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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Extracellular miRNAs in saliva as a reflection of the body's response to particulate matter exposure in children

Promotor :
dr. Michelle PLUSQUIN

Copromotor :
Prof. dr. Tim NAWROT

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Annette Vriens

Proefschrift ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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Acknowledgements

Scientific research can only be learned by doing it yourself, making mistakes and overthinking the problems you have crossed. Moments when you feel completely overwhelmed and are scared for what's ahead – read: analyse and interpret your data – are most definitely a part of it. However, real scientific research also comes with joy and satisfaction when suddenly the 'Eureka-feeling' strikes you and everything – or at least something – becomes clear but also when deadlines are reached. Though I believe research is a skill you have to learn by doing it yourself, it is not an autodidactic skill and I cannot deny that **dr. Michelle Plusquin** has taught me much that I could not have learned myself. Michelle, thank you for all the time you took to guide me through my path of becoming a researcher. You always challenged me to work and think independently, which was not always easy but I can definitely say that I benefit from it. It was a great opportunity to work with someone as kind and challenging as you. I can only say: your enthusiasm for research is infectious and helped me through difficult times. Thank you for all your help and the possibilities you gave me!

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Lastly, I especially want to thank my parents for the encouragements during my studies as well as during this internship.

List of abbreviations

Ago2:	Argonaut 2
BaP:	benzo(a)pyrene
BMI:	body mass index
BP:	blood pressure
CAD:	coronary artery disease
CAT:	catalase
Cdk	cyclin-dependent kinase
CDKN:	cyclin-dependent kinase inhibitor
cDNA:	complement DNA
COGNAC:	cognition and air pollution in children
Ct:	cycle treshold
DAB:	diaminobenzidine
DEP:	diesel exhaust particle
Dis:	distance
DWTD:	distance-weighted traffic density
DNA:	deoxyribonucleic acid
ELISA:	enzyme-linked immunosorbent assay
EM:	electron microscopy
eNOS:	endothelial nitric oxide synthase
EPC:	endothelial progenitor cell
ESCAPE:	European Study of Cohorts for Air Pollution Effects
ETS:	environmental tobacco smoke
EV:	extracellular vesicles
FDR:	false discovery rate
FP7:	the seventh Framework Programme for research and technological development
GIS:	geographical information system
HRP:	horseradish peroxidase
IRC:	interrun calibrator
ISEV:	international society for extracellular vesicles
IQR:	interquartile range
mRNA:	messenger RNA
miRNA:	micro-ribonucleic acid
MIQE:	minimum information for publication of quantitative real-time PCR experiments
NES:	neurobehavioral evaluation system
NF:	normalization factor
NF-κB:	nuclear factor kappa-light-chain-enhancer of activated B cells
NO:	nitric oxide
NTC:	no template control
O_2^-:	superoxide
$ONOO^-$:	peroxynitrite
PBS:	phosphate buffered saline

PCR:	polymerase chain-reaction
PM:	particulate matter
$PM_{2.5}$:	PM with diameter < 2.5 nanometer
PM_{10}:	PM with diameter < 10 nanometer
qPCR:	quantitative polymerase chain-reaction
RNA:	ribonucleic acid
ROS:	reactive oxygen species
RT:	reverse transcription
RT-qPCR:	real-time qPCR
SAS:	statistical analysis software
SHS:	second-hand tobacco smoke
SD:	standard deviation
snoRNA:	small nucleolar RNA
SNP:	single nucleotide polymorphisms
SOD:	superoxide dismutase
TD:	traffic density
TSP:	total suspended particles
UFP:	ultrafine particles
UV:	ultraviolet
VEGF:	vascular endothelial growth factor
VSMC:	vascular smooth muscle cell

Abstract

Background: Particulate matter (PM) is a mixture of small particles causing air pollution. Exposure to PM is known to affect human health by inducing systemic inflammation and oxidative stress. Intercellular communication by transport of nucleic acids via microvesicles and exosomes plays a role in the development of pathologies such as cardiovascular disorders and cancer. Transfer of miRNAs and mRNAs can cause a phenotypic shift in recipient cells. Recently, in a highly exposed population, PM was shown to affect miRNA expression in microvesicles. In this project, three PM-sensitive miRNAs were investigated in salivary exosomes of children using two repeated measurements.

Methods: Saliva was collected from healthy children at three different time points using the Oragene RNA collection method. Two repeated measurements of 91 children were used. Differential centrifugation and ultracentrifugation steps were applied for exosome isolation from the saliva. Total RNA enriched with small RNAs was isolated from the extracellular fraction. cDNA was synthesized using a megaplex pool of stem-loop primers specific for human miRNAs. Using Taqman microRNA assays, the expression levels of *miR222*, *miR146a* and *miR155* were profiled. The effect of normalization of the expression levels to protein content of the extracellular fraction, RNA and miRNA yield and spiked-in *cel-miR39* as a control for technical variation was studied. Multivariate-adjusted mixed models with correction for false discovery rate were applied to estimate the effect of PM exposure on miRNA expression.

Results: CD63-positive vesicular structures were found in the ultracentrifuged saliva samples, though vesicles were found also larger than <100 nm. Considering different quantification strategies of miRNAs within this extracellular fraction, the use of an external spike-in miRNA, which is not influenced by PM exposure as the protein and RNA content was shown to be the best normalizer. After adjustment for potential covariates, an interquartile range (IQR) increase in UFP was associated with a 20.8% (± 7.2%) increase in *miR*222 expression for indoor exposure during the study visit (P=0.025) and 33.8% (± 8.8%) for outdoor UFP concentrations (P=0.0054). In a univariate model between *miR222* and blood pressure (BP), a significant association between could be found (P=0.0326 for systolic BP and P=0.0297 for diastolic BP). After adjustment for covariates only a trend was observed (P<0.1).

Conclusion: Saliva has not been used before in epidemiological research for identifying molecular effects of air pollution. Within this project the use of saliva as a non-invasive biological specimen is confirmed for finding early alterations in miRNA expression in association with air pollution. However we found that isolating exosomes as described in literature, also yields larger vesicles. Within this extracellular fraction, *miR222* was found to be differentially expressed, suggesting its involvement in the PM-related oxidative stress and vascular disorders, such as atherosclerosis, already early in life.

