.2. Measurement of lung respiratory function and collection of biological samples

Initially, it was planned to collect blood samples, defined as the gold standard for biomarker investigation, to compare with the results obtained with noninvasive samples. However, after discussion with the doctors from the CLB, this step was removed, due to multiple reasons (see WP5 Task 5.1), resulting in sampling of urine and saliva only.

Prior to the start of the study, all practical preparations were done (see Figure 3). The instruments needed for the measurement of the lung parameters were purchased and trainings regarding their correct use were organized. A coded labeling system was set up and the personal codes were labelled on each sample recipient and document to ensure the anonymity of the participating children throughout the whole study (detailed in task 1.3).

3. Preparing the field study: preparation of all the consumables, sample recipients, installation of the material and of trailer on the school site in Diepenbeek

Frequent communication between Sciensano and the CLB ensured a clear planning of the day of the field study. The recipients needed for the sampling were labelled with the anonymous codes and all material was installed in a dedicated room of the school one day before the study. The day of the study, the portable air pollution instruments were installed in the different locations in and outside the school. The CLB identified each participating child and gave him/her a unique identification code on a sticker, to ensure the anonymity of each child towards the investigation team. The children were called out of the classes in small groups, and lead into the "investigation" room where the examination took place. The coded saliva samples were collected in the investigation room and the coded urine samples of the boys and girls were collected in separate bath rooms. A colored sticker was added to the individual code sticker of each child after each examination or sampling to ensure a proper follow-up. All the examinations, tests and samples collected on the day of the study are summarized in tabl IV.

Table IV: Overview of examinations, tests and samples collected for each child on the day of the field study.

Examinations	**Purpose**
Weight & height	General health parameter
Blood pressure	Investigation of correlation with air pollution
Spirometry	Investigation of respiratory health
FeNO test	Investigation of respiratory health
Sample collections	
Saliva sample (DNA) (2 samples)	Genetic and epigenetic (methylation) biomarker investigation
Saliva sample (RNA)	Epigenetic (miRNA) biomarker investigation
Urine sample	Protein biomarker investigation

The "second morning" urine samples of the children, collected with a 125 ml sample vessel (VWR, Leuven, Belgium) were immediately aliquoted and maintained in a cooled container until final storage at -80°C later that day. No preservative was added to avoid potential impact on downstream analyses.

Two saliva samples were collected using Oragene DNA tubes (DNA Genotek, Ottawa, Ontario, Canada). This allowed the saliva to be stored at room temperature for a prolonged time (for up to 5 years, according to the manufacturer), maintaining the integrity of the DNA as it contains reagents to preserve the high molecular weight DNA by inhibiting degradation and preventing bacterial growth (Nunes et al. 2012). One saliva sample was collected using the Oragene RNA tube (DNA Genotek, Ottawa, Ontari, Canada), where 2 mL of saliva was collected into the Oragene RNA vial

and once the cap tightened, RNA was released from the cells and stabilized at room temperature, until storage at -20°C at the end of the study. The saliva samples were collected at least 30 minutes after the start of the school and/or after the break to avoid any traces of drinking and/or eating in the samples. All children were able to give urine and saliva samples, except for one child of the school in Diepenbeek having difficulties to spit. In between the saliva and urine collections, the length and weight of the child were measured by the CLB, as well as the blood pressure by making five consecutive readings using an automated blood pressure instrument (Stabilo Graph, Germany) with a pediatric cuff. The first blood pressure measurement was excluded. The mean of the remaining blood pressure measurements was used for analysis. Furthermore, the fractional exhaled NO (feNO) was measured with the NOBreath monitor (Bedfond, Maidstone, UK), following the protocol established by the manufacturer. Before starting the measurements of the feNO, the ambient NO was measured to ensure appropriate measuring conditions (with ambient NO preferably <5 parts per billion (ppb) but not more than 35 ppb). Subsequently, at least three measurements were done with one disposable single-use mouthpiece recommended for each patient. Similarly to the collection of saliva, no eating or drinking was allowed just before the measurement. Once the feNO test was completed, the lung function was measured. This spirometry test was performed using the Pocket-Spiro® MPM100 (MEC, Belgium), following the guidelines provided during the organized training by the manufacturer. Three measurements were done, from which the best one was automatically selected by the MEC PDI software. From the spirometry test, the forced expiratory volume in 1 second (FEV1), the forced vital capacity (FVC) and the peak expiratory flow (PEF) values of each child could be obtained.

Eventually, all the examinations and sample collections were performed within a school day, for each school. One of the main bottlenecks was the spirometer test. Well trained staff was definitely needed to perform this test and the difficult use of the nose clip by the children during the tests sometimes led to uninterpretable results. The FeNO tests and saliva sampling were quite straight forward but valuable time was lost during the 30 minutes of waiting after the school start/break (when children had been eating and/or drinking) and during which it was not always possible to do any of the tests or sample collections. A main bottleneck of both lung parameter tests was the missing information regarding the ethnicity of the child (Pellegrino et al. 2005; Scanlon and Shriver 2010), which was obtained from the questionnaire.

Conclusion WP2

The goal of this work package was to gather all samples and additional information regarding the (respiratory) health of children and to collect information about their air pollution exposure. During the whole work flow of the field study, special attention was dedicated to the encountered practical bottlenecks. Noninvasive samples (urine and saliva) were collected from the children without any major difficulties. Blood was not included in the sampling. Information on the children's respiratory health was collected through the health-related questions from the questionnaire and by the measurement of lung parameters. The feNO and the respiratory function were measured, the latter being more difficult to measure in children. The effect of a difference in exposure of several air pollutions due to location or time point was envisaged. The air pollution parameters were measured by the combined use of a stationary trailer and the portable Airbeam monitors, providing complementary air pollution data on each school site. A trailer was installed on the school site of Diepenbeek, but no trailer was available on the school site of Molenbeek due to logistical and practical reasons. Nevertheless, a trailer was readily available 700 m away, sharing data on the measured air pollutants. Cautious interpretation was however needed, due to the microenvironment of the trailer being different than at the school. The portable Airbeams yielded complementary data about the in- and outside PM values. However, the Airbeam has technical weaknesses resulting into data difficult to interpret. This was in contrast with the portable BC monitor, monitoring accurately the black carbon at the urban school site. Eventually, differences in air pollutant concentrations were observed between the two locations and between time points. This allowed to find relevant associations between air pollutants and measured biomarkers (see further in WP3).

WP3. Measurement of the effect of air pollution on health and how genetics may influence the response to these pollutants

As previously described, the study was focused on the investigation of the effect of a difference in (instead of critical levels of) exposure of PM and O_3, mainly due to practical reasons. Additionally, levels of other pollutants, such as black carbon and NOx, were also taken into account. A database, created in the context of another study, was used to investigate a number of relevant protein and genetic biomarker candidates.

Task 3.1 Measurement of CC16 protein in urine samples as biomarker of air pollutants

A major biomarker of interest of pulmonary health is the Club cell secretory protein (CC16). This small protein (16 kD) is produced by the club cells in the distal bronchioles of the lung, by the nasal epithelium cells and all along the trachea-bronchial tree (Broeckaert and Bernard 2000; Hermans and Bernard 1999; Benson et al. 2007). Once secreted in the epithelial lining fluid of the respiratory tract, it will leak in small amounts across the airway epithelium into the blood. There it is rapidly cleared by glomerular filtration (Hermans and Bernard 1999), findings its way into urine. CC16 reflects the integrity of the pulmonary barrier and can be used to evaluate the impact on health of several air pollutants. This protein has been described as a marker of respiratory diseases like asthma, allergic rhinitis and COPD but could also be used as a biomarker of adverse effects on upper/lower airways, linked to air pollution and exposure to lung irritants such as chlorine, smoke and chemicals (Bernard et al. 2017; Wang et al. 2017; Halatek et al. 2014; Rava et al. 2015; Lindahl et al. 2004). This protective role of CC16 in the respiratory tract is supported by a range of studies in children or adults associating lower levels of serum CC16 with decreased lung function and increased risks of wheezing, asthma, bronchiolitis, allergic rhinitis, lung cancer and chronic obstructive pulmonary disease (Ingrid A. Laing et al. 2000; Lomas et al. 2008; Bernard, Nickmilder, and Dumont 2015; Guerra et al. 2015). Additionally, studies have shown that the CC16 level is also influenced by a genetic factor. One of the most investigated identified polymorphisms in the CC16 gene, is characterized by an adenine to a guanine substitution at position 38 (A38G). Some studies have reported associations between this polymorphism and both reduced serum levels of CC16 and an increased incidence of asthma (Ingrid A. Laing et al. 2000; Mansur et al. 2002). In this study, blood was not available, but urinary CC16 was investigated as a surrogate for serum CC16.

The main challenge, when measuring the small proteins such as CC16 in urine, is the proper adjustment for physiological confounders influencing the urinary excretion, including both dilution and tubular reabsorption capacity. Creatinine is often used but does not take into account the tubular reabsorption of the protein, correcting only for the dilution. Therefore, three other proteins were selected as potential adjustment candidates due to their similar biochemical properties and/or size as CC16. Finally, the challenge of renal handling was overcome in this study by selecting the appropriate small protein with similar biochemical properties for adjustment. This was demonstrated by results obtained when investigating the impact of the genetic factor A38G SNP on the measured urinary CC16 (discussed in Task 3.2).

In the context of another project, a mass spectrometry protocol was developed to quantify CC16, as well as eight other proteins of interest, using a Multiple reaction monitoring (MRM) method. This method, which is described in an ongoing publication (Nauwelaerts et al., 2020 (a), *ongoing)* allowed relative quantification of most proteins except for three proteins, due to technical and biological hurdles. The proteins were measured in all urine samples collected during the different phases of the field experiment and the samples obtained from the COGNAC biobank (n = 233). For the correct interpretation of the data, it was important to adjust for renal handling but also for sex and age. Exclusion of boys, older than 10 years old, was needed, as they might already be at prepuberal stage, leading to additional CC16 production by the prostate.

The association between the measured protein concentrations and the measured air pollutants, the lung respiratory function and other parameters recorded in the questionnaire taking into account potential confounders was investigated by statistical analysis. Children from the COGNAC study, whose urinary proteins were analysed, were exposed to an average concentration of PM2.5 of 20 µg/m³, which is below the hourly WHO guideline (of 25µg/m³). Interestingly, the analysis of the results showed that an increase in PM2.5 was associated with an increase of urinary CC16 for

children. This suggests that increases of PM2.5 could provoke increased inflammation in the lungs, translated by an increase of CC16.

