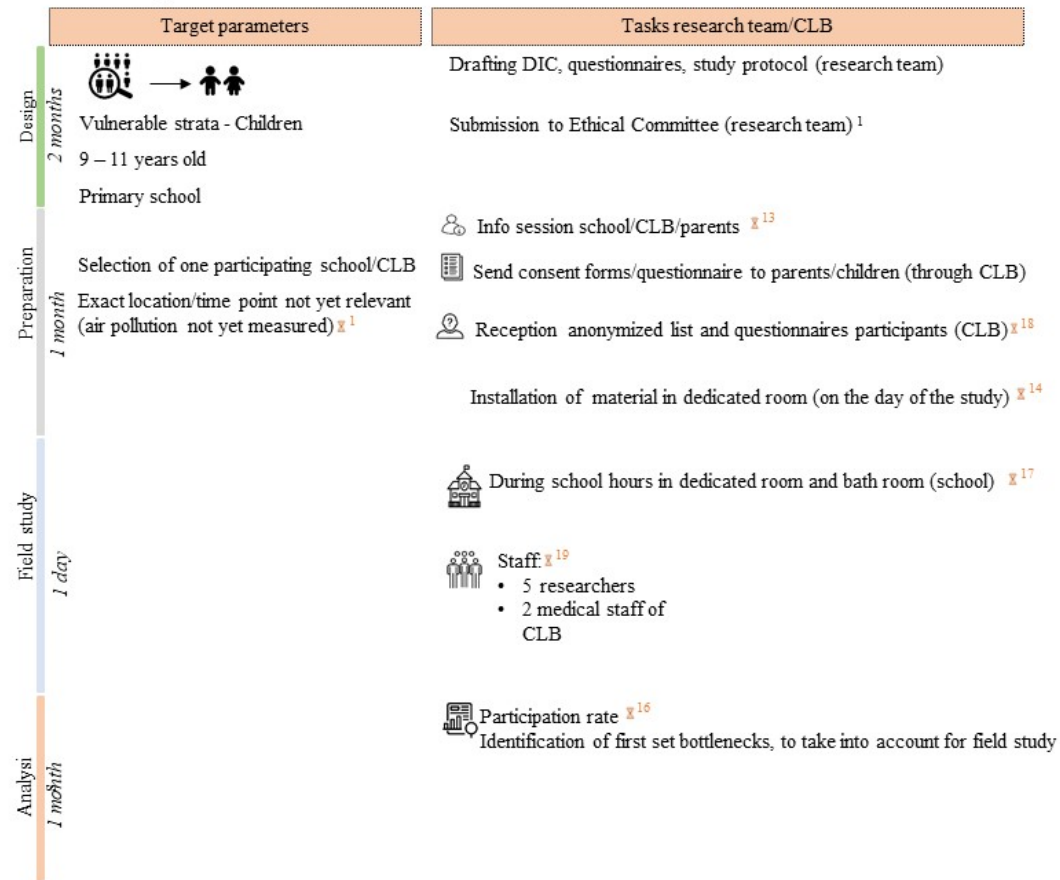