Samenvatting

Fijn stof bestaat uit een mengsel van kleine partikels die bijdragen tot luchtvervuiling. Blootstelling aan fijn stof kan leiden tot de vorming van ontstekingen en oxidatieve stress, deze kunnen op hun beurt verschillende aspecten van de gezondheid treffen en zorgen voor de ontwikkeling van aandoeningen of bijdragen tot een verhoogde kans op aandoeningen. Er is echter nog onvoldoende inzicht in de mechanismen waarlangs fijn stof de gezondheidstoestand kan wijzigen. De communicatie tussen verschillende cellen kan gebeuren via het transport van genetische informatie, zoals RNA en miRNAs, door middel van extracellulaire vesikels (i.e. exosomen en microvesikels). Fusie van deze vesikels met de gastcellen zorgt voor de inductie van deze genetische informatie waardoor het fenotype van de gastcel kan wijzigen. Deze vorm van intercellulaire communicatie is betrokken in de vorming van kanker en cardiovasculaire aandoeningen. Recent is aangetoond dat blootstelling aan fijn stof de miRNA expressie in extracellulaire vesikels uit plasma beïnvloedt.

Dit project bestudeert de expressie van miRNAs in de exosomale fractie uit speeksel afkomstig van kinderen in relatie met blootstelling aan fijn stof. Binnen de COGNAC studie werd er speeksel verzameld van 337 gezonde kinderen op drie verschillende tijdstippen. Speeksel afgenomen op twee verschillende tijdstippen van 90 kinderen werd gebruikt voor dit project. Door middel van ultracentrifugatie werden exosomen geïsoleerd uit speeksel dat in Oragene® RNA containers (i.e. voor stabilisatie van het RNA in de stalen) werd verzameld. Het totaal RNA met inclusie van kleine RNA species werd verkregen door een kolomgebaseerde extractie en cDNA specifiek voor humane miRNAs werd gemaakt met gebruik van haarspeldstructuur primers. De expressie van *miR-222*, *miR-146a* en *miR-155* werd gemeten met specifieke assays op basis van de Taqman chemie. Statistische analyse om het effect van blootstelling aan fijn stof op miRNA expressie te schatten werd uitgevoerd a.d.h.v. SAS 9.3 Software.

De gebruikte techniek voor het isoleren van exosomen uit speeksel, werd al frequent gebruikt in de literatuur. De aanwezigheid van exosomen kon niet aangetoond worden aan de hand van CD9 maar er werden wel CD63-positieve vesikels gevonden op EM beelden. Er was een aanzienlijke opbrengst van RNA en miRNA in de stalen, maar het is nog niet bevestigd dat deze uitsluitend van exosomale afkomstig zijn. Toepassen van andere technieken kunnen hieromtrent uitsluitsel bieden. In de verkregen extracellulaire factie van het speeksel, was het mogelijk om veranderingen in *miR222* expressie in associatie met fijn stof blootstelling op te sporen. *miR222* is betrokken in verschillende processen die bij kunnen dragen tot een verstoorde werking van endotheelcellen wat kan resulteren in een verlies van de vasculaire integriteit. Dit suggereert een mogelijke rol voor *miR222* in de oxidatieve stress-gerelateerde ontwikkeling van cardiovasculaire aandoeningen als gevolg van luchtvervuiling. Met dit onderzoek wordt ook het gebruik van speeksel in moleculair epidemiologisch onderzoek ondersteund.

1. Introduction

1.1. Air pollution

Air pollution can have both natural and anthropogenic origins though most pollution is caused by mankind.[1] Combustion processes of fossil fuels are the main source of air pollution. Air pollution consists out of gaseous, volatile and particulate pollutants. Particulate matter (PM) can be further divided according to particle size into coarse ($PM_{2.5-10}$ diameter < 10 µm), fine ($PM_{2.5}$ diameter < 2.5 µm) and ultrafine (UFP diameter < 100 nm) particles.[1] Urban air pollution includes traffic and industrial activity as such urban life increases PM exposure and associated health risks.[1] As combustion of fossil fuels is an important source of air pollution, traffic is major contributor to PM exposure.[1]

1.1.1. Air pollution and human health

Epidemiological evidence indicates that PM is predominantly responsible for air pollution associated health effects.[1] Upon exposure, the particles can be inhaled by the respiratory system. Penetration of the particles is determined by size, as such smaller particles can penetrate more deeply into the lungs. The particles can be translocated into the bloodstream by crossing the alveolar membrane or the nasal epithelium and by this way, particles can reach different tissues. Particles can trigger responses at the sites of entrance to the body or alter the responses of the autonomic nervous system.[2]

The response to PM predominantly affects inflammatory pathways and pathways relevant in oxidative stress.[3] When changes in these pathways cannot be compensated, this can be a trigger for disease development. Both chronic and acute exposure to PM is associated with an increased morbidity and mortality. Recent findings by the European Study of Cohorts for Air Pollution (ESCAPE*)[4] pointed out an increased risk at the population level for lung cancer[5], pneumonia during childhood[6], as well as decreased lung function.[7] Still, the exact mechanisms leading to systemic inflammation and the role of inflammation in disease development remain elusive. Furthermore, Nawrot and colleagues showed that contribution to traffic is an important trigger in the development of cardiovascular disease.[8] This contributed to the establishment of the adverse effects of traffic-related exposure. Research has shown that even brief exposure to traffic-related air pollution can induce short-term health effects.[9]

In order to guarantee safety of human health and the environment, the European Commission has developed standard values for PM (Table 1) and other air pollutants. If concentrations exceed these values human health might be at stake and measures to lower the air pollution should be taken.[10]

* The ESCAPE study is a project funded by the European Union (FP7). This study aims to investigate the long-term effects of exposure to air pollution on human health in Europe by combing data of several European cohort studies. Aims of the study are divided in four areas, which each have another point of focus: 1) development of a methodology for long-term exposure assessment, 2) study the association between exposure and mortality and chronic diseases, which are potentially associated with long term exposure, 3) investigate an exposure-response relationship for a) adverse perinatal outcomes b) respiratory disease c) cardiovascular disease d) cancer and all-cause and cause-specific mortality, 4) development of a database with quantitative estimates of the impacts of long-term exposure for all these endpoints. [4]

Table 1: European Air Quality Standards for particulate matter (PM)[11]			
Pollutant	**Concentration**	**Averaging period**	**Permitted exceedences each year**
$PM_{2.5}$	25 µg/m³	1 year	n/a
PM_{10}	50 µg/m³	24 hours	35
	40 µg/m³	1 year	n/a
$PM_{2.5}$ = fine particulate matter; PM_{10} = coarse particulate matter			