Additionally to the association between CC16 and PM2.5, statistical analysis also revealed an interesting positive association between the urinary CC16 and the measured feNO. Indeed, an increase of the urinary CC16 level was observed with increasing feNO. This confirms the role of CC16 as biomarker of lung inflammation. Therefore, CC16 can be proposed as a replacement analysis for feNO measurement in some cases. This would also circumvent some of the practical drawbacks of the feNO test, such as the large variations in the 'normal' ranges of feNO in children and the use of different commercially available technologies to measure feNO (Mandon et al. 2012; Dweik et al. 2011). Detailed findings concerning the gene polymorphism results (task 3.2) will be presented in a publication (Nauwelaerts et al., 2020 (b), *ongoing)*

Task 3.2 Measurement of gene polymorphism

Previous studies have described the genetic influence on environmental stressors associated with the development of asthma and airway hyper-responsiveness. Two main single nucleotide polymorphisms (SNPs) were selected to be tested, namely the CC16 A38G and the UGRP1-112G/A. CC16 A38G (rs3741240) is located in the CC16 gene, elaborated above, and is associated with decreased lung function and with increased susceptibility to asthma and other lung and inflammatory diseases (K. Hong et al. 2013; Zhao et al. 2013; Candelaria et al. 2005; Mukherjee, Zhang, and Chilton 2007). The SNP rs1368408 is located in the promoter of the Secretoglobin family 3A member 2 gene (also called uteroglobin-related protein 1 (UGRP1) gene) playing an important role in secreting lung surfactant protein, and has been shown to result in susceptibility for asthma (Niimi et al. 2002; Xie et al. 2014; Kim et al. 2017). Serum is the gold standard for genotyping experiments. This project proposed to use noninvasive alternatives such as saliva or urine, collected during the field study. Commercial kits were used to extract total urinary DNA and salivary DNA respectively from urine collected in the test phase and from saliva collected in the test phase and phase 1 of the follow-up study. Extracted urinary and salivary DNA from the test phase were used to compare the genotyping outcomes of both SNPs for both biofluids. The results indicated that a better genotyping outcome was obtained with salivary DNA compared to urinary DNA. More details of these findings were described in a publication (Nauwelaerts et al., 2020 (d)).

Based on these results, the genotyping experiments of the follow-up study were performed on the saliva samples, collected from the children in phase 1. Almost all the collected saliva samples (40 out of 41) were successfully genotyped. The genotyping results (CC16 A38G: 58% wild-type, 25% heterozygous, 18% homozygous mutant; UGRP1 -112A/G: 75% wild-type, 25% heterozygous, 0.01% homozygous mutant) followed a similar trend as the expected allele frequencies described by the 1000 genome Project (CC16 A38G: 45% wild-type, 44% heterozygous, 11% homozygous mutants ; UGRP1 -112A/G 78% wild-type and 21% heterozygous, 0.014% homozygous mutant)(The 1000 Genomes Project Consortium 2011). The slight deviation can be due to the differences in heterogeneity of ethnicity of the population compared to the 1000 Genome Project and to the relatively small sample size of our study.

Some studies have reported associations between CC16 A38G and reduced serum levels of CC16 as well as increased incidence of asthma (Ingrid A. Laing et al. 2000; Mansur et al. 2002; Sengler et al. 2003; Candelaria et al. 2005; I. A. Laing et al. 2009; Ku et al. 2011; Taniguchi et al. 2013). When adjusting the urinary CC16 and excluding boys older than 10 years old (for their potential prepuberal confounding effect), the urinary CC16 levels were significantly reduced in children with the 38AA/AG genotypes as compared to those with the wild type 38GG genotype. These findings were observed in the samples of the field study as well as in those of the COGNAC biobank. This decrease of urinary CC16 most likely mirrors the decrease of serum CC16 reported by Laing et al. in subjects with the 38GG genotype (Ingrid A. Laing et al. 2000). Based on the similar tendency of reduced CC16 levels observed in serum and in urine, urine might be proposed as a proxy for serum to measure changing concentrations of CC16. This makes urine possibly a good source for protein biomarkers, related to respiratory health, potentially replacing blood (when not available, and if appropriate adjustment done) in the context of large scale epidemiological studies. However, it also means that, when measuring the concentrations of urinary CC16, the CC16 A38G SNP should be investigated as well and that cautious interpretation of the outcome is needed. The

CC16 A38G genotype could be a confounding effect, influencing the measured urinary CC16 level outcome.

As mentioned in Task 3.1 (and in an ongoing publication Nauwelaerts et al., 2020 (c)*)*, the results obtained from the COGNAC samples showed that PM2.5 was positively associated with urinary CC16. However, when stratifying the samples per genotype outcome of the CC16 A38G SNP, a difference in urinary CC16 increase was observed. Indeed, statistical analysis indicated that the measured urinary CC16 showed almost no increase in children with the 38AG (heterozygous) or 38AA (homozygous mutant) genotype. This was in contrast with the children with a 38GG (wild type) genotype, who showed a significant increase of urinary CC16 associated with an increase of PM2.5. Thus, this positive correlation with PM2.5 is mainly driven by children with the 38GG CC16 genotype. For the other two genotypes (A/A and A/G) holding the mutant allele, there is no increase in CC16 protein concentration when there is an increase in air pollution. Regarding the UGRP1 SNP -112A/G, preliminary statistical analysis didn't reveal any significant associations between the SNP and the respiratory health.

Conclusion WP3

In this work package, potential genetic and protein biomarkers were investigated in the collected samples of the field study and the selected samples of the available COGNAC biobank, using developed and validated methods in non-invasive samples. When comparing both samples, salivary DNA yielded better genotyping results than urinary DNA, making it the preferred noninvasive matrix for genotyping purposes in large scale epidemiological studies. However, when increasing the urinary DNA input, the genotype call rate increased. These findings were described in a publication (Nauwelaerts et al., 2020 (d)). Until now, most of the protein biomarker studies were performed using serum using immunological assays. In order to facilitate large scale studies for epidemiological analysis, a multiple reaction monitoring (MRM) technology, previously validated (described in an ongoing publication, Nauwelaerts et al., 2020(a), *ongoing*) was successfully used to measure several potential respiratory health protein biomarkers simultaneously in urine Amongst them, and most importantly, was the measurement of urinary CC16. This protein reflects the integrity of the pulmonary barrier and can be used to evaluate the impact on health of several air pollutants. The method also allowed the successful identification of a small protein, suitable to adjust for the effect of diuresis and renal handling. Interestingly, the COGNAC results, showed that an increase in PM2.5 was associated with an increase of urinary CC16. This outcome was strengthened, when stratifying the samples per genotype outcome. An important positive correlation was observed in children with the 38GG CC16 genotype while for the other two genotypes (A/A and A/G) holding the mutant allele, almost no increase was measured. From this we can conclude that cautious interpretation is needed when investigating urinary CC16. The CC16 A38G genotype could play a confounding effect, influencing the level of measured urinary CC16 (Nauwelaerts et al., 2020(b), *ongoing*).

Statistical analysis of the field study results also revealed an interesting positive association between the urinary CC16 and the measured feNO, where an increase of the urinary CC16 was observed with an increase in feNO, potentially indicating an increase of lung inflammation. The integrated results suggest that air pollution provokes lung inflammation, which can be noninvasively measured with adjusted urinary CC16. This is confirmed by the positive correlation between feNO and CC16, strengthening the role of urinary CC16 as biomarker of lung inflammation. The measurement of urinary CC16 is therefore proposed as a replacement analysis for the feNO test, circumventing some of the practical drawbacks encountered with this measurement.

WP4. Measurement of epigenetic biomarkers in response to ozone and PM

The epigenome has been shown to be an important target of environment-induced modifications and therefore to be a novel source of biomarkers (J. Hong and Khurana Hershey 2012). In this study we focused on the investigation of miRNAs and methylation biomarker candidates, using non-invasive samples.

Based on the selected analysis methods and in the given context of investigating the effect on pulmonary health, urine was found to be unsuitable for studying biomarkers related to epigenetic changes (see WP5 task 5.1). Therefore, the investigation was narrowed down to the analysis of the saliva samples from the field study and of the buccal DNA from the COGNAC biobank.

Task 4.1 Measurement of methylation biomarker candidates in buccal DNA

piNOS was one of the investigated methylation biomarkers, previously reported to be differentially methylated as a response to air pollution exposure in children (Tarantini et al. 2009). Based on the biomarker database, created in the context of another study, additional methylation candidates were also included in the study (TET-1 and foxp3). Studies have observed differences in methylation level of these targets that were associated with traffic pollution or wheezing and asthma (Nadeau et al. 2010; Brunst et al. 2013; Runyon et al. 2012; Baccarelli et al. 2012; Ding et al. 2017; Somineni et al. 2016). The selected method used in this study included a first step of bisulfite treatment of the DNA, consisting of converting methylated cytosines into uracil. As partner of the project, the research team of T. Nawrot (UHasselt), tested the methylation measurement assay. The DNA was bisulfite treated with the EZ-DNA Methylation-Gold Kit (Zymo Research, USA), which is the gold standard for bisulfite conversion, followed by amplification and pyrosequencing, generating successful results for each methylation target.

This successful protocol was tested on the DNA samples from the field study (summer phase) and the buccal DNA from the COGNAC. The latest commercialized EZ Lightning Kit (Zymo Research, USA) was used for bisulfite treatment of the DNA, ensuring the most rapid bisulfite conversion with a minimal volume of sample. The bisulphite converted DNA was transferred to the UHasselt for pyrosequencing. Unfortunately, no satisfactory results were obtained. Several reasons could explain this: the low input DNA quantities (2 ng), the relatively old COGNAC samples (from 2011-2014) or the newly tested Lightning kit, not yet described in publications as the new gold standard for bisulfite conversion of DNA. In contrast, additional in-house samples, used as controls during pyrosequencing, yielded successful methylation results. These fresh DNA samples were bisulfite converted with the EZ-DNA Methylation-Gold Kit (Zymo Research, USA). Therefore, using this kit followed by pyrosequencing, seems to be a successful method for analysing the methylation biomarker candidates. Unfortunately, given the time-frame, it was not possible to confirm this again with the samples of the field study. Future experiments are planned to analyse a selection of the readily available DNA of phase 1 as well as on additional –to be extracted- DNA samples of phase 2. Different input quantities of bisulfite converted DNA will be sent to UHasselt for pyrosequencing. A change of methylation between the different time points and/or locations, can confirm the suitability of one or more of these methylation candidates as epigenetic biomarker of exposure to air pollution in children.

Task 4.2 Measurement of miRNAs in saliva

Based on the biomarker database, created in the context of another study, miR-222 and miR-146a in the extracellular fraction of saliva were selected as potential biomarkers related to air pollutant exposure. Respectively playing a role in vascular biology and in inflammation (De Lucia et al. 2017; Ding et al. 2017; Olivieri et al. 2013), studies have shown they are differentially expressed as a response to PM and air pollution (Vriens et al. 2016; Krauskopf et al. 2019; Tsamou et al. 2018; Motta et al. 2013; Zhong et al. 2019; Hou et al. 2018). The final workflow was investigated in the context of another study. It was based on a comparable protocol used in Vriens et al., where the same two extracellular salivary miRNAs were investigated in the COGNAC biobank. In contrast with the time consuming step of ultracentrifugation, used in Vriens et al., commercial kits were used for the extraction of the extracellular fraction of a control saliva sample. The different kits lead to the isolation of extracellular fractions containing different size ranges of extracellular vesicles and exosomes. The exogenous control cel-miR-39 was spiked, in order to follow-up the technical

variations during the isolation and extraction steps. Total RNA was isolated with a commercial kit and characterized and quantified. Finally, a 2-step real-time qPCR could detect the specific miRNA expression of miR-146a, miR-222, cel-miR-39 and miR-191. The latter was analysed and used as an endogenous control, due to its stable expression in saliva (Gai et al. 2018).