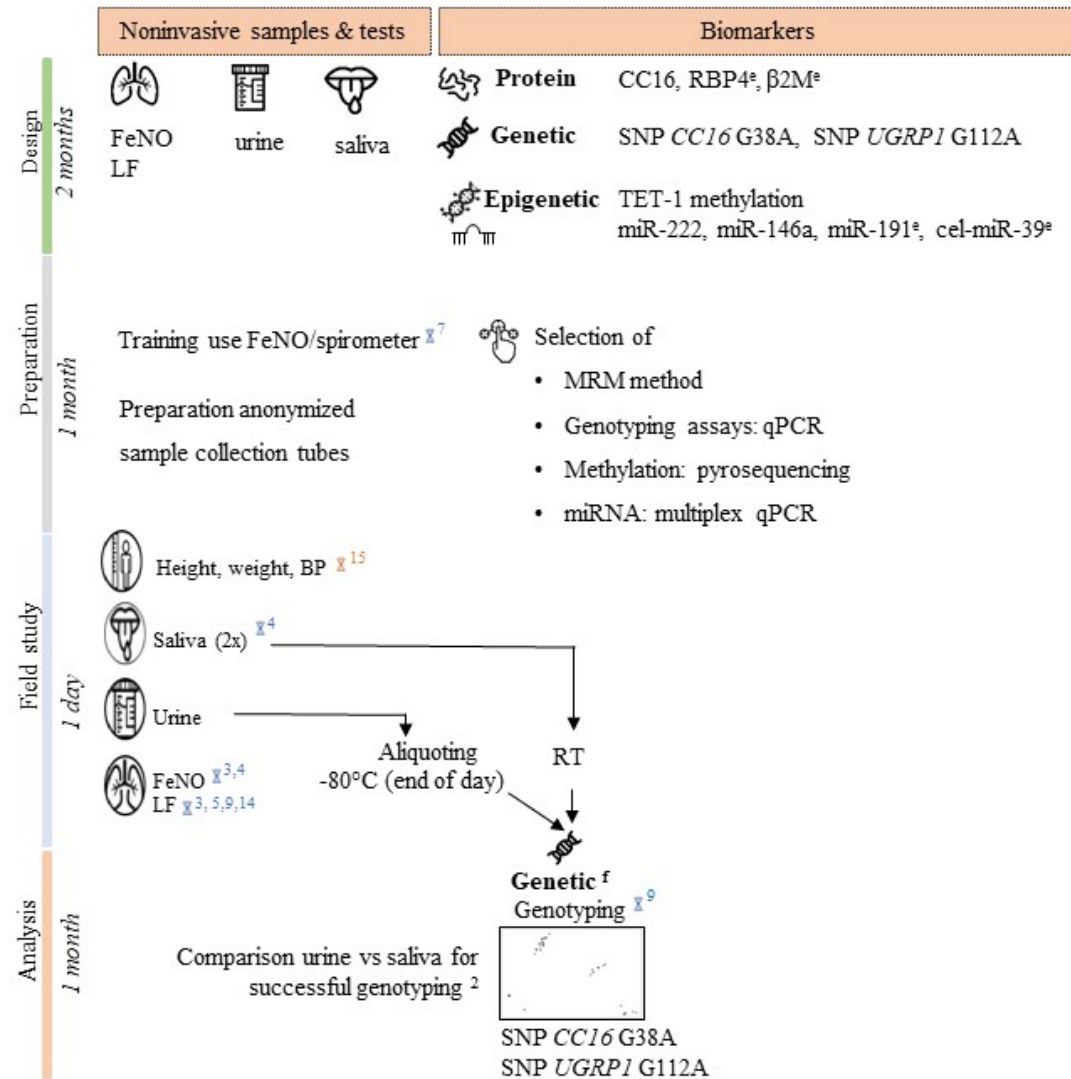