1.1.2. Children as a vulnerable subpopulation

Childhood is a critical point in life for exposure to PM and the development of health effects in adulthood. Susceptibility for PM-associated health effects is influenced by many factors such as social status, age, gender, race, pre-existing disease, housing characteristics etc.[12] Children are at greater risk due to their immature state and different exposure patterns compared to adults.[13, 14]

In children, the liver and immune system are not fully developed and cannot fully cope with the toxic load of PM. Furthermore, the lower lung function together with not fully developed lung epithelium results in greater particle deposition. Air toxins and PM can pass the lung epithelium more easily, which implicates a higher PM exposure compared to adults. Indeed, research has shown a 50% higher deposition of particles in children than in adults.[13]

Generally, children spend more time outside and schools are often located near big roads. Breathing frequency can be higher since children are active during school breaks and this also influences particle deposition. Taken together children get easily in contact with traffic-related PM, especially in the morning and after school hours, when traffic peaks during rush hours. This can result in high exposure and higher health risks. Ventilation of occupied classrooms influences the concentration of indoor particles by deposition of outdoor particles inside.[15]

Very recently it was shown that exposure to PM levels below current air quality standards can affect children's health.[5, 7] Results from a European cohort study (i.e. ESCAPE) further established effects of air pollution exposure in children on lung function.[7] Lung function was shown to decrease with increased exposure to fine particles ($PM_{2.5}$), but not with exposure to larger particles.[7] Results from this study indicate that exposure in early life can affect lung function later in childhood. Lung function during childhood is generally a predictor for health throughout life. This demonstrates that even with current policies for air quality human health is not ensured.

1.2. The 'omics'- revolution

'Omics' are scientific disciplines focusing on a specific subset of biological molecules. The 'omics' revolution has led to the possibility to investigate the effects of air pollution exposure – as well as other stressors – on numerous levels. PM can interact with human health on different levels; e.g. the genome, the transcriptome, the epigenome, miRNAome, etc. (Figure 1).[16]

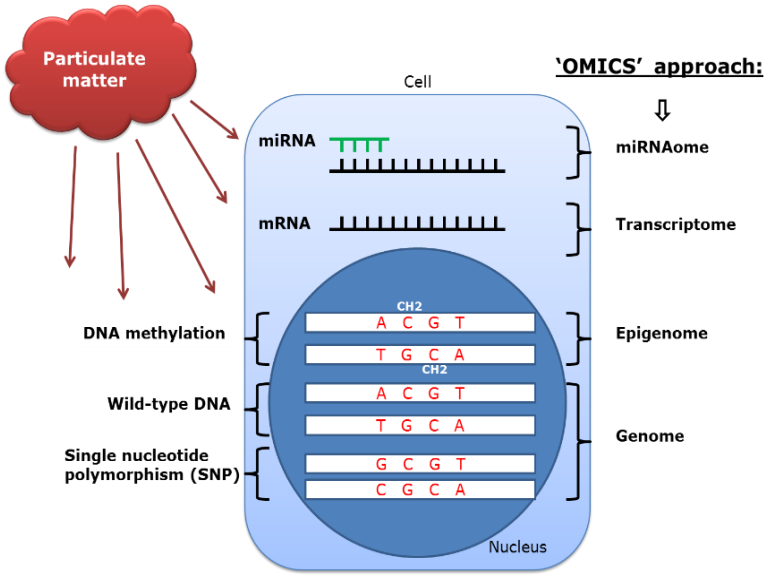

Figure 1: Particulate matter (PM) exert its effects on many different levels. The 'omics' approach is an easy way to demonstrate and investigate the different levels of effects. Responses to PM can be found in DNA methylation patterns, genome expression, transcriptome expression and the expression of miRNAs. Furthermore, responses to PM can be influenced by the presence of certain single nucleotide polymorphisms (SNPs) which can render subjects more susceptible to the effects of PM exposure. Additionally, investigating responses on different levels might provide more insights into the disease mechanisms associated with PM exposure and lead to a broader view of these gene-environment interactions. – Figure adapted from Holloway et al. (2012).[16]

1.2.1. MicroRNAs and their contribution to disease

miRNAs are short, non-coding RNAs encoded in genomic DNA. miRNAs play an important role in cellular functioning by regulating the expression of protein-coding genes. Upon contact with gene transcripts of its targets, miRNAs will inhibit gene expression by repression of translation or by degradation and cleavage of the messenger RNAs (mRNAs). An important feature of miRNAs is that they can have multiple functional targets. One miRNA can thus regulate the expression of numerous targets. Additionally, one target can be regulated by more than one miRNA.[17, 18]

Dysregulated miRNA expression can easily affect the functioning of molecular pathways. They have been implicated in the regulation of a wide variety of biological processes as well as disease development. As such, changes in miRNA expression are shown to be involved in inflammation[19] and cardiovascular disease development.[20, 21] Especially in susceptible subpopulations, changes in miRNA expression might be an underlying factor for disease development.[17, 18] This makes miRNAs interesting biomarker candidates for elucidating disease mechanisms associated with PM exposure.

1.2.2. MicroRNAs and environmental exposure

Exposure to environmental pollutants can affect miRNA expression and hereby induce a reprogramming of the gene expression which renders individuals more susceptible for disease development.[16] Exposure to cigarette smoke[22-25], diesel exhaust particles (DEP)[26]; benzo(a)pyrene (BaP)[27] and PM[28] were shown to regulate the expression of miRNAs. However, up till now there are only few studies which investigated the effect of PM on miRNA expression in humans.[18, 28-31]

Since systemic inflammation and oxidative stress play a critical role in the development of PM-associated health effects, miRNAs involved in these responses (Figure 2A) might respond to PM exposure.[32] Up to now, four studies investigated miRNA expression in association with PM exposure in humans[28-31] and indeed, some of the miRNAs sensitive to PM exposure are involved in inflammation or oxidative stress (Figure 2B). Specific miRNAs (*miR-1*, *miR-126*, *miR-135a*, *miR-146a*, *miR-155*,

miR-21, *miR-222* and *miR-9*), which are involved in inflammation, atherosclerosis and endothelial dysfunction are down-regulated in elderly upon short-term $PM_{2.5}$ exposure below air quality standards.[30] Other studies investigated the short-term effects in an occupational exposed population of foundry workers and observed effects on *miR-21*, *miR-222*,[28] *miR-let-7g*, *miR-29a*, *miR-146a* and *miR-421*.[31]

Recently, miRNA content of microvesicles was shown to be altered upon short-term occupational PM exposure. The expression of *miR-302c* and *miR-128* was significantly up-regulated in microvesicles isolated from plasma of foundry workers in a steel plant.[29]