All miRNAs were successfully detected, indicating that the selected workflow for the quantification of a these specific miRNAs was suitable for future larger scale studies. Unfortunately, due to the limited time frame, it was not possible to confirm this on additional samples and if a correlation may be found between the expression of the miRNA and the air pollution exposure of the children. However, this workflow will be applied on the saliva samples collected during the field study (planned in the context of another project to continue). Statistical analysis will reveal if a significant correlation exists between miRNA and the measured air pollution.

Conclusions WP4

This work package focused on the measurement of potential epigenetic biomarkers, including a number of miRNAs and methylation candidates, previously described to play a role in the response to air pollutant exposure and in respiratory health. Saliva samples were found to be suitable for this analysis. Three methylation candidate biomarkers (TET-1, piNOS, foxp3) were investigated in buccal DNA. Studies have shown changes in their methylation levels when correlated with traffic pollution or wheezing and asthma. The method for measuring the methylation level of these targets, based on bisulfite conversion of buccal DNA followed by pyrosequencing, was selected and successfully performed on test samples. The detection of two specific miRNAs (miR-222 and miR-146a) in the extracellular fraction of saliva was performed. Several studies have shown they are differentially expressed as a response to PM and air pollution. Moreover, these miRNAs were already quantified in the COGNAC biobank by UHasselt, giving an insight on the used methods and results. The method was further fine-tuned and included the measurement of an exogenous and endogenous miRNA control. All miRNAs were successfully detected in test samples, indicating that the selected workflow could be retained for future larger scale studies. Both workflows will be applied on a selection of the field study samples (in the context of another project to continue). Unfortunately, due to the limited time frame, it was not possible to see if a correlation may be found between the expression of the methylation status of the gene and/or the miRNA and the air pollution exposure of the children.

WP5. Evaluation of feasibility & design of large scale epidemiological studies

This work package addresses one of the main objectives of the whole PMOlluGENix-V2 project, i.e. to deliver the evidence-based results in order to design a future large scale epidemiological study. A number of bottlenecks, affecting future large scale studies, needed to be identified and tackled. In this work package these limiting factors were analysed and the most efficient approach giving a maximal result with a minimal effort (cost, time, manpower) was proposed as part of the optimal design of future large scale epidemiological studies.

Task 5.1 Evaluation of the use of noninvasive samples as valuable source of biomarkers

Different biofluids can be used as source of biomarkers for respiratory health. An overview of the advantages and the inconveniences of the discussed biofluids in this report is given in table V. Using blood samples is the gold standard source of many biomarkers at many levels (protein, genetic, epigenetic) and is needed to validate the biomarkers found in noninvasive samples. Initially, blood sampling was planned in the set-up of the field study. However, certain limitations hampered this possibility. Firstly, both the Ethical Committee and the parents of the participating children were not expected to easily accept the blood collection, especially in the context of an epidemiological study. Blood sampling can cause anxiety and/or physical discomfort, particularly in vulnerable populations like small children. This reluctance for acceptance was confirmed by the responses collected from the follow-up letters, distributed to the parents at the end of the study. Between 47% to 70% of the parents did not agree to let their child participate if blood were asked,

even in case the personal results (including for instance the blood type) of their child would be shared with them. Secondly, professional training of phlebotomy is needed, as well as additional equipment and infrastructure, thereby hampering sampling in a more epidemiological setting.

Table V: Evaluation of the use of different biofluids for investigation of biomarkers (protein, genetic and epigenetic)

	Blood	Saliva	Urine
Protein	**+++**	**NA**	**+++**
Genetic	**+++**	**+++**	**+/-**
Epigenetic	**+++**	**+++**	**-**
Advantages	Gold standard	Noninvasive Genetic/methylation: stable at room temperature	Noninvasive Cheap
Disadvantages	Quite invasive Large scale study Medical staff needed Difficult agreement of - Ethics committee - Parents	No drink/food before sampling miRNA: - Quick processing - specific storage conditions (-20°C)	Adequate adjustment needed Timing sampling (preferably 1st or 2nd urine) Specific storage conditions - genetic : -80°C → budget - protein: -20°C

+++: suitable for this application; +/-: not ideal, but useful if no other choice, - : not suitable in this context and with selected methods. NA: not applicable

In contrast, the sampling of urine and saliva was straightforward, noninvasive and easily accepted. When using a specific collecting device, the saliva was stored under the required conditions to ensure stable salivary DNA for years, even at room temperature, therefore making it an appropriate source of genetic biomarkers. This has been confirmed by previous studies, confirming that the saliva sample was of such high quality that it could be used as an alternative to blood DNA in epidemiological studies (Nunes et al. 2012; Abraham et al. 2012; Rylander-Rudqvist et al. 2006). The only small drawback in the study was to respect the timing of saliva collection, as no drinking or eating was allowed before sampling. Similarly, the timing was also important for the urine sampling, where the collection of first or second morning urine is preferred. Collecting urine is cheap and large volumes are available. However, as discussed in WP3 Task 3.2 and in Nauwelaerts et al. (2020(d)), it is not the preferred matrix for genotyping assays, yielding inferior results compared to the salivary DNA. Therefore, saliva is the preferred noninvasive matrix for genotyping purposes in large scale epidemiological studies. Only in particular cases using urine could nevertheless still be considered useful. For instance when using older, already existing and valuable biobanks, and for which it is not possible anymore to obtain the saliva of the corresponding subjects. In this case, the use of urine allows to perform genotyping experiments with acceptable results, particularly in context of larger epidemiological studies, where a small genotype calling drop-out is acceptable. Nevertheless, in case urinary DNA is needed for genotyping, specific storage conditions (-80°C freezers) are needed to maximally preserve the DNA, which necessitates a certain budget. Importantly, the choice of the matrix also depends on whether additional biomarkers in other fields (proteomics, epigenetics, etc.) have to be measured simultaneously with the genotypes. Indeed, urine is mostly an important source of protein biomarkers. The main challenge of using urine is the need for correct adjustment for renal handling and diuresis. By identifying the adequate adjustment protein, this hurdle was tackled. This was confirmed by observing a decrease of adjusted urinary CC16, similarly as described in blood (Ingrid A. Laing et al. 2000), for individuals with the CC16 SNP 38AA and 38AG genotype.

Also for the epigenetic investigation, the different biofluids were evaluated. In the context of investigating epigenetic changes related to the air pollution exposure and their effect on respiratory health, urine was found not be a suitable matrix. As urine contains blood cells, epithelial cells,

malignant cells it is difficult to identify the origin of the epigenetic change, which is known to be tissue-specific. This was in contrast with saliva, containing mainly epithelial cells. Therefore, buccal DNA, extracted from saliva, was found to be an interesting source for investigating the methylation patterns, related to changes of air pollution or wheezing and asthma (see WP4 Task 4.1). Additionally, potential miRNA biomarkers in the extracellular fraction of saliva were investigated. However, specific collecting devices and storage conditions are needed for preserving the miRNA and a relatively quick processing (less than 6 months) of the collected samples is requested. In conclusion, when stored properly, and in the context of the impact of air pollution on respiratory health, saliva is an ideal source for both for methylation and miRNA investigation in epidemiological large scale studies, using the methods described above.

Task 5.2 Evaluation of use of MRM-technology

The quantification of potential protein biomarkers was done using the MRM technology. The advantages and bottlenecks of this method are summarized in table VI. This technique is ideal for monitoring the abundance of a set predefined target proteins over many samples, in a high throughput and multiplex set-up. It can detect proteins over a wide dynamic range (4-5 orders of magnitude), is very sensitive and contains a high degree of reproducibility and repeatability. However, this method can only be used with prior knowledge about the target proteins. Additionally, technical or biological challenges, inherent to a protein, can sometimes lead to unsuccessful measurement. Once validated, as was the case in this study, the MRM method is a time and cost efficient way for measuring multiple proteins in a high number of samples. Additionally, the MRM method allowed to quantify the adjustment protein, which previously failed in another study by using immunoassays. Its biochemical property of degradation at acidic pH was not an issue for the quantification with the MRM method (based on measurement of protein fragments), contrarily to the immunoassay.

Table VI: Advantages and bottlenecks of the MRM-technology

	MRM
Advantages	Cost-and time-efficient High throughput Multiplex High sensitivity Dynamic range High repeatability & robustness Works with degraded proteins
Disadvantages	Time consuming method development Prior knowledge of proteins needed Sometimes biological and technical challenges inherent to the protein

Task 5.3 Evaluation of using “one point” study versus “two points” study design

The main goal of this task was to use the measured biomarker data from the field study to compare a “one point” versus a “two points” study design. In a “one point” study, the measurement of parameters and biomarkers in a population exposed to air pollution is compared to a “reference” value measured for a similar population without exposure. The “two points” study uses the same population in a follow-up setting in order to compare parameters and biomarkers measured in exposed and non-exposed conditions.

In the “one point” design of our study, the outcome of 19 children in Brussels (“exposed”) was compared to the outcome of 23 children in Diepenbeek (“reference”). Biomarkers were measured to investigate a difference of exposure both in summer and winter. This resulted in unpaired data from two independent populations residing in different locations in a cross-sectional study set-up. The difference in exposure was due to the difference in location (rural versus urban). In the “two point study”, biomarker changes of the 42 children were measured at two time points, resulting in a set of paired observations due to repeated measurements (i.e. each subject is measured at two different times). The difference in exposure was due to the difference in time point (summer versus winter).

To compare both designs, simulations were used to estimate the power of each design. Performing simulations allowed to compare the two study designs that in practice are non-comparable as they are characterized by a different sample size and effect size. Both designs are simulated to have the same sample size and effect size in order to mimic a situation where the difference in power can only be explained by the difference in study design.

In order to illustrate this, urinary CC16 measurements in both set-ups were compared. For the "one point" study, a hypothetical situation was created assuming 50 subjects within each group (urban and rural area), resulting in a total of 100 subjects and consequently, 100 measurements. A simulation was done using an unpaired t-test and lead to the estimation of a power of 32%.
To mimic the "two point" study, paired data with a given correlation were simulated. A realistic Pearson correlation coefficient of 0.60, based on the actual measurements in the field study on both time points, was used to simulate the paired samples. Using a paired t-test, the estimated power to detect the same effect size as for the "one point" study, was 65%, when assuming a sample size of 50 children with measurements at two time points (i.e. 100 measurements). As the same parameters (means, standard deviations, and number of measurements) were fixed for both the one-point and the two point study, the difference in power (32% vs. 65%, respectively) can be explained by the study design (unpaired samples vs. paired samples, respectively). These results are not surprising. The "two point" study design reduces intersubject variability by the use of paired data obtained from measuring each subject twice. This consistency in test subjects using a repeated measurements design is more powerful at showing a relationship between the independent and dependent variables compared to an independent groups design, if the variables are correlated. The greater the correlation, the higher the power of the test. Consequently, less measurements are often needed in a "two point" design compared to a "one point" study to detect the same effect with a given power. An overview of the strengths and weaknesses of each study design are summarized in Table VII.