Supplementary files

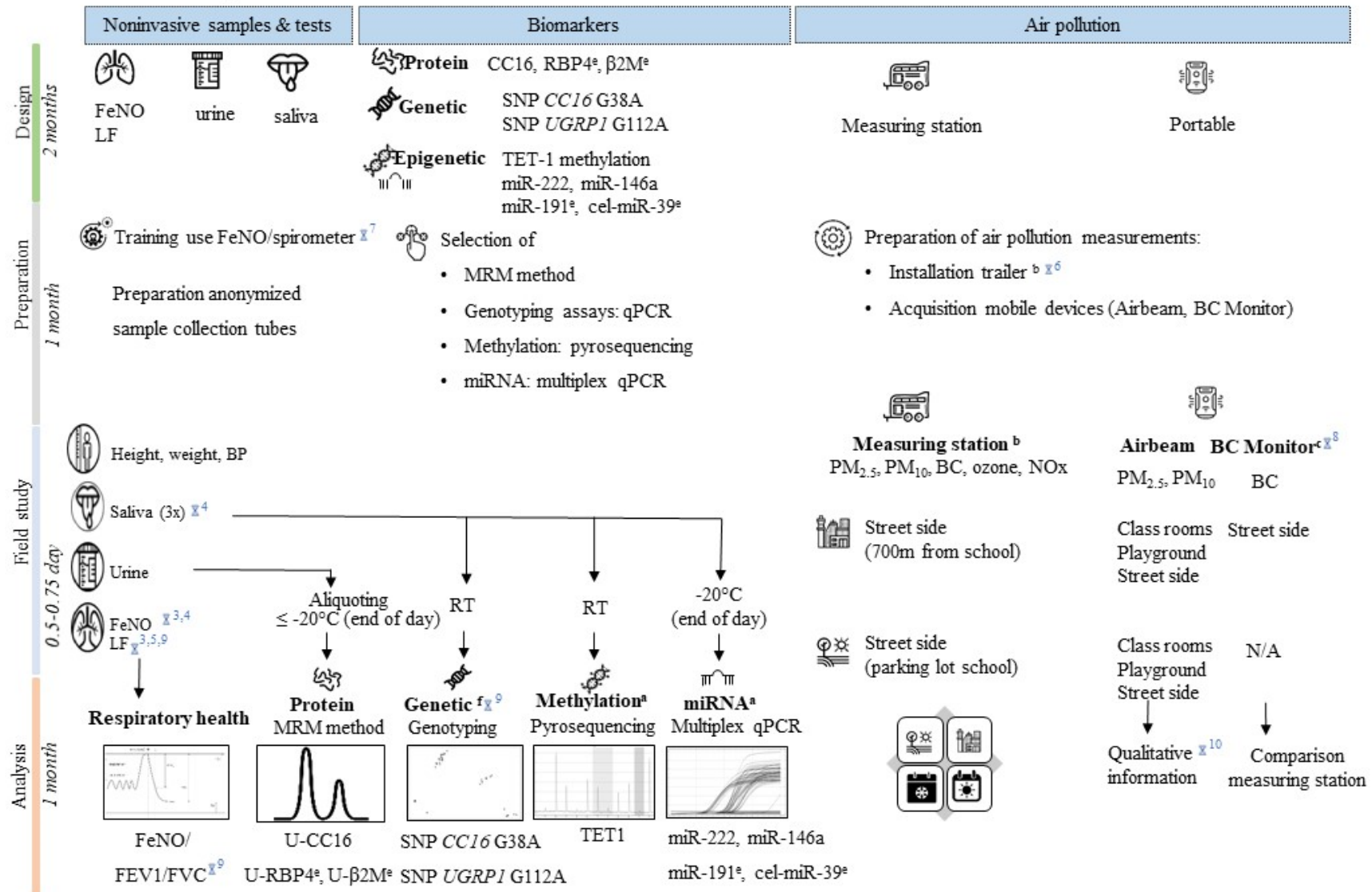
A Test phase



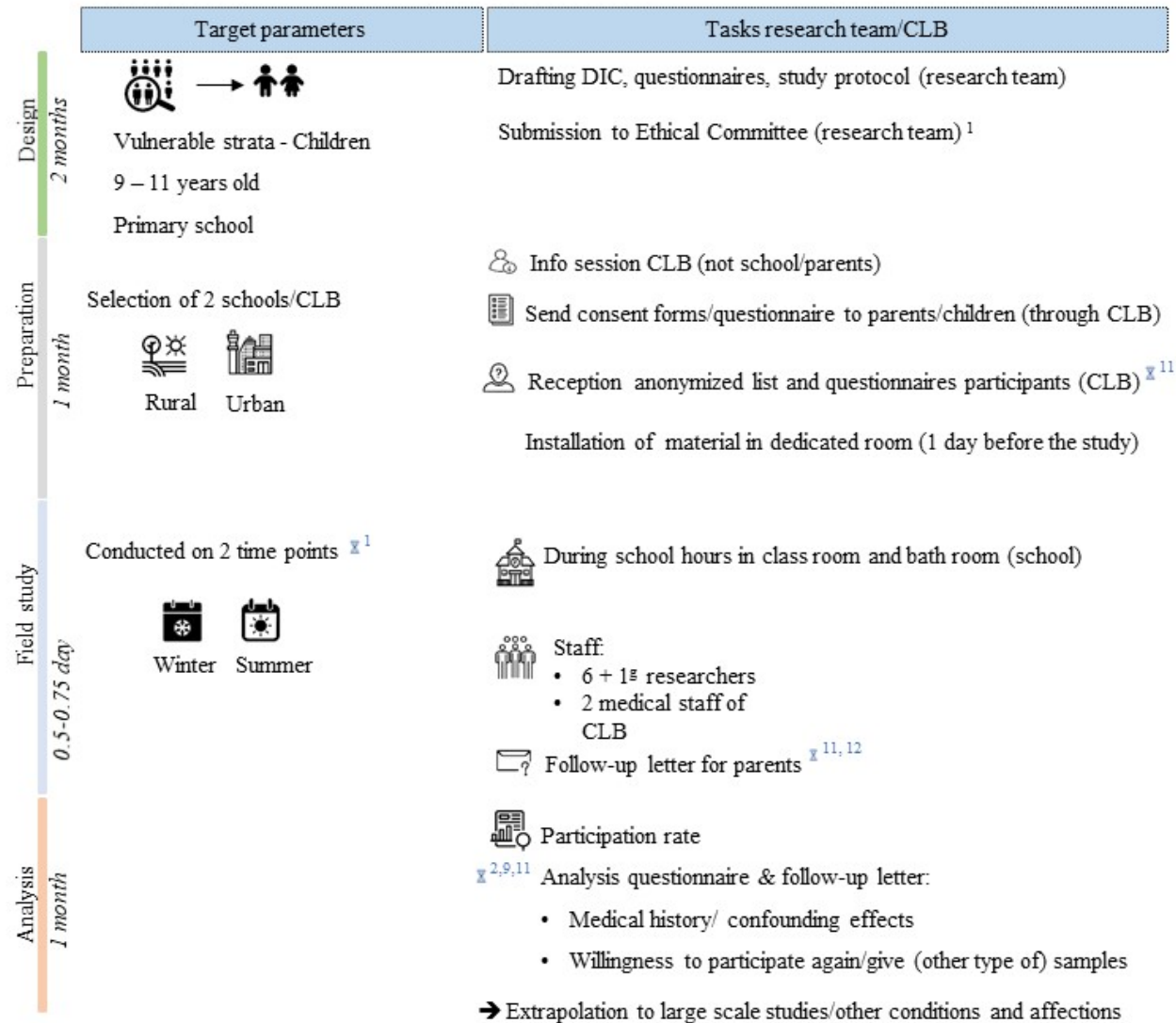
B Test phase



C Field study



D Field study



E Bottlenecks ⌚

- ¹ Timing: take into account holiday periods/tight schedule of school. Not possible to perform the study in the two schools on the same day due to schedule, number of tests and examinations and limited staff
- ² Significant amount of data missing due to (1) questions not completed, (2) limited number of forms collected
- ³ Time consuming
- ⁴ No food or drink 30 minutes before test/sampling
- ⁵ Difficult to perform in children
- ⁶ Difficult to install in every location, especially urban setting with limited open space available
- ⁷ Extensive training needed for its use
- ⁸ Limited battery life
- ⁹ No information available on ethnicity of child
- ¹⁰ Technical characteristics disable the use for absolute pollutant values, only qualitative information available
- ¹¹ Limited time between distribution and collection of the documents
- ¹² Limited number of forms collected in urban school
- ¹³ limited enthusiasm of participating school/CLB → field study: select motivated school and/or organize additional info session, including for the parents to increase willingness to contribute and participate to this study.
- ¹⁴ Much time : lost due to installation material in class room on the day of study → field study: install all equipment at least one day before study
- ¹⁵ BP measurement not successful due to technical issue → field study: need of specific pediatric arm cuff
- ¹⁶ Participation rate is 65 %. Reasons for (non-) motivation to participate? → field study: collect more information by distributing follow-up letter ¹⁷