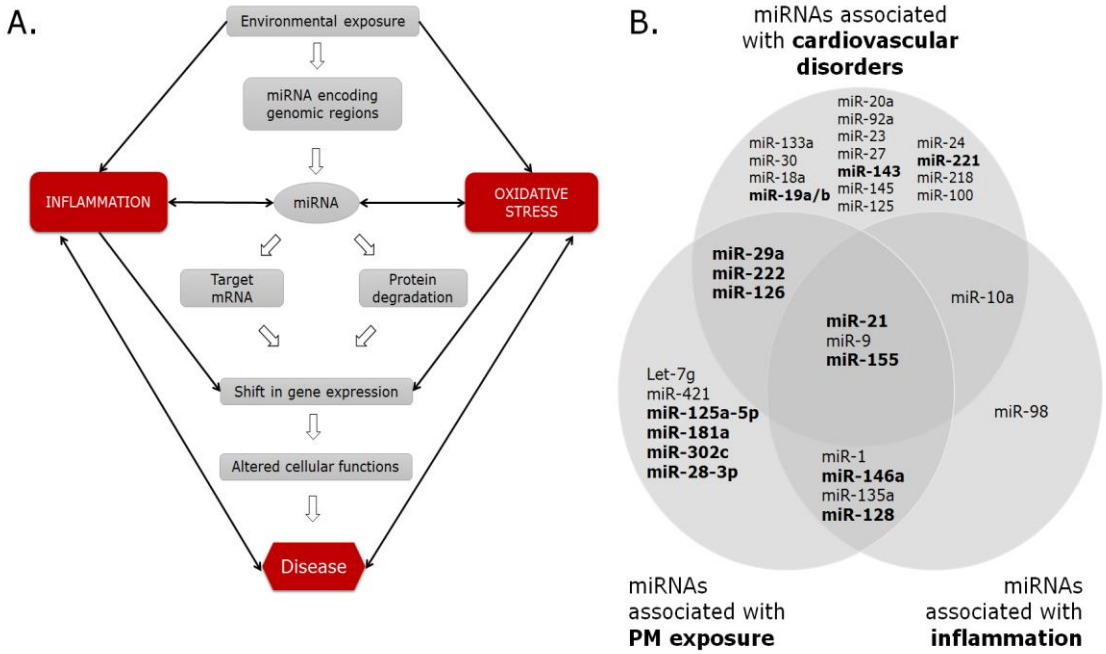

Figure 2: (A) The role for miRNAs in disease development associated with particulate matter exposure. – Figure adapted from Zhou et al (2011).[17] **(B)** The diagram illustrates miRNAs generally associated with cardiovascular disorders, such as endothelial dysfunction, atherosclerosis or heart failure, and inflammation. miRNAome analysis showed that some of these miRNAs were responsive to PM exposure. The miRNAs depicted in bold were shown to be present in microvesicles. – Data from Tijssen et al (2012)[21], McDonald et al (2012)[20], Schroen et al (2012)[19], Bollati et al (2010, 2014)[28,29], Fossati et al (2014)[30], Motta et al (2013)[31], Hulsmans et al (2013)[35].

These data suggest a role for miRNAs in PM-associated responses on a short term with sufficient relevancy for further investigation. In order to fully understand the role of miRNAs as a mediator of the health effects associated with PM exposure, more studies are needed.

1.2.3. Exosomes: important information in nanopackages

Exosomes are small vesicles containing cytoplasmic content and as such nucleic acids, secreted by a wide variety of cells. They differ from secreted microvesicles since they are not pinched off from the cell membrane. Intraluminal vesicles will be formed in late endosomes, which will move up to the cell surface. Fusion of such multivesicular structures with the plasma membrane results in the release of the intraluminal vesicles. The released exosomes can be identified by specific surface markers (CD63; CD9; CD81 and TSG101) or morphologically according to their size of 30 to 100 nm.[33]

When exosomes, which are enriched for specific cytoplasmic nucleic acids such as miRNAs and RNAs in complex with proteins, are released into the microenvironment they might regulate intercellular communication.[33] The presence of exosomes in bodily fluids supports its role in shuttling information between cells.[34] This type of communication is involved in tumorigenesis as well as in inflammation and cardiovascular disease development.[35] Microvesicular transport is already shown to be associated with the transfer of miRNAs involved in inflammation and subsequent onset of atherosclerosis.[35] Bollati and colleagues found an altered expression of microvesicular miRNAs in association with PM[29], this suggests a role for microvesicular communication in the mechanisms of PM-induced health effects.

1.3. Saliva, a mirror of the body's health status

Cohort studies with a population of children, may make use of non-invasive biological matrices when collecting biological samples. With the use of a non-invasive biological matrix to obtain genetic information from study subjects, people will be more likely to participate in the study and as such this will result in higher response rates.[36] Additionally, in a study with adults was shown that the donation of saliva was preferred instead of the donation of urine or blood.[37]

Saliva is a mixture of secretions from intrinsic and extrinsic salivary glands, nasal and bronchial secretions, different biological molecules and microbiota.[38] It is shown that cellular content from saliva consists of leukocytes as well as oral epithelial cells[39], which reach the saliva through intracellular and extracellular routes. This makes saliva a suitable alternative for blood in the monitoring of physiological and pathological states in humans. Furthermore, the composition of saliva can be affected by disease states.[40, 41] It had been suggested that saliva can reflect the body's health status with comparable accuracy as blood.[40, 42] Nowadays, saliva is widely used as a tool for diagnosing various pathological conditions[40] and is an emerging tool in biomarker search[43, 44] Saliva can be obtained non-invasively and collection is relatively inexpensive, safe and easy, compared to blood samples. Over the last decade, many researchers have focused on the use of saliva as a biological matrix. However, the use of salivary genetics in epidemiologic research is still limited.

1.4. The situation and aim of the Master's thesis

This Master's thesis is part of the ongoing COGNAC study (cognition and air pollution in children), which investigates the effects of PM exposure on cognition and cardiovascular health in primary school children. In this study, cognitive performance tests and cardiovascular parameters as well as biological information of enrolled subjects was collected. Buccal swabs, urine and saliva samples were obtained to investigate the effects of PM exposure on different platforms following the 'omics'-approach. Focusing on different platforms might result in a more integrated view on the effects of PM exposure.