Table VII: Comparison of one point versus two points study

"two point" study	**"one point" study**
Paired data	Unpaired data
Need of specific design to target same children at multiple time points (i.e. schools) → limited choice	No need of specific design to target children of same age
Less samples needed (if variables are correlated)	More samples needed
Subjects serve as their own control, decreasing the risk of confounding (less background variation)	Comparison of two independent groups increasing the risk of confounding (more background variation)
Expensive	Quick
Time consuming	Cheap
Risk of sample drop-out	

The "one point" versus "two point" study design was also applied on the feNO measurements in the children of the field study and lead to interesting findings. The "one point" design showed that the measured feNO values, children from Molenbeek (urban) had both in summer and winter significantly higher feNO values compared to the children in Diepenbeek (rural). The "two point" design demonstrated that the feNO values were also higher for all children in the winter compared to the summer. This might suggest that children in the city are more susceptible to show

inflammation in the lungs, due to the surrounding air pollution. Additionally, we could expect that the air pollution (except ozone) is stronger in the winter (Cichowicz, Wielgosiński, and Fetter 2017), thus yielding a stronger effect and feNO outcome. However, no direct correlation was found between the feNO and the air pollution.

From our experience in the field, we can conclude that the "two point" study set-up is preferred, because each child can serve as its own control. Indeed, "pairing" the data provides a way the eliminate the "background" variation, by focusing on the net difference between each member of the pair. This is in contrast with the "one point" study, where the comparison is made between two groups of "exposed" (urban) versus "unexposed" (rural) children. This is especially challenging here, due to the limited air pollution exposure differences that were observed between the different locations, possibly leading to a limited effect size. Comparing the outcome in the same individuals will minimize the possible variations in measured outcome, provoked by other factors than air pollution exposure. Indeed, each individual can be exposed to a multitude of additional confounding factors or diseases (asthma, allergy, ..) which could have an impact on the measured outcome, risking to dilute the effect of air pollution exposure. These confounding factors can be identified through the questionnaires and can be adjusted for, but it makes the comparison of the measured biomarkers between two different populations (in case of "one point" study), with different sets of confounding factors, more challenging. Nevertheless, the "two point "study study-design also shows some weaknesses. Children can move away or wish to no longer participate, which would disrupt a part of the study. These studies are generally more expensive and time-consuming.

Task 5.4 Complete design of future large scale epidemiological study

A retrospective evaluation allowed to integrate all the obtained results to discuss (during a workshop) and evaluate the feasibility of the different sampling/technologies/biomarker approaches for applying them in a large scale epidemiological study. Two schools in two distinct locations were selected and the children, 9-11 years old, were investigated at two different time points. Six months were needed for the set-up of the field study, including the submission to the ethical committee and the selection of the locations, the schools, the targeted study group and the desired time points. The measuring trailer was installed and calibrated and the portable air monitors were selected. Info sessions were organized, the DIC and questionnaires were distributed. The exact date of the field study was set at least one month in advance. On the examination day, all samples and tests were performed within 6 hours with a staff of 6 people, examining around 20 children of one school. Maximally three days later, an identical process was executed in the other school. Subsequent data measurement and analysis of the different biomarkers and of the performed examinations required at least six months to be completed. The whole workflow of phase 1 and phase 2 was conducted fluently and without major problems, yielding interpretable results on three levels of biomarkers of children exposed to different levels of air pollutants.

However, when extrapolating to a larger scale epidemiological study, increasing the number of sites, the number of time points and the number of children, this set-up reveals to be very challenging. It requires supplementary measuring instruments (spirometer and FeNO), more staff and more time, which is practically and budget-wise less feasible. Alternatively, the concept of **auto-sampling** and **auto-measurement** via portable instruments is be proposed (and compared with the the conducted small scale study design in table VIII). After a thorough explanation at the start of the study, urine can be collected from children at multiple time points in the schools, where it should be stored at -20°C. The saliva, needed for genotyping, can be collected only once, whenever convenient, and can be stored at room temperature. The general health parameters (weight, height) can be measured during the yearly examination at the CLB. In case the epigenetic biomarkers need to be investigated, additional saliva sampling on different time points can easily be included. Especially for the methylation experiments, the saliva samples can stay at room temperature, in contrast with the saliva samples intended for miRNA investigation (-20°C storage). As the measurement of the feNO and spirometry are quite time consuming, necessitating trained staff and multiple instruments, based on the results of this project, it is proposed to omit them from the workflow and to focus on the measurement of urinary CC16. This protein is an interesting alternative for feNO, as found in this study, as a positive association between the feNO and urinary

CC16 (see WP3 Task 3.1) has been observed. Both parameters indicate a possible increase of inflammation in the lungs. This adapted workflow allows the substantial increase in number of sites, time points and children. The repeated measures gives additional information on the dynamic effect of air pollution. However, to succeed in this, there is a need of motivated schools, a minimal training of the school staff and additional infrastructure (storage cabinets and freezers).

Table VIII: overview of conducted small scale field study design versus proposed extrapolation to large scale field study design

	Small scale field study design	**Extrapolation to large scale design**
Locations	Two (schools)	Multiple (home?/school?/CLB?)
Sample collection	Urine and saliva sampled by research team	Autosampling of urine and saliva
Respiratory health	Nonivasive biomarkers FeNO and spirometry	Noninvasive biomarkers (as surrogate for FeNO)
Air pollution	Fixed trailer Portable Airbeams	Performant portable air monitors

Another important challenge is the measurement of air pollution on a large scale. Ideally, the use of portable monitors is preferred, as it gives information on the dynamic effect of air pollution, about in-and outside pollution and about the differences in different micro-environments. However, the Airbeams used in this study, are suitable for qualitative measurement of PM and sensibilizing the population, but less for quantitative studies. Indeed, when investigating the impact of an exposure to a certain PM level, precise measurements are needed. Further investigation of more performant alternatives is required.

All of these findings were presented on the 8th of November 2019, during an interactive workshop that took place at Eurostation II, as part of the closure of the project. The workshop was organised with the working group “Heat and ozone peaks”, coordinated by Irceline. During this interactive workshop the final findings of the project were communicated. The importance of biomarkers for monitoring the effect of air pollution and its impact on health was put into context. This lead to a discussion between the partners and several organisations (various cabinets, Federal Public Service of Public Health, Environment Brussels, Royal Meteorological Institute, Ircel-Celine) and allowed transmission of expertise between experts in the field. The feasibility of the developed strategy was evaluated in order to provide the missing scientific knowledge, necessary for the preparation, implementation and evaluation of federal policies/strategies for conducting future large scale epidemiological studies about the efficient biomonitoring of the respiratory health in children impacted by the air pollution. The workshop was concluded with a discussion session, initiated by a series of multiple choice questions. The use of an interactive poll web application (Slido) allowed the audience to respond immediately and anonymously, leading to a group discussion. The feasibility of conducting this type of large scale epidemiological integrated studies and related ethical concepts were discussed. Several bottlenecks encountered in the study were pointed out and constructive feedback was given regarding the future set-up of this type of study at large scale. Amongst other topics, the choice of conducting this study in collaborating schools was questioned, as this requires efforts from the teachers which exceed their typical responsibilities. Schools are often solicited to participate in all types of studies, but are they the appropriate place and is it the teacher’s role to invest time in this? If not in schools, how would the children be targeted and followed-up over several time points? Autosampling at school or at home was proposed but other

alternatives can be investigated as well. If the study is spread over a number of years and depending on the number of required repeated measurements, urine and saliva could also be collected by the CLB, during their yearly consultation. This would only implicate the collaboration of the CLB, with limited additional work, as most of the examinations (weight, height, urine sampling) are done anyway. Another option could be the collaboration with boarding schools, gathering all children in the same location during but also after school. Ethical aspects of sharing anonymous results and whole genome sequencing were brought up and lead to an interesting debate. Another discussion point was the pertinence of this type of conducted study. It was suggested that there is already enough evidence about the impact of air pollution on the respiratory health of children. The focus should more be on informing the policy makers and providing them all scientific knowledge necessary for the preparation, implementation and evaluation of federal policies. Finally, the hypothetical expansion to other types of studies using the methodology from this study, was also elaborated during the discussion at the workshop. Additional modifications would be required to target other vulnerable groups like elderly, pregnant women and patients with heart, lung or other diseases.

Conclusions WP5

The main objective of the whole PMOlluGENix-V2 project was to deliver evidence-based results in order to design a future large scale epidemiological study, here specifically investigating the effect of air pollution on the respiratory health of children by the measurement of noninvasive biomarkers. To achieve this goal, a small scale field study was conducted, allowing to go through the whole workflow of designing and preparing the study, conducting the actual field work, measuring the biomarkers and performing the integrated analysis. Along all the different steps of the workflow, bottlenecks were identified to assess the feasibility of this type of study in a larger scale set-up. A distinction can be made between a proposed design for any large scale study and the points proposed specifically in the context of an epidemiological study related to air pollution.

The measurement of biomarkers in an integrative way (protein, genetic and epigenetic level) is valuable for any large scale study, as each of these separate levels does not capture the entire biological complexity. Combining them provides a more comprehensive and complementary view of biology and disease. Blood samples are considered to be the gold standard source for many biomarkers, but were not an option in this field study, due to practical and ethical reasons, especially when examining children. Urine and saliva were found to be valuable and complementary noninvasive alternatives for biomarker investigation at different levels. Saliva was the perfect surrogate of blood for genotyping. Urine yielded inferior results compared to saliva for genotyping, but can nevertheless be considered useful, particularly in case no other samples are available. Urine is especially useful as an important source of protein biomarkers and the MRM method allowed the successful and efficient quantification of multiple protein biomarkers, with proper adjustment. Saliva and urine samples can also be interesting sources of epigenetic biomarkers, taking into account that the choice the epigenetic change, which is tissue-specific, depends on the investigated subject (i.e. air pollution, cancer, etc).These findings lead to the proposal of combined sampling of saliva and urine as alternative to blood, resulting in an integrated analysis of biomarkers at different levels and offering complementary information. Additionally, autosampling of these noninvasive samples can be considered, in order to reduce the staff and time needed. Targeting children through schools seems the most straightforward way to approach them. However, this requires working with committed schools and motivated teachers. Schools are often solicited to participate in multiple studies. Taking into account all additional administration and organization, these type of studies require efforts from the teachers that exceed their typical responsibilities. Therefore, other alternatives should also be envisaged, such as sampling at home, collaborating exclusively with the CLB during the annual examination or working with boarding schools. Comparing the “one point” versus the “two point” approach gave an additional insight on how to design a large scale study. Although both set-ups have their advantages and their weaknesses, the “two point” study set-up is preferred, as each individual can serve as its own reference. The pairing of the data provides a way the eliminate the “background” variation, and shows a higher power than an unpaired “one point” study. Consequently, less measurements are

often needed in a “two-point” design compared to a “one-point” study to show the same significance. Nevertheless, the “two point “study study-design also shows some weaknesses. It can be more time consuming and expensive. Furthermore, a drop-out at the level of the individuals can diminish the strength of the study.