Not practical to perform sampling and measurements in class room, interferes with class and sport lessons and other activities (tests) → field study: conduct study in separate dedicated room. Increase communication with school to avoid tests and sport lessons during day of field study
- ¹⁸ Limited time between distribution and collection of the documents. Reception of documents even on the day of the study (loss of time) → field study: increase timing between distribution and reception of documents. Avoid reception of documents on the day of the study
- ¹⁹ CLB workload too high on the day of the field study → field study: limit the workload of the CLB to the minimum. Increase the researchers staff.

Figure S1: Detailed overview of the test phase (panels A and B), the field study (panels C and D) and the encountered bottlenecks (panel E). Description of the design, the preparation and the conduction of the field study and its test phase. The different tasks of the field study were conducted by members of the research team and medical staff members of the CLB. Noninvasive tests were performed, including the measurement of height, weight, blood pressure, spirometry and FeNO. Noninvasive samples (saliva and urine) were collected in which biomarkers were measured at different levels (protein, genetic and epigenetic), using cost-effective high throughput methods. The air pollution exposure at the school was measured, using stationary and portable methods.

¹ The study protocol, questionnaires, DIC of the test phase (see Figure S1 (B)) were slightly modified after the test phase. An addendum was submitted to and accepted by the Ethical Committee before the start of the field study.

² S. J. D. Nauwelaerts et al., “Selection of a noninvasive source of human DNA envisaging genotyping assays in epidemiological studies: Urine or saliva?,” J Biomol Tech, p. jbt.20-3101-004, Apr. 2020, doi: 10.7171/jbt.20-3101-004.

^a : on selection of samples

^b : only in rural school

^c : only in urban school

^d : additional urine samples collected for 3 children on day of field study in other school by additional staff member of the R&D team

^e : measured to adjust

^f : DNA extracted only on saliva samples collected during first time point of the field study

^g : one extra person of the research team needed in the other school for collecting the additional urine samples

β2M: beta-2-microglobulin; BC: black carbon, BP: blood pressure, CC16: Club cell protein; CLB: Pupil guidance center; DIC: Document of informed consent; FeNO: fractional exhaled nitric oxide; FVC: Forced vital capacity; FEV1: amount of air you can force from your lungs in one second; miR: micro RNA; MRM: multiple reaction monitoring; N/A: not applicable; NOx: nitrogen oxides; PM: particulate matter; PM_{2.5}: PM of diameter less than 2,5 micron; PM₁₀: PM of diameter less than 10 micron; RBP4: retinol binding protein 4; RT: room temperature; SNP: single nucleotide polymorphism; TET-1: Ten-eleven translocation methylcytosine dioxygenase 1; U-CC16: urinary CC16; U-β2M: urinary β2M; U-RBP4: urinary RBP4; UGRP1: Uteroglobin related protein 1

Created using icons from the Noun Project (see Supplementary file S7 for more details)