Within the scope of this study, the hypothesis was put forward that PM exposure can affect miRNA expression and that this can be reflected in differential packaging of miRNA into exosomes, in order to communicate effects to other cells (Figure 3). Therefore, it was hypothesized that PM exposure can affect the miRNA expression in salivary exosomes of primary school children.

In order to test this following **objectives** are postulated.

1. Optimization of the isolation of exosomes from saliva and the extraction of RNAs from the salivary exosomes.
2. Development of a proper normalization strategy for extracellular miRNA expression.
3. Determination of the expression of a subset of PM-sensitive miRNAs in exosomes, in association with exposure to PM.

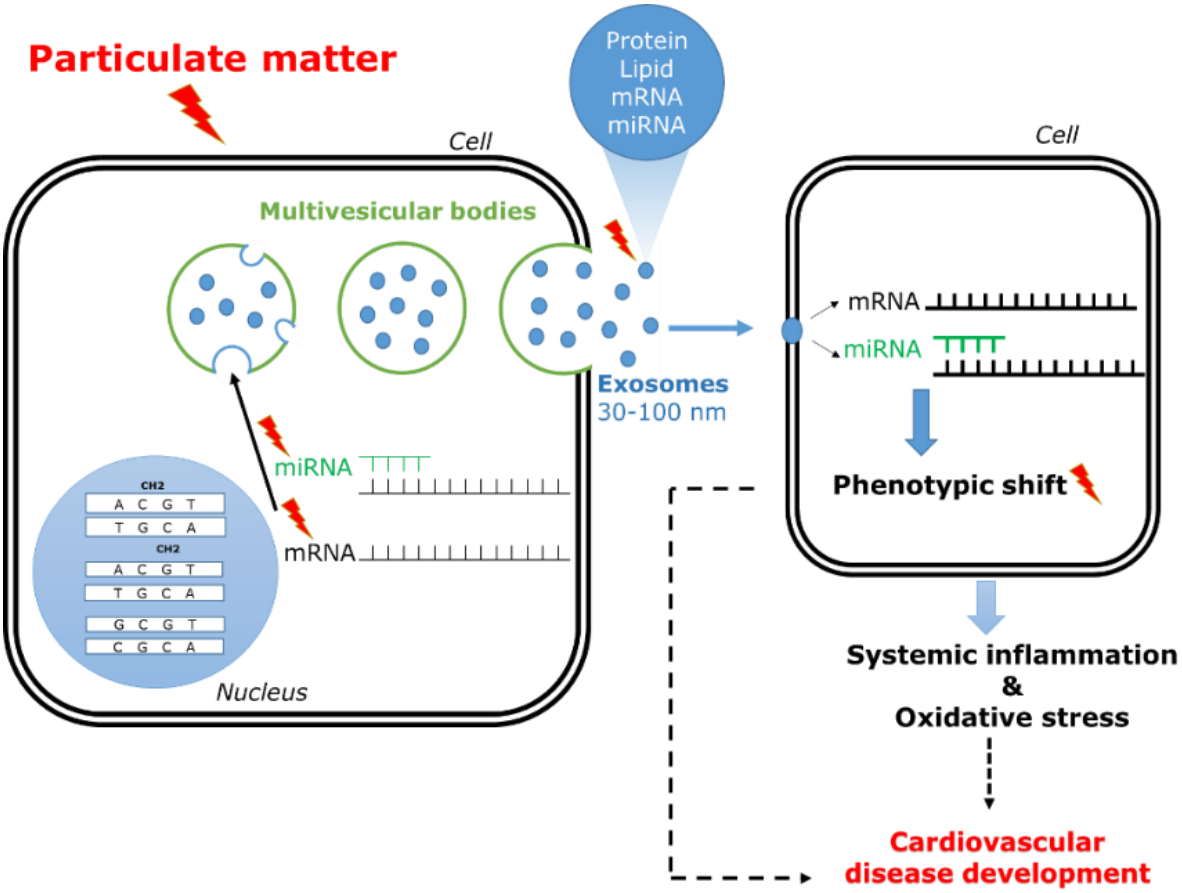

Figure 3: Visual representation of the hypothesis. Particulate matter (PM) can exert effects on different molecular levels. Genomic DNA can be affected (e.g alterations DNA methylation status) though presence of single nucleotide polymorphisms (SNPs) might modify the effects. Furthermore, there can be changes in the expression of several mRNAs and non-coding RNAs, including miRNAs. These changes can result in a change of phenotype in the cell itself. However, cellular information such as proteins, lipids and RNAs can be specifically packaged in exosomes in order to communicate the effect of certain stressors to other cells. Fusion of exosomes with their target cells will result in the release of cellular information into the target cell and can cause a phenotypic shift in this cell. I hypothesize that PM exposure will lead to differential packaging of cellular content into exosomes which can be reflected by changes in the RNA load in these microvesicles. Exosomal intercellular communication of PM-associated effects might be a mechanism involved in transferring the signals to cells and tissues in the periphery and contribute to disease development.

2. Materials and methods

2.1. Study population and Ethics statement

The schools included in the COGNAC study are located in three cities in Belgium: Tienen, Zonhoven and Kiewit. All children from the fourth, fifth and sixth grade of the primary schools received an information letter with an invitation to participate in the study. Generally, 43.4% of the invited children participated in the study (52.2% in Zonhoven, 38% in Kiewit and 33.3% in Tienen). The participants were between 9 and 12 years old at the moment of the sampling. Subjects enrolled in the COGNAC study donated saliva samples during the fieldwork. In order to investigate the effects of PM exposure, three repeated samplings were performed. This allows each study subject to act as its own control situation. Information on material situation, housing conditions, exposure to tobacco smoke, etc. was assessed via questionnaires. Written informed consent from the parents was obtained before onset of the study. The COGNAC study was approved by the Medical Ethics Committee of Hasselt University and Ziekenhuis Oost-Limburg.

2.2. Exposure assessment

Exposure parameters for both PM exposure as well as long-term residential traffic exposure were present for each individual. Parameters for short-term exposure were measured in the room during all tests with a PM measuring tool (including PM_{10}; PM_{7};$PM_{2,5}$; PM_{1} and total suspended particles (TSP)) (MetOne Aerocet-531) and a measuring tool for UFP (Aerasense Nanotracer; Philips). Outside measurements were carried out during the periods in which the children are outside (i.e. before school hours and during breaks for 10 minutes). Using these outside measurements, an average concentration from 9 a.m. until 12 p.m. was calculated for PM_{10}; $PM_{2.5}$ and UFP. Parameters for residential traffic exposure were obtained using geographical information systems (GIS), which provide information on the presence of important roads nearby the residency. Calculated parameters are the distance of the residency to freeways (i.e. N-roads) and highways (i.e. E-roads); traffic density within 50 meters, 100 meters, 200 meters and 500 meters buffers as well as a distance-weighted traffic density which takes into account the larger contribution of the nearest roads. These parameters were based on the current address, which was via questionnaires.