In the specific context of this study related to air pollution, genetic and protein biomarkers related to respiratory health and effect of air pollution exposure were successfully analysed in resp. saliva and urine. Saliva was also found to be an interesting source of epigenetic biomarkers, and methods were successfully tested for the detection of relevant miRNAs and methylation biomarkers related to the effect of air pollution or respiratory health. All these results lead to an integrated analysis of biomarkers at different levels, cross linking the effect of air pollution at different levels of the body. Additionally, the analysis of these specific urinary and salivary biomarkers was proposed as an alternative to the lung parameter measurements. These time consuming and challenging tests in children (especially in the spirometry test) could then be omitted and replaced by the biomarker measurement. Ideally, the air pollution exposure is measured by combining data from a stationary validated measuring trailer with personal portable air monitors, allowing the dynamic monitoring of personalized exposure to air pollution inside and outside. However, a measuring campaign with a trailer is costly and not always possible due to practical reasons. Moreover, air pollution in micro-environments are not always taken into account when using trailors. Alternatively, additional relevant information can be collected using spatial temporal interpolation methods, modelling the daily exposure levels of PM. The use of the portable Airbeams can give a qualitative insight on differences of PM levels in and outside the schools and between school sites. However, due to the limited technology, they are not suitable for quantitative measurements. Alternative commercially more powerful portable systems should be investigated in the future. Evidently, the use of portable air pollution monitors are a promising way to link personal exposure to measurement of biomarkers collected through noninvasive samples.

Performing this small scale field study complemented with the investigation of an existing biobank, showed that successful measurement of noninvasive protein and genetic biomarkers was found to be correlated already with low levels (below the WHO thresholds) of air pollution and/or respiratory health.

Scientific valorisation

- Oral Presentation: ”PMOlluGENix-V2: Strategy to evaluate health risks of air pollution episodes in vulnerable individuals”
    - Name of the congress/meeting/event: Workshop – PMOLLUGENIX-V2
    - Date: 08/11/2019
    - Location: Eurostation, Brussels , in collaboration with working group “Heat and ozone peaks”(coordinator: Irceline)
    - Authors: Sarah Nauwelaerts, Koen De Cremer, Jordy Vercauteren, Natalia Bustos Sierra, Sigrid De Keersmaecker, Nancy Roosens

- Poster: “Strategy to Evaluate Health Risks of Short-term Exposure of Air Pollution in Vulnerable Individuals”
    - Name of the congress/meeting/event: Etats Generaux de l’Air de Bruxelles Resistance is in the Air: Citizens, science and air pollution: International interdisciplinary symposium
    - Date: 25-26/04/2019
    - Location: Brussels, Belgium)
    - Authors: Sarah Nauwelaerts, Koen De Cremer, Alfred Bernard, Natalia Bustos Sierra, Tim Nawrot, Jordy Vercauteren, Christophe Stroobants, Nancy Roosens*, Sigrid C.J. De Keersmaecker* (* equal contribution)

- Oral Presentation: “Strategy to Evaluate Health Risks of Short-term Exposure of Air Pollution in Vulnerable Individuals”
    - Name of the congress/meeting/event: ICEPPH 20th International Conference on Environmental Pollution and Public Health
    - Date: 17/09/2018
    - Location: Hotel NH Roma Villa Carpegna, Rome, Italy
    - Authors: Sarah Nauwelaerts, Koen De Cremer, Alfred Bernard, Meredith Verlooy, Kristel Heremans, Natalia Bustos Sierra, Katrien Tersago, Tim Nawrot, Jordy Vercauteren, Christophe Stroobants, Sigrid C.J. De Keersmaecker*, Nancy Roosens* (* equal contribution)

- Peer-reviewed publications can be found in 5. Publications.

Scientific valorisation made accessible to the general public

- *Planned: Oral presentation: session explaining the general results of the field study in both schools in Molenbeek and Diepenbeek*
    - Date: Spring of 2020
    - Location: Vier Winden Basisschool (Molenbeek) and Basisschool Rooierheide (Diepenbeek)
    - Speaker: Sarah Nauwelaerts

- Oral presentation Straffe Koffie: info session explaining the goal of the field study
    - Name of the congress/meeting/event: Straffe Koffie moment
    - Date: 20/06/2018
    - Location: Vier Winden Basisschoool (Molenbeek)
    - Speaker: Sarah Nauwelaerts

- Oral presentation: “Development of noninvasive biomarkers to monitor respiratory health and to evaluate health risks of air pollution episodes in young children”
    - Name of the congress/meeting/event: Scientific Lunch
    - Date: 18/10/2017
    - Location: Sciensano (Elsene)
    - Speaker: Sarah Nauwelaerts

4. PERSPECTIVES

Based on this study the design of a workflow to investigate the effect of air pollution on the respiratory health of children in future large-scale settings was proposed. This design includes the advantages of the use of noninvasive samples, such as urine and saliva, to measure biomarkers, which are valuable tools in the context of large scale epidemiological studies, where blood collection, especially in children, is not accepted. Moreover, by integrating the investigation at different levels, i.e. proteinaceous, genetic and epigenetic, the design optimizes the use of potential health biomarkers by giving an insight on inter-related or complementary relationships and roles of various types of molecules in cells of an organism.

Children were defined as a particular vulnerable population in the context of air pollution and respiratory health. However, this target group is not that easy to include in field studies. In this study, the examinations took place in schools in collaboration with the CLB. Schools are often chosen to conduct this type of study because of practical reasons. However, as experienced during this study, the more motivated and committed the schools are, the more efficiently the study can be conducted. Therefore, for further similar studies, other set-ups should be considered, as schools are often, maybe already too often, solicited for all types of studies, requesting additional time and efforts from them, which could have a negative impact on their motivation to participate. This could imply to select children to participate which are not necessarily grouped at one specific place (like was the case in the school). Autosampling of the biofluids is therefore proposed, in combination with self-monitoring of air pollution. This will also allow to have a dynamic view on the self-exposure of the child, not only in school but also at home and during transport. Therefore the use of low-cost, mobile, sensitive and specific air pollution monitors, capable of monitoring in-and outside air pollution in a continuous way should be used. Despite the availability of a number of commercial monitors, they often show a size- and sensitivity trade-off due to their limited technologies. Further investigation (or even future development of this technology) is needed to select the most suitable instruments.

In this study, genetic biomarkers were integrated into the interpretation of how an environmental exposure (i.e. air pollution) affects the population (i.e. children) health (i.e. respiratory health). Therefore, this study fits within the philosophy of public health genomics. However, the focus of this feasibility study was limited (because of budget and time restrictions) to the investigation of two genetic biomarkers (SNPs) that were known to be related to respiratory health. As the use of new high throughput techniques (e.g. sequencing technologies) permits the simultaneous study of many thousands of SNPs, future large scale studies should aim to investigate a larger spectrum of genetic biomarkers. Alternatively, given the ongoing public debate in the willingness of getting your genome sequenced (even already at birth), this information might already be available upfront the study. It will then come down to ethical issues, whether these data will be available to be used. The implementation of genotyping into public health policies and services could nevertheless contribute to the population health by giving rise to personalized medicine but also to precision public health. Therefore, these kinds of studies, involving genetic biomarkers, should be encouraged in the future, and this not only for the exposure to air pollution. These studies will allow to find new trends and correlations involving complex interactions in relation to diseases, exposures, susceptibility and health outcomes at population level.(El-Khoury et al. 2016; Khoury 2015) Identifying the impact of genomic variants in specific groups and to target those with specific genomic risks, with tailor-made and relevant interventions and lifestyle modifications, will result in a more cost-efficient and effective monitoring and in better prevention strategies (The Human Genomics Strategy Group 2012; Burton, Jackson, and Abubakar 2014). It is therefore the role of the government and policy makers to ensure and allow the effective implementation of the technologies used to generate the genomic based knowledge and integrated study approach into the health system. Conduction of this type of feasibility studies should facilitate their decision making.

The concept of public health genomics has started to be taken into account in the Belgian public health policy. Indeed, every four years, the national health information survey (HIS) is conducted, involving ~10.000 persons (age 18-64) living in Belgium, with the aim to get a general overview of the public health status in our country. In the past, this study was only based on a questionnaire

concerning the health status, lifestyle, health care use and socio-demographic characteristics. However, the last national health survey was extended with a physical examination and blood sample collection (Health Examination Survey (HES)). The final goal is to link the HES data with the information obtained from the HIS and to collect information among a representative sample of the total population by the collection of objective health measures. Part of the collection will also be analyzed for their genotype, with the aim to link specific genetic biomarkers, with specific health status information. However, only blood is collected in this study, which involved a large budget and time investment. Based on the PMOlluGENix-V2results, it can be proposed that future large scale studies of this type should include (additional) noninvasive samples and biomarker measurements. This might also increase the participation rate and allow to generate a more complete and integrated view on the population's health, and maybe not only of a the general target population (as is now the case for HES), but also of more 'vulnerable' target groups (e.g. children, elderly) for which blood measurements are even a more technical or ethical issue. Indeed, similarly to the PMOlluGENix-V2 study, there is a need to implement these types of large scale investigations on specific, vulnerable or at-risk groups, such as children but also elderly and pregnant women. In a far but ideal future, it can even be considered to couple the HES to specific exposure measurements (e.g. based on portable systems), to arrive to an integrated, evidence-based view on the population health status linked to specific exposures. This will be needed to take the appropriate actions by the policy makers to support a pro-active public health policy.

However, already at smaller scale, but by conducting an integrative study, using noninvasive biomarkers, this study showed that even levels of PM2.5 below the WHO guidelines can impact the respiratory health of children. Therefore, these evidence-based data need to be used and to be taken into account when policy makers enact the law establishing the goals for air quality, targeting lower thresholds than applicable now.

5. PUBLICATIONS

Accepted publications

- S.J.D Nauwelaerts, D. Van Geel, M. Delvoye, K. De Cremer, A. Bernard, N.H.C. Roosens, and S.C.J. De Keersmaecker. Selection of a Noninvasive Source of Human DNA Envisaging Genotyping Assays in Epidemiological Studies: Urine or Saliva? Journal of Biomolecular Techniques 2020(d). 31(1).

Planned publications

- Nauwelaerts et al., 2020(b) Feasibility study of measuring the effects of air pollution on the respiratory health of children in future large scale studies *(provisionary title) - ongoing*

- Nauwelaerts et al., 2020(c) Urinary CC16 as a noninvasive biomarker of effect and exposure to low levels of PM *(provisionary title) – ongoing*

Abstracts

- S. Nauwelaerts, K. De Cremer, A. Bernard, M. Verlooy, K. Heremans, N.B. Sierra, K. Tersago, T. Nawrot, J. Vercauteren, C. Stroobants, S.C.J. De Keersmaecker, and N.H.C. Roosens. Strategy to Evaluate Health Risks of Short-term Exposure of Air Pollution in Vulnerable Individuals. WASET: 20th International Conference on Environmental Pollution and Public Health; 09/17-18/2019; Rome/Italy.