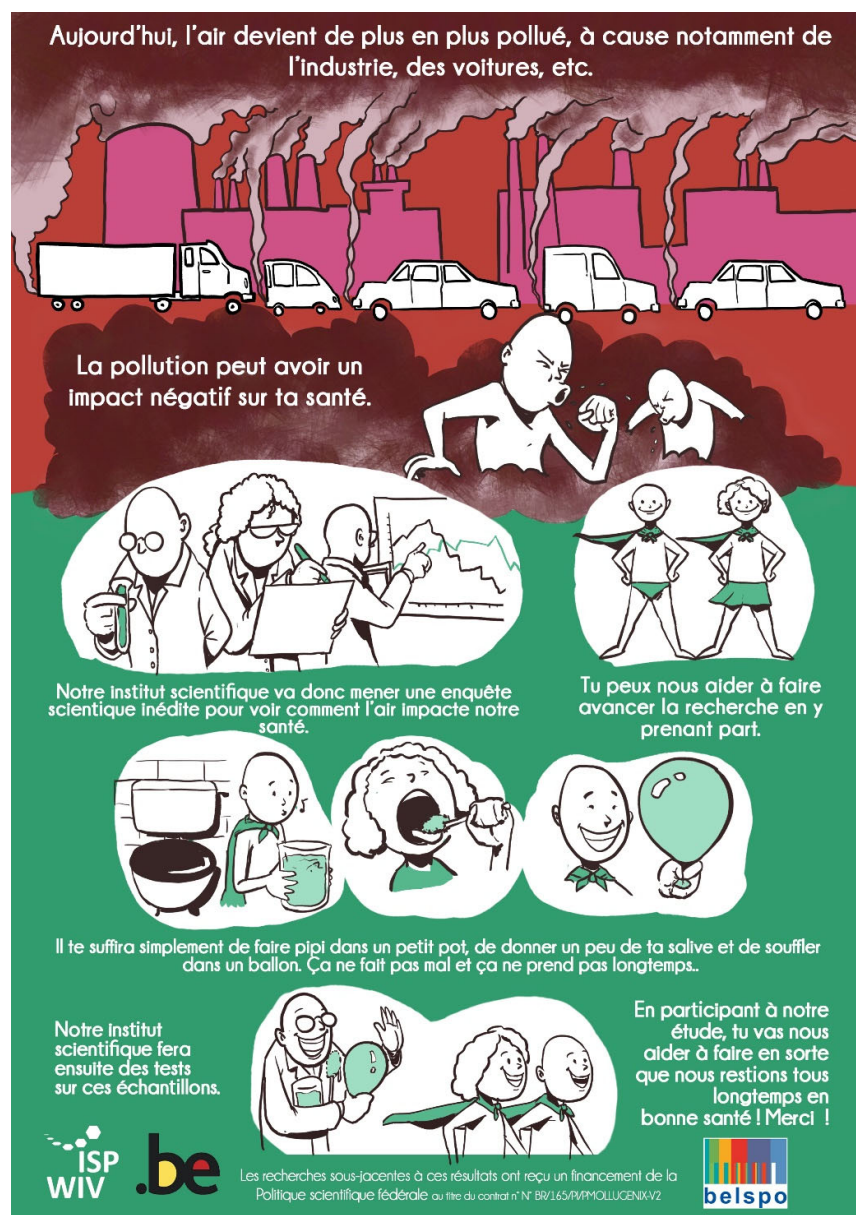


Figure S2: Graphical simplified document of informed consent (assent) to be signed by the children before accepting to participate to the field study. Figure made by J. Van Haute.

Table S1: PCR and sequencing primers for TET1 methylation

| Primer | Sequence (5' → 3') |
|------------|--------------------------------------|
| Forward | TGTGTTATGATTGTTGGAAGAAGTA |
| Reverse | BIOTIN-ATTCCTAACTCTAAACTAAATATCTACAA |
| Sequencing | TTTTTATTTTTTTATTTATAAAGTG |

Table S2: Comparison of different factors affecting choice of monitoring site.