2.3. Saliva sampling

Before sampling, subjects refrained at least 30 minutes from eating, drinking or hygiene procedures. Additionally, they rinsed three times with tap water to avoid contamination of the samples by food residues. Saliva samples were collected using the RNA collection method. Subjects had to insert 2 mL of whole saliva into the Oragene® RNA self-collecting kit (DNA Genotek) (Figure 4). When closing the container, a preservation liquid is released from the cap. By shaking the containers, the preservation liquid is mixed with the saliva. This liquid ensures RNA stability upon storage at room temperature for two months while inhibiting nuclease activity. However, within 6 hours after sampling the samples were stored at -20°C until further analysis.

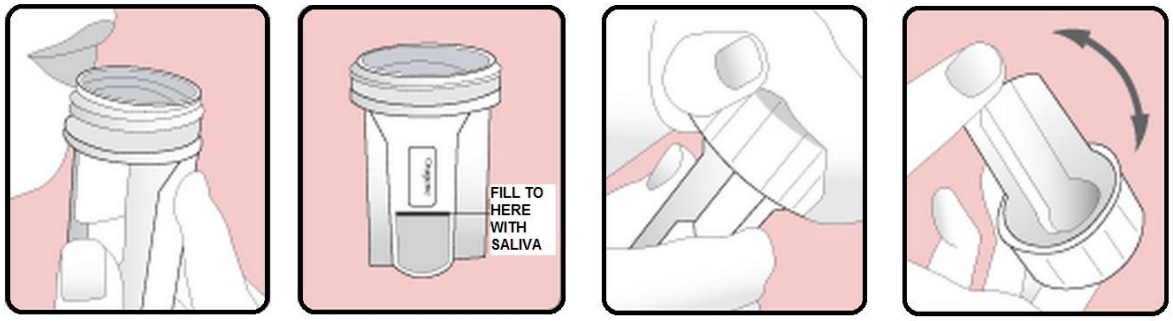

Figure 4: Sampling method using the Oragene® RNA self-collection kit. 2mL whole saliva is donated, as such the bar with 'Fill to here with saliva' will be reached. Next, the cap is firmly closed and RNA stabilization solution is released from the cap. This ensures integrity of the RNA upon storage at room temperature. The RNA stabilization solution has to be properly mixed with the saliva by shaking the container for at least 10 seconds.

2.4. Assessment of cardiovascular parameters

During fieldwork, heart rate and blood pressure of the study volunteers was measured using an ambulatory blood pressure monitor (STABIL-O-Graph). In order to rule out effects of nervousness, excitement, etc. on blood pressure and heart rate, the measurements were carried out five times. From every subject, the average of three independent repeated measurements (i.e. the last three) was used for the analysis. Repeated measurements by subject were carried out at the same day of the week and time to avoid diurnal variation of blood pressure.

2.5. Exosome and RNA isolation

The exosomal fraction was isolated from the saliva by ultracentrifugation[45] and used for RNA isolation. Briefly, the Oragene® containers containing samples were incubated at 50 °C for one hour in a water bath. Afterwards, aliquots of 1 mL were made and samples were incubated at 90 °C for 15 minutes. 40 µL of neutralizer solution was added for better pelleting of debris. Next, samples were centrifuged at 1 500x *g* for 10 minutes to eliminate cells from the saliva. Supernatant was centrifuged at 16 000x *g* for 20 minutes to pellet dead cells, cell organelles and debris from the supernatant. For pelleting of the exosomes, supernatant was centrifuged at 160 000x *g* for one hour (Optima LE-80K Ultracentrifuge; Beckman). To eliminate contaminating proteins from the exosome pellet, the pellet was washed with 1x phosphate-buffered saline (PBS) (pH 7,4) and centrifuged at 160 000x *g* for one hour. All centrifugation steps were carried out at 4 °C. Polyallomer tubes (Beckmann) for ultracentrifugation were pre-treated with RNA*Zap* (Ambion) to eliminate RNAse activity. Exosome pellets were suspended in RNAse-free water.

Different RNA isolation techniques (i.e. TRIzol-based RNA isolation, phenol-chloroform isolation and the miRNeasy mini kit) were compared for the isolation of RNA and miRNA from the exosomes (technical information on the TRIzol-based an phenol-chloroform isolation techniques is provided in the supplemental information). Exosomes were lysed using QIAzol Lysis Reagent (Qiagen) and total RNA was extracted using the miRNeasy Mini kit (Qiagen) following the manufacturer's protocol. Quantification of the total RNA and purity of the samples was assessed using spectrophotometry (UV-vis Nanodrop Spectrophotometer). The amount of nucleic acids was quantified by measuring the

light absorbance at 260 nm. Purity of the samples was estimated using the ratios of the absorbance at different wavelengths: A260/A280 for protein contamination and A260/A230 for solvent contamination of the samples. Additionally, RNA and miRNA concentration, were quantified using the Qubit Assays (Invitrogen) following the manual. Presence of the miRNA was evaluated using the Small RNA kit with the Agilent 2100 Bioanalyzer. Manufacturer's protocol was followed.

2.6. Exosome characterisation

Firstly, exosome pellets were analyzed by electron microscopy (EM) for the presence of vesicles of the size range of 30 nm to 100 nm. Afterwards, the presence of exosomes was evaluated using the exosome-specific CD9 enzyme-linked immunosorbent assay (ELISA) as well as a CD63 immunostaining using diaminobenzidine (DAB) for EM visualization.

2.6.1. Confirmation of vesicles by electron microscopy

After exosome isolation, the pellet was dissolved in a small amount (i.e. 30 µL) of PBS (pH 7,4). Samples were used for fixation. Briefly, drops of the samples were placed on Parafilm and positioned on a carbon coated nickel grid. Next, the samples were fixed using 2 % paraformaldehyde and incubated for 10 minutes on the grid. Using 2.5% glutaraldehyde the samples were post-fixed and incubated on the grid for 10 minutes. Afterwards, the grid was washed using deionized water. The samples were contrasted using 2 % uranyl acetate and incubated for 15 minutes to the grid. Lastly, samples were embedded by incubating for 10 minutes with 0.13 % methyl cellulose and 0.4 % uranyl acetate. Excess liquids were removed and the grid was air dried for 5 minutes. Afterwards, preparations were examined with a Philips EM 208S transmission electron microscope operating at 80 kV and digitized with a Morada 3.0 TEM camera controlled by iTEM FEI (version 5.0) software from Olympus Soft Imaging Solutions GmbH.