- S. Nauwelaerts, K. De Cremer, A. Bernard, N. Bustos Sierra, T. Nawrot, J. Vercauteren, C. Stroobants, N.H.C. Roosens, and S.C.J. De Keersmaecker. Strategy to Evaluate Health Risks of Short-term Exposure of Air Pollution in Vulnerable Individuals. Brusselair: Etats Généraux de l'Air de Bruxelles, Resistance is in the Air: Citizens, science and air pollution. International interdisciplinary symposium; 04/25-27/2019; Brussels/Belgium

6. ACKNOWLEDGEMENTS

The authors would like to thank the Belgian Federal Science Policy Office (BELSPO) and Sciensano who funded this research through respectively the project PMOLLUGENIX-V2 (BELSPO, BR/165/PI/ PMOLLUGENIX) and the research project Respikid (Sciensano PJ/RP). The authors would also like to thank the colleagues Maud Delvoye, Mathieu Gand, Dirk Van Geel and Els Vandermassen from Transversal Activities in Applied Genomics (TAG) at Sciensano for their help and implication in the preparation of the field study; Berta Tenas Ureña from the University of Vic for helping with the biomarker measurements during her internship in TAG at Sciensano and the Sequencing platform of TAG for running the sequencing reactions. They are grateful for the statistical analysis performed by Nina Van Goethem from the department of Public Health and Genome at Sciensano. Finally, they would like to thank all the participating schools and the CLB for their enthusiastic collaboration during the field study. This included the following schools: De Notelaar (Boutersem), de Vier Winden Basisschool (Molenbeek), Basisschool Rooierheide (Diepenbeek). The participating CLB and the medical staff present were: CLB Go Leuven-Tienen, CLB Pieter Breughel (Wenda Van Engeland, Ann Meeus) and Vrij CLB Limburg afdeling Hasselt (Sigrid Bauters).

1. REFERENCES

Abraham, Jean E, Mel J Maranian, Inmaculada Spiteri, Roslin Russell, Susan Ingle, Craig Luccarini, Helena M Earl, Paul P D Pharoah, Alison M Dunning, and Carlos Caldas. 2012. “Saliva Samples Are a Viable Alternative to Blood Samples as a Source of DNA for High Throughput Genotyping.” *BMC Medical Genomics* 5 (19): 1–6.

Acevedo, Nathalie, Lovisa E. Reinius, Dario Greco, Anna Gref, Christina Orsmark-Pietras, Helena Persson, Göran Pershagen, et al. 2015. “Risk of Childhood Asthma Is Associated with CpG-Site Polymorphisms, Regional DNA Methylation and MRNA Levels at the GSDMB/ORMDL3 Locus.” *Human Molecular Genetics* 24 (3): 875–90. https://doi.org/10.1093/hmg/ddu479.

Akhabir, Loubna, and Andrew J. Sandford. 2011. “Genome-Wide Association Studies for Discovery of Genes Involved in Asthma.” *Respirology* 16 (3): 396–406. https://doi.org/10.1111/j.1440-1843.2011.01939.x.

Akhtar, Rais, and Cosimo Palagiano, eds. 2018. *Climate Change and Air Pollution*. 1st ed. Springer Climate. Menai, Asutralia: Springer International Publishing. https://doi.org/10.1007/978-3-319-61346-8.

Annesi-Maesano, Isabella, Dan Norback, Jan Zielinski, Alfred Bernard, Cristina Gratziou, Torben Sigsgaard, Piersante Sestini, and Giovanni Viegi. 2013. “Geriatric Study in Europe on Health Effects of Air Quality in Nursing Homes (GERIE Study) Profile: Objectives, Study Protocol and Descriptive Data.” *Multidisciplinary Respiratory Medicine* 8 (1): 1–10. https://doi.org/10.1186/2049-6958-8-71.

Baccarelli, Andrea, Franca Rusconi, Valentina Bollati, Dolores Catelan, Lifang Hou, Fabio Barbone, Pier Alberto Bertazzi, and Annibale Biggeri. 2012. “Nasal Cell DNA Methylation, Inflammation, Lung Function and Wheezing in Children with Asthma.” *Epigenomics* 4 (1): 91–100. https://doi.org/10.2217/epi.11.106.Nasal.

Barraza-Villarreal, Albino, Jordi Sunyer, Leticia Hernandez-Cadena, Maria Consuelo Escamilla-Nunñez, Juan Jose Sienra-Monge, Matiana Ramírez-Aguilar, Marlene Cortez-Lugo, et al. 2008. “Air Pollution, Airway Inflammation, and Lung Function in a Cohort Study of Mexico City Schoolchildren.” *Environmental Health Perspectives* 116 (6): 832–38. https://doi.org/10.1289/ehp.10926.

Benson, M., M. Fransson, T. Martinsson, Å T. Naluai, R. Uddman, and L. O. Cardell. 2007. “Inverse Relation between Nasal Fluid Clara Cell Protein 16 Levels and Symptoms and Signs of Rhinitis in Allergen-Challenged Patients with Intermittent Allergic Rhinitis.” *Allergy: European Journal of Allergy and Clinical Immunology* 62 (2): 178–83. https://doi.org/10.1111/j.1398-9995.2006.01264.x.

Bernard, Alfred, Marc Nickmilder, and Xavier Dumont. 2015. “Chlorinated Pool Attendance, Airway

Epithelium Defects and the Risks of Allergic Diseases in Adolescents: Interrelationships Revealed by Circulating Biomarkers." *Environmental Research* 140: 119–26. https://doi.org/10.1016/j.envres.2015.03.034.

Bernard, Alfred, Antonia Sardella, Catherine Voisin, and Xavier Dumont. 2017. "Nasal Epithelium Injury by Chlorination Products and Other Stressors Predicts Persistent Sensitization to Aeroallergens in Young Schoolchildren." *Environmental Research* 158 (June): 145–52. https://doi.org/10.1016/j.envres.2017.06.009.

Broeckaert, F., and A. Bernard. 2000. "Clara Cell Secretory Protein (CC16): Characteristics and Perspectives as Lung Peripheral Biomarker." *Clinical and Experimental Allergy* 30 (4): 469–75. https://doi.org/10.1046/j.1365-2222.2000.00760.x.

Brunst, Kelly J, D Ph, Yuet-kin Leung, Patrick H Ryan, and K Gurjit. 2013. "FOXP3 Hypermethylation Is Associated with Diesel Exhaust Exposure and Risk for Childhood Asthma Kelly." *J Allergy Clin Immunol.* 131 (2): 592–94. https://doi.org/10.1016/j.jaci.2012.10.042.FOXP3.

Burton, H, C Jackson, and I Abubakar. 2014. "The Impact of Genomics on Public Health Practice." *British Medical Bulletin* 0 (0): 1–10.

Bustos Sierra, Natalia, and Tommi Aikainen. 2017. "Rapport over de Surveillance van de Mortaliteit Door Alle Oorzaken in Belgie in de Zomer van 2017." Vol. 20.

Candelaria, Pierre V., Vibeke Backer, Ingrid A. Laing, Celeste Porsbjerg, Steen Nepper-Christensen, Nick De Klerk, Jack Goldblatt, and Peter N. Le Souëf. 2005. "Association between Asthma-Related Phenotypes and the CC16 A38G Polymorphism in an Unselected Population of Young Adult Danes." *Immunogenetics* 57 (1–2): 25–32.

Chen, Yan, Gary W.K. Wong, and Jing Li. 2016. "Environmental Exposure and Genetic Predisposition as Risk Factors for Asthma in China." *Allergy, Asthma and Immunology Research* 8 (2): 92–100. https://doi.org/10.4168/aair.2016.8.2.92.

Choi, You Jung, Sun Hwa Kim, Si Hyuck Kang, Sun Young Kim, Ok Jin Kim, Chang Hwan Yoon, Hae Young Lee, Tae Jin Youn, In Ho Chae, and Cheol Ho Kim. 2019. "Short-Term Effects of Air Pollution on Blood Pressure." *Scientific Reports* 9 (1): 1–8. https://doi.org/10.1038/s41598-019-56413-y.

Cichowicz, Robert, Grzegorz Wielgosiński, and Wojciech Fetter. 2017. "Dispersion of Atmospheric Air Pollution in Summer and Winter Season." *Environmental Monitoring and Assessment* 189 (12). https://doi.org/10.1007/s10661-017-6319-2.

Collart, Philippe, Dominique Dubourg, Alain Levêque, Natalia Bustos Sierra, and Yves Coppieters. 2018. "Short-Term Effects of Nitrogen Dioxide on Hospital Admissions for Cardiovascular Disease in Wallonia, Belgium." *International Journal of Cardiology* 255 (2): 231–36. https://doi.org/10.1016/j.ijcard.2017.12.058.

Ding, Shengguang, Haitao Huang, Yiming Xu, Hao Zhu, and Chongjun Zhong. 2017. "MiR-222 in Cardiovascular Diseases: Physiology and Pathology." *BioMed Research International* 2017 (Figure 1). https://doi.org/10.1155/2017/4962426.

Dweik, Raed A, Peter B Boggs, Serpil C Erzurum, Charles G Irvin, Margaret W Leigh, Jon O Lundberg, Anna-Carin Olin, Alan L Plummer, and D Robin Taylor. 2011. "American Thoracic Society Documents An Official ATS Clinical Practice Guideline: Interpretation of Exhaled Nitric Oxide Levels (FE NO) for Clinical Applications Executive Summary Introduction Methods Committee Composition, Meetings, and Document Preparat." *Am J Respir Crit Care Med* 184: 602–15. https://doi.org/10.1164/rccm.912011ST.

El-Khoury, Victoria, Sandrine Pierson, Tony Kaoma, François Bernardin, and Guy Berchem. 2016. "Assessing Cellular and Circulating MiRNA Recovery: The Impact of the RNA Isolation Method and the Quantity of Input Material." *Scientific Reports* 6 (December 2015): 1–14. https://doi.org/10.1038/srep19529.

European Environment Agency. 2019. "Air Quality in Europe - Report." https://doi.org/10.2800/62459.

Favé, Marie Julie, Fabien C. Lamaze, David Soave, Alan Hodgkinson, Héloïse Gauvin, Vanessa Bruat, Jean Christophe Grenier, et al. 2018. "Gene-by-Environment Interactions in Urban Populations Modulate Risk Phenotypes." *Nature Communications* 9 (1). https://doi.org/10.1038/s41467-018-03202-2.

Gai, Chiara, Francesco Camussi, Roberto Broccoletti, Alessio Gambino, Marco Cabras, Luca Molinaro, Stefano Carossa, Giovanni Camussi, and Paolo G. Arduino. 2018. "Salivary

Extracellular Vesicle-Associated MiRNAs as Potential Biomarkers in Oral Squamous Cell Carcinoma." *BMC Cancer* 18 (1): 1–11. https://doi.org/10.1186/s12885-018-4364-z.

Guarnieri, Michael, and John R Balmes. 2014. "Outdoor Air Pollution and Asthma." *The Lancet* 383 (9928): 1581–92. https://doi.org/10.1016/S0140-6736(14)60617-6.

Guerra, Stefano, Marilyn Halonen, Monica M Vasquez, Amber Spangenberg, Debra A. Stern, Wayne J. Morgan, Anne L. Wright, et al. 2015. "The Relation of Circulating CC16 to Lung Function Growth, Decline, and Development of COPD across the Lifespan." *Lancet Respir Med.* 3 (8): 613–20.