| Determining factors | (1) school | (2) summer/scouts camps (with overnight stay) | (3) playground camps (without overnight stay) |
|--|---|--|---|
| same children over different time points with different exposure | Yes, mandatory presence | Not always same children participating to each (yearly) camp, might depend on parents' holiday schedule Only in the summer (limited exposure differences) | Not always same children participating, might depend on parents' holiday schedule |
| different locations with contrasting pollutants | yes (urban and rural) | usually rural or woody | yes (can be urban or rural) |
| easy collaboration with medical staff | yes, due to collaboration with CLB (in Belgium) | no, recruiting additional medical staff can be envisaged but more expensive to organize Other third party (such as the camp supervisors or scout leaders) could be envisaged but more challenging to organize | no, recruiting additional medical staff can be envisaged but more expensive to organize Other third party (such as the camp supervisors) could be envisaged but more challenging to organize |
| anonymity guaranteed | yes, due collaboration with CLB (in Belgium) | | |
| grouped in age categories | yes, with smaller age differences than in (2) and (3), more convenient for this type of study | usually broader age categories | usually broader age categories |
| flexible day time schedule to conduct study | No: class schedule to take into account | Uncertain: potential camp activities could interfere with the field study schedule | Uncertain: potential camp activities could interfere with the field study schedule |
| flexible period to conduct study | School holidays have to be taken into account, but study possible during large period in the year | No: fixed camp dates but only during (summer) school holidays | No: fixed camp dates, but only during school holidays |
| easy installation of monitoring station | depends on available space in the selected locations. | depends on available space in the selected locations. Specific electric requirements more difficult to meet when camp location is in woody area | depends on available space in the selected locations. |

Table S3: Profile and micro-environment of the urban and rural school and possible surrounding emission sources.

| Characteristics | Urban school | Rural school |
|--|---|---|
| type of street where school is located | street canyon, quite busy street and environment | along large/wide road, not so busy |
| type of area | densely populated, residential area | scarcely populated area with woods and farmland |
| type of building | low energy school | classic building |
| distance to highway or national road | 450m (circular highway) | 450m (national road) |
| distance to industry | 250m from canal*(presence of construction companies and brewery, recycling companies along the canal) | 8.5 km from industrial area |

| | | |
|---------------|---|-----|
| other aspects | motivated school, participation to other air quality campaigns. Recent action: reduced car passage during morning and evening rush hour | N/A |
|---------------|---|-----|

* measuring station located near canal

Table S4: Summary of successful sampling, measurements and analyses of the different biomarkers.

| | Urban | | Rural | |
|------------------------------------|--------------|--------------|-----------------------|--------------|
| | time point 1 | time point 2 | time point 1 | time point 2 |
| Measurements | | | | |
| Height and weight | 20 (100%) | 20 (100%) | 20 (100%) | 20 (100%) |
| Blood Pressure | 18 (95%) | 19 (100%) | 23 (100%) | 23 (100%) |
| FeNO | 19 (100%) | 19 (100%) | 23 (100%) | 21 (91%) |
| Lung function ^c | 19 (100%) | 19 (100%) | 23 (100%) | 23 (100%) |
| Sampling | | | | |
| Urine | | | | |
| Main collection | 19 (100%) | 19 (100%) | 23 (100%) | 23 (100%) |
| Additional collection ^a | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) |
| Saliva | | | | |
| saliva collection 1 | 19 (100%) | 19 (100%) | 22 (96%) | 22 (96%) |
| saliva collection 2 | 19 (100%) | 19 (100%) | 22 (96%) | 22 (96%) |
| saliva collection 3 ^b | 19 (100%) | N/A | 22 (96%) | N/A |
| Biomarker investigation | | | | |
| Genetic^g | | | | |
| <i>CC16</i> SNP G38A | 19 (100%) | N/A | 21 (95%) ^c | N/A |
| <i>UGRP1</i> SNP G112A | 19 (100%) | N/A | 21 (95%) ^c | N/A |
| Protein^h | | | | |
| U-CC16 protein | 18 (95%) | 19 (100%) | 21 (91%) | 20 (87%) |
| U-β2M protein | 18 (95%) | 19 (100%) | 22 (96%) | 23 (100%) |
| U-RBP4 protein | 18 (95%) | 19 (100%) | 22 (96%) | 23 (100%) |
| Epigenetic | | | | |
| Methylation^{di} | | | | |
| TET-1 | 3 (100%) | 0 | 3 (100%) | 0 |
| miRNA^{ej} | | | | |
| miR-146a | 1 (100%) | 1 (100%) | 1 (100%) | 1 (100%) |
| miR-222 | 1 (100%) | 1 (100%) | 1 (100%) | 1 (100%) |
| miR-191 | 1 (100%) | 1 (100%) | 1 (100%) | 1 (100%) |
| cel-miR-39 | 1 (100%) | 1 (100%) | 1 (100%) | 1 (100%) |