2.6.2. Immunohistochemical staining of exosomes for electron microscopy

After exosome isolation, the pellet was resolved in 260 µL of RNAse-free water. Only 30 µL of the resuspended pellet was using for fixation. All products used for fixation of the samples were at room temperature. Briefly, drops of the resuspended exosomes were placed on Parafilm and positioned on a carbon coated nickel grid for 60 minutes. Next, the samples were fixed using 2 % paraformaldehyde (in PBS) and incubated for 10 minutes on the grid. Afterwards, the grid was washed using deionized water. Firstly, a peroxidase block to block possible red blood cells in the samples incubated for 20 minutes on the grid. Samples were washed three times using deionized water. Next, a goat anti-rabbit antibody incubated for 20 minutes on the samples to avoid non-specificity of the primary antibody. The grid was washed using deionized water. Next, the CD63-specific primary antibody (rabbit anti-human anti-CD63 diluted to 0.05 µg/µL; Abcam ab68418) incubated for 40 minutes on the grid. The grid was washed using 0.1 % BSA (in PBS) in order to avoid non-specific interactions. The peroxidase-labelled polymer (i.e. the secondary antibody; EnVision+ system-HRP (DAB); Dako) incubated for 40 minutes in the grid. Next, samples were washed using PBS. Subsequent, the grids

incubated with DAB and the substrate chromogen for 10 minutes. Samples were washed with deionized water. Post-fixation and contrasting of the samples was carried out as before (see section 2.6.1). Afterwards, preparations were examined using EM (as mentioned in section 2.6.2.).

2.6.3. Exosome-specific ELISA

Exosomes are characterised and quantified using a CD9-specific sandwich ELISA plate-based immunoassay (ExoTEST; Gentaur). After isolation of the exosomes by samples, ultracentrifugation, a mixed sample was obtained by mixing 10 % of the resuspended exosome pellet of nine different samples. In order to gain information on the detection limits of the assay for the salivary exosome the absorbance of a three-fold serial dilution series was employed. The manufacturer's protocol was followed. Briefly, lyophilized exosome standards were reconstituted in 100 µL milliQ water and 100 µL 1x PBS (pH 7.4) and a two-fold serial dilution was loaded in duplicate on the immunoplate. A three-fold serial dilution series of the mixed sample was loaded in eight wells. The plate was washed three times using the washing buffer provided with the kit. The primary antibody was loaded to the plate and incubated for 20 minutes at room temperature while shaking at 120 rpm. Afterwards, it incubated two hours at 4 °C. The plate was washed three times before the secondary antibody was applied to the immunoplate. The plate incubated for 20 minutes while shaking at 120 rpm at room temperature before it was incubated one hour at 4 °C. The plate was washed trice and substrate solution was added to the plate. This incubated in the dark until a blue colour was visible. Lastly, stop solution was added in order to stop the reaction and the colour changed from blue to yellow. Fluorescence measurements were carried out on a FLUOstar Omega plate reader at 450 nm and 570 nm within 10 minutes. The absorbance at 570 nm was subtracted from the absorbance at 450 nm and plotted against the concentration of exosomes, which is expressed in ng/µL.

2.7. miRNA expression analysis

Expression is evaluated in a miRNA-specific manner, using individual assays. Six miRNAs were selected for expression analysis in salivary exosomes (Table 2).

2.7.1. miRNA selection

Six miRNAs (i.e. *miR-128*, *miR-9*, *miR-21*, *miR-146a, miR-222 and miR-155*) were selected based on previous reports indicating PM-sensitivity of the miRNAs.[28-31]

Table 2: Sequences for the selected miRNAs, their miRBase accession number and TaqMan assay.

miRNA	miRBase Accession number	Mature miRNA Sequence	TaqMan miRNA assay
hsa-miR-21-5p	MIMAT0000076	UAGCUUAUCAGACUGAUGUUGA	ID 397
hsa-miR146a-5p	MIMAT0000449	UGAGAACUGAAUUCCAUGGGUU	ID 468
hsa-miR-222-3p	MIMAT0000279	AGCUACAUCUGGCUACUGGGU	ID 512
hsa-miR-128a-3p	MIMAT0000424	UCACAGUGAACCGGUCUCUUU	ID 2216
hsa-miR-155-5p	MIMAT0000646	UUAAUGCUAAUCGUGAUAGGGGU	ID 2623
hsa-miR-9-5p	MIMAT0000441	UCUUUGGUUAUCUAGCUGUAUGA	ID 583

2.7.2. miRNA quantification

A two-step real-time quantitative PCR (RT-qPCR) was carried out for quantification of specific miRNAs using TaqMan miRNA assays (Life Technologies). In the reverse transcription (RT) step, cDNA will be synthesized from 125 ng of total RNA samples (i.e. with the exception of samples with a lower concentration) using looped primers (Megaplex RT primers Human Pool A; Life Technologies) and reagents from the TaqMan microRNA RT kit (Life Technologies). cDNA synthesis was carried out on a PCR gradient thermal cycler (TC-5000; Techne, UK) 40 cycles of two minutes at 16°C, one minute at 42 °C and one second at 50°C. The reverse transcriptase enzyme was inactivated by incubation for five minutes at 85°C. Afterwards, the samples were hold at 4°C until the end of the run and stored at -20°C afterwards.

The qPCR step was performed in an 8 µL reaction volume using 384-well plates. Reactions contained the TaqMan miRNA assay (Life Technologies), TaqMan Fast Advanced Master mix (Life Technologies), RNAse-free water and cDNA samples. qPCR was carried out on ABI 7900HT sequence detection system (Applied Biosystems) using following thermal cycling: 10 minutes at 95 °C followed by 40 cycles of 15 seconds at 95 °C and one minute at 60 °C. All runs were performed in triplicate and a no-template control (NTC) was used as a negative control. The MIQE guidelines[46] were taken in account and six interrun calibrators (IRC) were be used. Raw qPCR data were analyzed using the SDS Relative Quantification software (version 2.3; Applied Biosystems).