Halatek, Tadeusz, Halina Sinczuk-Walczak, Beata Janasik, Malgorzata Trzcinka-Ochocka, Renata Winnicka, and Wojciech Wasowicz. 2014. "Health Effects and Arsenic Species in Urine of Copper Smelter Workers." *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering* 49 (7): 787–97. https://doi.org/10.1080/10934529.2014.882207.

Hermans, Cedric, and Alfred Bernard. 1999. "Lung Epithelium-Specific Proteins. Characteristics and Potential Applications as Markers." *Am J Respir Crit Care Med* 159: 646–78.

Hong, Ji, and Gurjit K. Khurana Hershey. 2012. "Genetic and Epigenetic Influence on the Response to Environmental Particulate Matter." *J Allergy Clin Immunol.* 129 (1): 33–41. https://doi.org/10.1038/jid.2014.371.

Hong, Kyungmo, Hyun-Kyung Kim, Mi-Jin Kang, Ho-Sung Yu, Byoung-Ju Kim, Young Ho Jung, Jinho Yu, and Soo-Jong Hong. 2013. "CC10 A38G Polymorphism (Rs3741240) Is Associated with Asthma Susceptibility and Bronchial Hyperresponsiveness Mediated by the Eosinophilic Inflammation in Korean Children." *Journal of Allergy and Clinical Immunology* 131 (2): AB205.

Hou, Tianfang, Jiping Liao, Cheng Zhang, Chao Sun, Xueying Li, and Guangfa Wang. 2018. "Elevated Expression of MiR-146, MiR-139 and MiR-340 Involved in Regulating Th1/Th2 Balance with Acute Exposure of Fine Particulate Matter in Mice." *International Immunopharmacology* 54 (November 2017): 68–77. https://doi.org/10.1016/j.intimp.2017.10.003.

IRCEL-CELINE. 2017. "Rapport Annuel 2017 de La Qualité de l'air En Belgique."

Jiang, Xu Qin, Xiao Dong Mei, and Di Feng. 2016. "Air Pollution and Chronic Airway Diseases: What Should People Know and Do?" *Journal of Thoracic Disease* 8 (1): E31–40. https://doi.org/10.3978/j.issn.2072-1439.2015.11.50.

Johnson, Jeremy D., and Wesley M. Theurer. 2014. "A Stepwise Approach to the Interpretation of Pulmonary Function Tests." *American Family Physician* 89 (5): 359–66.

Khoury, Muin J. 2015. "Planning for the Future of Epidemiology in the Era of Big Data and Precision Medicine." *American Journal of Epidemiology* 182 (12): 977–79. https://doi.org/10.1093/aje/kwv228.

Kim, Su Kang, Hosik Seok, Hae Jeong Park, Kyuup Han, Sang Wook Kang, Ju Yeon Ban, Hee-Jae Jung, et al. 2017. "Association between Secretoglobin Family 3A Member 2 (SCGB3A2) Gene Polymorphisms and Asthma in a Korean Population." *Medical Science Monitor : International Medical Journal of Experimental and Clinical Research* 23: 1880–85.

Krauskopf, Julian, Karin van Veldhoven, Marc Chadeau-Hyam, Roel Vermeulen, Glòria Carrasco-Turigas, Mark Nieuwenhuijsen, Paolo Vineis, Theo M. de Kok, and Jos C. Kleinjans. 2019. "Short-Term Exposure to Traffic-Related Air Pollution Reveals a Compound-Specific Circulating MiRNA Profile Indicating Multiple Disease Risks." *Environment International* 128 (April): 193–200. https://doi.org/10.1016/j.envint.2019.04.063.

Ku, M. S., H. L. Sun, K. H. Lu, J. N. Sheu, H. S. Lee, S. F. Yang, and K. H. Lue. 2011. "The CC16 A38G Polymorphism Is Associated with the Development of Asthma in Children with Allergic Rhinitis." *Clinical and Experimental Allergy* 41 (6): 794–800. https://doi.org/10.1111/j.1365-2222.2010.03679.x.

Laing, I. A., N. H. De Klerk, S. W. Turner, P. K. Judge, C. M. Hayden, L. I. Landau, J. Goldblatt, and P. N. Le Souëf. 2009. "Cross-Sectional and Longitudinal Association of the Secretoglobin 1A1 Gene A38G Polymorphism with Asthma Phenotype in the Perth Infant Asthma Follow-up Cohort." *Clinical and Experimental Allergy* 39 (1): 62–71. https://doi.org/10.1111/j.1365-2222.2008.03102.x.

Laing, Ingrid A., Cedric Hermans, Alfred Bernard, Paul R. Burton, Jack Goldblatt, and Peter N. Le Souëf. 2000. "Association between Plasma CC16 Levels, the A38G Polymorphism, and Asthma." *American Journal of Respiratory and Critical Care Medicine* 161 (1): 124–27.

https://doi.org/10.1164/ajrccm.161.1.9904073.

Langie, S.A.S., K.S. Szic, K. Declerck, S. Traen, G. Koppen, G. Van Camp, G. Schoeters, W.V. Berghe, and P. De Boever. 2016. "Whole-Genome Saliva and Blood DNA Methylation Profiling in Individuals with a Respiratory Allergy." *PLoS ONE* 11 (3): 1–17. https://doi.org/10.1371/journal.pone.0151109.

Li, Jinhui, Shengzhi Sun, Robert Tang, Hong Qiu, Qingyuan Huang, Tonya G. Mason, and Linwei Tian. 2016. "Major Air Pollutants and Risk of COPD Exacerbations: A Systematic Review and Meta-Analysis." *International Journal of COPD* 11 (1): 3079–91. https://doi.org/10.2147/COPD.S122282.

Lindahl, Mats, Kristina Irander, Christer Tagesson, and Bengt Ståhlbom. 2004. "Nasal Lavage Fluid and Proteomics as Means to Identify the Effects of the Irritating Epoxy Chemical Dimethylbenzylamine." *Biomarkers* 9 (1): 56–70. https://doi.org/10.1080/13547500410001662005.

Lomas, D. A., E. K. Silverman, L. D. Edwards, B. E. Miller, H. O. Coxson, and R. Tal-Singer. 2008. "Evaluation of Serum CC-16 as a Biomarker for COPD in the ECLIPSE Cohort." *Thorax* 63 (12): 1058–63. https://doi.org/10.1136/thx.2008.102574.

Lucia, Claudio De, Klara Komici, Giulia Borghetti, Grazia Daniela Femminella, Leonardo Bencivenga, Alessandro Cannavo, Graziamaria Corbi, et al. 2017. "MicroRNA in Cardiovascular Aging and Age-Related Cardiovascular Diseases." *Frontiers of Medicine* 4 (June). https://doi.org/10.3389/fmed.2017.00074.

Mandon, Julien, Marieann Högman, Peter J. F. M. Merkus, Jan van Amsterdam, Frans J. M. Harren, and Simona M. Cristescu. 2012. "Exhaled Nitric Oxide Monitoring by Quantum Cascade Laser: Comparison with Chemiluminescent and Electrochemical Sensors." *Journal of Biomedical Optics* 17 (1): 017003. https://doi.org/10.1117/1.jbo.17.1.017003.

Mansur, A. H., A. A. Fryer, M. Hepple, R. C. Strange, and M. A. Spiteri. 2002. "An Association Study between the Clara Cell Secretory Protein CC16 A38G Polymorphism and Asthma Phenotypes." *Clinical and Experimental Allergy* 32 (7): 994–99. https://doi.org/10.1046/j.1365-2222.2002.01426.x.

Michels, Nathalie, Barbara Vanaelst, Krishna Vyncke, Isabelle Sioen, Inge Huybrechts, Tineke De Vriendt, and Stefaan De Henauw. 2012. "Children's Body Composition and Stress – the ChiBS Study: Aims, Design, Methods, Population and Participation Characteristics." *Archives of Public Health* 70 (1): 1. https://doi.org/10.1186/0778-7367-70-17.

Motta, Valeria, Laura Angelici, Francesco Nordio, Valentina Bollati, Serena Fossati, Fabio Frascati, Valentina Tinaglia, Pier Alberto Bertazzi, Cristina Battaglia, and Andrea A. Baccarelli. 2013. "Integrative Analysis of Mirna and Inflammatory Gene Expression after Acute Particulate Matter Exposure." *Toxicological Sciences* 132 (2): 307–16. https://doi.org/10.1093/toxsci/kft013.

Mukherjee, Anil B., Zhongjian Zhang, and Beverly S. Chilton. 2007. "Uteroglobin: A Steroid-Inducible Immunomodulatory Protein That Founded the Secretoglobin Superfamily." *Endocrine Reviews* 28 (7): 707–25.

Nadeau, Kari, Cameron McDonald-Hyman, Elizabeth M. Noth, Boriana Pratt, S. Katharine Hammond, John Balmes, and Ira Tager. 2010. "Ambient Air Pollution Impairs Regulatory T-Cell Function in Asthma." *Journal of Allergy and Clinical Immunology* 126 (4). https://doi.org/10.1016/j.jaci.2010.08.008.

Nauwelaerts et al., 2020(a): Method development and validation for the quantification of urinary protein biomarkers related to respiratory health in children *(provisionary title) - ongoing*

Nauwelaerts et al., 2020(b): Feasibility study of measuring the effects of air pollution on the respiratory health of children in future large scale studies *(provisionary title) - ongoing*

Nauwelaerts et al., 2020(c): Urinary CC16 as a noninvasive biomarker of effect and exposure to low levels of PM *(provisionary title) – ongoing*

Nauwelaerts, Sarah J D, Dirk Van Geel, Maud Delvoye, Koen De Cremer, Alfred Bernard, Nancy H C Roosens, and Sigrid C J De Keersmaecker. 2020(d). "Selection of a Noninvasive Source of Human DNA Envisaging Genotyping Assays in Epidemiological Studies : Urine or Saliva ?" *Journal of Biomolecular Techniques* 1 (31): 1–9. https://doi.org/10.7171/jbt.20-3101-004.

Niimi, T, M Munakata, C L Keck-Waggoner, N C Popescu, R C Levitt, M Hisada, and S Kimura. 2002. "A Polymorphism in the Human UGRP1 Gene Promoter That Regulates Transcription Is Associated with an Increased Risk of Asthma." *Am J Hum Genet* 70 (3): 718-25.

Nunes, Ana P, Isabel O Oliveira, Betânia R Santos, Cristini Millech, Liziane P Silva, David A González, Pedro C Hallal, Ana M B Menezes, Cora L Araújo, and Fernando C Barros. 2012. "Quality of DNA Extracted from Saliva Samples Collected with the Oragene ™ DNA Self-Collection Kit." *BMC Medical Research Methodology* 12 (65).

Olivieri, Fabiola, Raffaella Lazzarini, Rina Recchioni, Fiorella Marcheselli, Maria Rita Rippo, Silvia Di Nuzzo, Maria Cristina Albertini, et al. 2013. "MiR-146a as Marker of Senescence-Associated pro-Inflammatory Status in Cells Involved in Vascular Remodelling." *Age* 35 (4): 1157–72. https://doi.org/10.1007/s11357-012-9440-8.