The percentages (%) are expressed in function of total of received/analyzed items (not total children initially participating)

^a Additional samples were collected from 3 children, randomly selected, during both phases of the study on the day of the field study conducted in the other area

^b Not collected on the second time point

^c from the 22 received saliva samples, one was not successfully genotyped, due to technical issue during DNA extraction

^d Analysis limited to randomly selected saliva samples from 3 children from urban school and 3 children from rural school, from whom a saliva sample was collected on time point 1 and time point 2

^e Analysis limited to randomly selected saliva samples from one children from an urban school and one children from a rural school, from whom a saliva sample was collected on time point 1 and time point 2

^f Measurement with nose clip challenging in children - repeated without nose clip

^g Successful analysis refers to the unbiased straightforward identification of the different genetic variants in high qualitative DNA samples using commercial genotyping assays [32]

^h Successful analysis refers to relative quantification of the different proteins using a validated MRM method, with values above the LOQ [19]

ⁱ Successful analysis refers to quantification of the methylation level (in %) using validated 'gold standard' methods of bisulphite treatment followed by pyrosequencing [52]

^j Successful analysis refers to the relative detection of different miRNAs in salivary extracellular fraction using commercial kits [42], [68]

N/A: not applicable (not collected, not measured)

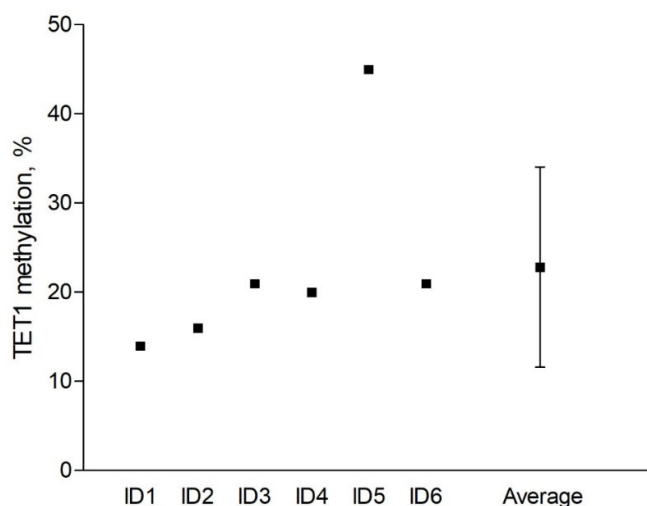


Figure S3: TET1 methylation (%) of 6 randomly chosen salivary DNA samples

Table S5: Cq-values (SD) representing the expression of miR-222 miR-146a, miR-191 (endogenous control) and cel-miR-39 (exogenous control) obtained by reverse transcription and quantitative real time PCR (RT-qPCR).

| Cq (SD) | cel-miR-39 | miR-146a | miR-191 | miR-222 |
|----------|--------------|--------------|--------------|--------------|
| sample 1 | 13.92 (0.21) | 22.82 (0.23) | 19.07 (0.19) | 19.74 (0.20) |
| sample 2 | 14.88 (0.57) | 22.78 (0.61) | 21.03 (0.45) | 20.08 (0.51) |
| sample 3 | 13.98 (0.04) | 23.32 (0.06) | 19.74 (0.08) | 20.76 (0.14) |
| sample 4 | 13.21 (0.17) | 24.25 (0.24) | 20.63 (0.13) | 21.40 (0.16) |