Orellano, Pablo, Nancy Quaranta, Julieta Reynoso, Brenda Balbi, and Julia Vasquez. 2017. "Effect of Outdoor Air Pollution on Asthma Exacerbations in Children and Adults: Systematic Review and Multilevel Meta-Analysis." *PLoS ONE* 12 (3): 1–15. https://doi.org/10.1371/journal.pone.0174050.

Pellegrino, R., G. Viegi, Vito Brusasco, R. O. Crapo, F. Burgos, R. Casaburi, A. Coates, et al. 2005. "Interpretative Strategies for Lung Function Tests." *European Respiratory Journal* 26 (5): 948–68. https://doi.org/10.1183/09031936.05.00035205.

Provost, Eline B., Agnès Chaumont, Michal Kicinski, Bianca Cox, Frans Fierens, Alfred Bernard, and Tim S. Nawrot. 2014. "Serum Levels of Club Cell Secretory Protein (Clara) and Short- and Long-Term Exposure to Particulate Air Pollution in Adolescents." *Environment International* 68: 66–70. https://doi.org/10.1016/j.envint.2014.03.011.

Puett, Robin C., Jaime E. Hart, Jeff D. Yanosky, Donna Spiegelman, Molin Wang, Jared A. Fisher, Biling Hong, and Francine Laden. 2014. "Particulate Matter Air Pollution Exposure, Distance to Road, and Incident Lung Cancer in the Nurses' Health Study Cohort." *Environmental Health Perspectives* 122 (9): 926–32. https://doi.org/10.1289/ehp.1307490.

Rava, Marta, Nicole Le Moual, Xavier Dumont, Stefano Guerra, Valerie Siroux, Benedicte Jacquemin, Francine Kauffmann, Alfred Bernard, and Rachel Nadif. 2015. "Serum Club Cell Protein 16 Is Associated with Asymptomatic Airway Responsiveness in Adults: Findings from the French Epidemiological Study on the Genetics and Environment of Asthma." *Respirology* 20 (8): 1198–1205. https://doi.org/10.1111/resp.12609.

Renzetti, G., G. Silvestre, C. D'Amario, E. Bottini, F. Gloria-Bottini, N. Bottini, A. Auais, M. K. Perez, and G. Piedimonte. 2009. "Less Air Pollution Leads to Rapid Reduction of Airway Inflammation and Improved Airway Function in Asthmatic Children." *PEDIATRICS* 123 (3): 1051–58. https://doi.org/10.1542/peds.2008-1153.

Runyon, R. Scott, Leslie M. Cachola, Nitya Rajeshuni, Tessa Hunter, Marco Garcia, Regina Ahn, Fred Lurmann, et al. 2012. "Asthma Discordance in Twins Is Linked to Epigenetic Modifications of T Cells." *PLoS ONE* 7 (11): 1–10. https://doi.org/10.1371/journal.pone.0048796.

Rylander-Rudqvist, Tove, Niclas Håkansson, Gunnel Tybring, and Alicja Wolk. 2006. "Quality and Quantity of Saliva DNA Obtained from the Self-Administrated Oragene Method - A Pilot Study on the Cohort of Swedish Men." *Cancer Epidemiology Biomarkers and Prevention* 15 (9): 1742–45.

Saenen, Nelly D., Hannelore Bové, Christian Steuwe, Maarten B.J. Roeffaers, Eline B. Provost, Wouter Lefebvre, Charlotte Vanpoucke, Marcel Ameloot, and Tim S. Nawrot. 2017. "Children's Urinary Environmental Carbon Load: A Novel Marker Reflecting Residential Ambient Air Pollution Exposure?" *American Journal of Respiratory and Critical Care Medicine* 196 (7): 873–81. https://doi.org/10.1164/rccm.201704-0797OC.

Samek, Lucyna. 2016. "Overall Human Mortality and Morbidity Due to Exposure to Air Pollution." *International Journal of Occupational Medicine and Environmental Health* 29 (3): 417–26. https://doi.org/10.13075/ijomeh.1896.00560.

Scanlon, Paul D., and Mark D. Shriver. 2010. "'Race Correction' in Pulmonary-Function Testing." *New England Journal of Medicine* 363 (4): 385–86. https://doi.org/10.1056/NEJMe1005902.

Schwartz, Joel. 2004. "Air Pollution and Children's Health" 113 (4).

Sengler, Claudia, Andrea Heinzmann, Silvija Pera Jerkic, Assia Haider, Christine Sommerfeld, Bodo Niggemann, Susanne Lau, et al. 2003. "Clara Cell Protein 16 (CC16) Gene Polymorphism Influences the Degree of Airway Responsiveness in Asthmatic Children." *Journal of Allergy and Clinical Immunology* 111 (3): 515–19. https://doi.org/10.1067/mai.2003.180.

Silva, Raquel A., J. Jason West, Yuqiang Zhang, Susan C. Anenberg, Jean François Lamarque,

Drew T. Shindell, William J. Collins, et al. 2013. “Global Premature Mortality Due to Anthropogenic Outdoor Air Pollution and the Contribution of Past Climate Change.” *Environmental Research Letters* 8 (3). https://doi.org/10.1088/1748-9326/8/3/034005.

Silveyra, Patricia, and Floros. 2013. “Air Pollution and Epigenetics: Effects on SP-A and Innate Host Defense in the Lung.” *Swiss Med Wkly* 142: 1–17. https://doi.org/doi:10.4414/smw.2012.13579.

Somineni, Hari K., Xue Zhang, Jocelyn M. Biagini Myers, Melinda Butsch Kovacic, Ashley Ulm, Noelle Jurcak, Patrick H. Ryan, Gurjit K. Khurana Hershey, and Hong Ji. 2016. “Ten-Eleven Translocation 1 (TET1) Methylation Is Associated with Childhood Asthma and Traffic-Related Air Pollution.” *Journal of Allergy and Clinical Immunology* 137 (3): 797-805.e5. https://doi.org/10.1016/j.jaci.2015.10.021.

Sughis, Muhammad, Tim S. Nawrot, Syed Ihsan-ul-Haque, Asad Amjad, and Benoit Nemery. 2012. “Blood Pressure and Particulate Air Pollution in Schoolchildren of Lahore, Pakistan.” *BMC Public Health* 12 (1): 378. https://doi.org/10.1186/1471-2458-12-378.

Taniguchi, Natsuko, Satoshi Konno, Takeshi Hattori, Akira Isada, Kaoruko Shimizu, Kenichi Shimizu, Noriharu Shijubo, Shau Ku Huang, Nobuyuki Hizawa, and Masaharu Nishimura. 2013. “The CC16 A38G Polymorphism Is Associated with Asymptomatic Airway Hyper-Responsiveness and Development of Late-Onset Asthma.” *Annals of Allergy, Asthma and Immunology* 111 (5): 376-381.e1. https://doi.org/10.1016/j.anai.2013.08.005.

Tarantini, Letizia, Matteo Bonzini, Pietro Apostoli, Valeria Pegoraro, Valentina Bollati, Barbara Marinelli, Laura Cantone, et al. 2009. “Effects of Particulate Matter on Genomic DNA Methylation Content and INOS Promoter Methylation.” *Environmental Health Perspectives* 117 (2): 217–22. https://doi.org/10.1289/ehp.11898.

The 1000 Genomes Project Consortium. 2011. “A Map of Human Genome Variation from Population Scale Sequencing.” *Nature* 467 (7319): 1061–73.

The Human Genomics Strategy Group. 2012. “Building on Our Inheritance: Genomic Technology in Healthcare.”

The National Center for Education Statistics. 2004. “National Center for Education Statistcs. The Condition of Education.” The U.S. D. Washington, DC. http://nces.ed.gov/pubsearch/pubsinfo.asp?pubid=2011033.

Tsamou, Maria, Karen Vrijens, Narjes Madhloum, Wouter Lefebvre, Charlotte Vanpoucke, and Tim S. Nawrot. 2018. “Air Pollution-Induced Placental Epigenetic Alterations in Early Life: A Candidate MiRNA Approach.” *Epigenetics* 13 (2): 135–46. https://doi.org/10.1080/15592294.2016.1155012.

U.S. Department of Health and Human Services. 2003. “National Institutes of Health: Outreach Notebook for the Inclusion of Women and Minorities as Subjects in Clinical Research. NIH Publication No. 03–7036.”

Vriens, Annette, Tim S. Nawrot, Nelly D. Saenen, Eline B. Provost, Michal Kicinski, Wouter Lefebvre, Charlotte Vanpoucke, et al. 2016. “Recent Exposure to Ultrafine Particles in School Children Alters MiR-222 Expression in the Extracellular Fraction of Saliva.” *Environmental Health: A Global Access Science Source* 15 (1): 1–9.

Vrijens, Karen, Valentina Bollati, and Tim S. Nawrot. 2015. “MicroRNAs as Potential Signatures of Environmental Exposure or Effect: A Systematic Review.” *Environmental Health Perspectives* 123 (5): 399–411. https://doi.org/10.1289/ehp.1408459.

Wang, Cuicui, Jing Cai, Renjie Chen, Jingjin Shi, Changyuan Yang, Huichu Li, Zhijing Lin, et al. 2017. “Personal Exposure to Fine Particulate Matter, Lung Function and Serum Club Cell Secretory Protein (Clara).” *Environmental Pollution* 225: 450–55. https://doi.org/10.1016/j.envpol.2017.02.068.

Wennlöf, Anita Hurtig, Agneta Yngve, and Michael Sjöström. 2003. “Sampling Procedure, Participation Rates and Representativeness in the Swedish Part of the European Youth Heart Study (EYHS).” *Public Health Nutrition* 6 (3): 291–98. https://doi.org/10.1079/phn2002425.

WHO Regional Office for Europe. 2004. “Health Aspects of Air Pollution Results from the WHO Project ‘Systematic Review of Health Aspects of Air Pollution in Europe.’”

Xie, Haojun, Muli Wu, Bin Shen, Yi Niu, Yating Huo, and Yuanxiong Cheng. 2014. “Association between the -112G/A Polymorphism of Uteroglobulin-Related Protein 1 Gene and Asthma Risk: A Meta-Analysis.” *Experimental and Therapeutic Medicine* 7 (3): 721–27.

Yang, I. A., K. M. Fong, P. V. Zimmerman, S. T. Holgate, and J. W. Holloway. 2009. “Genetic

Susceptibility to the Respiratory Effects of Air Pollution." *Postgraduate Medical Journal* 85 (1006): 428–36. https://doi.org/10.1136/thx.2007.079426.

Zhao, Guangri, Xiaodan Lin, Ming Zhou, and Jian Zhao. 2013. "Association between CC10 38A/G Polymorphism and Asthma Risk: A Meta-Analysis." *Pakistan Journal of Medical Sciences* 29 (6): 1439–43.

Zhong, Yijue, Jiping Liao, Yan Hu, Yunxia Wang, Chao Sun, Cheng Zhang, and Guangfa Wang. 2019. "PM2.5 Upregulates MicroRNA-146a-3p and Induces M1 Polarization in RAW264.7 Cells by Targeting Sirtuin1." *International Journal of Medical Sciences* 16 (3): 384–93. https://doi.org/10.7150/ijms.30084.