2.7.3. PCR amplification efficiency

The PCR amplification efficiency was determined for each assay using a serial dilution series. For all samples, the cycle at which the fluorescence threshold was exceeded (Ct) (i.e. dependent variable) was plotted against the logarithm of the concentration (i.e. independent variable). Hereby, a calibration curve was generated. The slope of the calibration curves was used to calculate the PCR amplification efficiency using following equation (1):

$$PCR\ efficiency = 10^{-1/slope} - 1 \quad (1)$$

Only the assays with good amplification efficiencies (i.e. between 90-110%) and which had a Ct below 35 were included in the analysis.

2.7.4. Normalization strategy

Several different variables were put forward as candidate normalizers for qPCR expression data. As such, the protein content, RNA and miRNA yield and the normalization factor (NF) of an external spike-in miRNA were evaluated as normalizers for correction of the sample input. Synthetic miRNA (Qiagen) was added to the samples to normalize for technical variation. After homogenization, samples were spiked-in with 250 fmol *C. elegans miR-39* as previously reported.[47] For proper normalization of qPCR data, all normalizers were compared with each other based on stability defined as a minimal variability between different subjects, effect of exposure and effect of normalization strategy on the study outcomes.

2.8. Statistics

All statistical analyses were carried out using SAS software (version 9.3; SAS Institute Inc.). Normality of the qPCR results and factors for normalization of the expression data was evaluated using both graphical and numerical methods, respectively QQ-plots and the Shapiro-Wilk test. In case of non-normal distribution, logarithmic transformation of the data was carried out. The effects of individual parameters for exposure and personal data, such as age, BMI, passive smoking status, etc. were estimated using univariate mixed models. Linear regression models taking in account multiple variables as well as possible confounders estimated the effect of exposure on miRNA expression using mixed models to correct for repeated measurement by school and subject. Confounders and covariates considered for entrance in the model were age, gender and school of the subject, factors which reflect socio-economic status of the subjects as well as passive smoking status, the day of the week and time of the measurements. The RNA content was included in the model as a covariate. Additional information on the choice of the covariates can be found in the supplemental information. Multivariate normal distribution of the correlated errors was evaluated using the QQ-plot of the residuals. In order to correct for multiple testing, the Benjamini Hochberg test for false-discovery rate (FDR) was carried out within analyses for each miRNA.

2.9. Pathway analysis

In order to comprehend the effect of possible changes in miRNA expression, targets of the miRNAs were identified using online prediction tools (TarBase and TargetScan). Whereas TargetScan predicts the miRNA targets based on an algorithm that searches for complementary sequences in the seed regions with respect to the conservation of the gene, TarBase gives a list of experimentally supported targets.

3. Results

3.1. Optimization total RNA extraction of salivary exosomes

3.1.1. Characterization of exosomes

The presence of exosomes can only be confirmed with high certainty based on immunologic techniques which target exosome-specific markers such as CD9 or CD63. Electron microscopic analysis of the pellet obtained from ultracentrifugation steps showed presence of vesicular structures in the same size order as exosomes, though larger vesicles were also present (Figure 5).

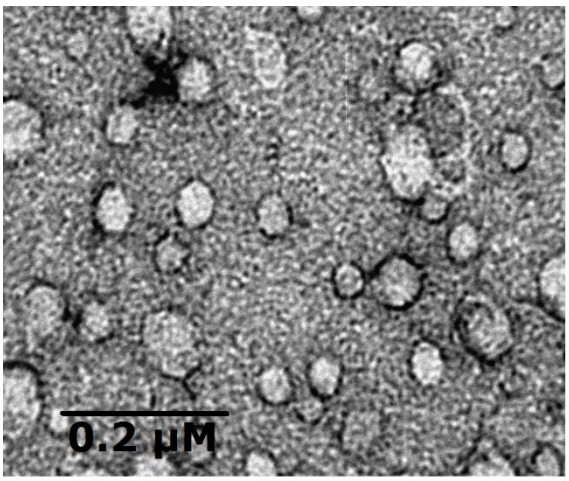

Figure 5: Electron micrograph of the pellet obtained after ultracentrifugation for exosome isolation, using 1mL of saliva from children collected in the Oragene® RNA stabilization containers. The pellet contains vesicular structures in the size order of exosomes (i.e. 30-100 nm diameter) as well as larger structures.

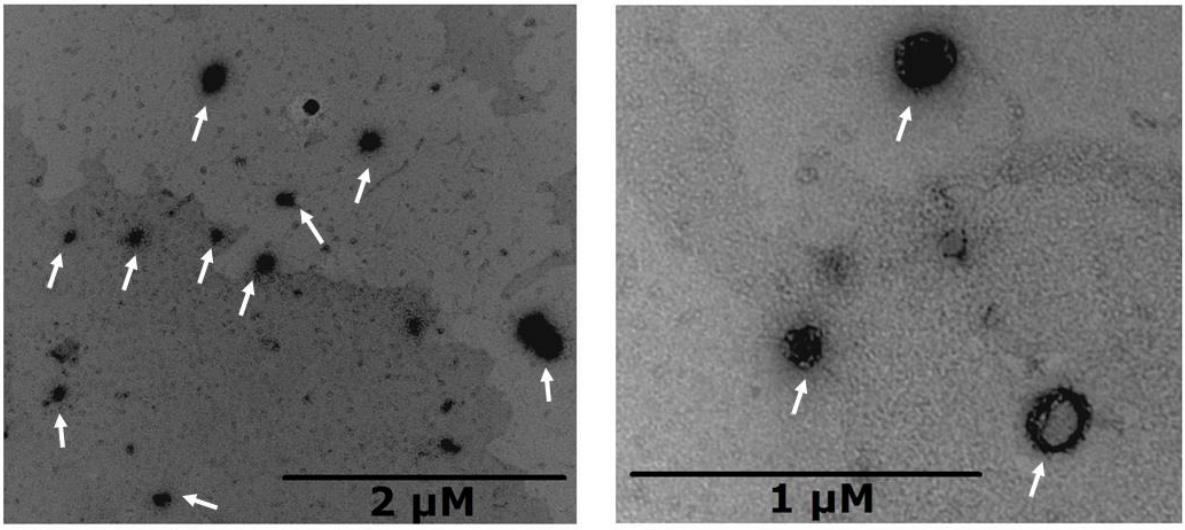

Figure 6: Electron micrograph of a CD63-DAB immunostained pellet obtained after ultracentrifugation for exosome isolation, using 1 mL of saliva from children collected in Oragene® RNA containers. White arrows indicate some of the CD63-positive vesicular structures.

On immunostained CD63-DAB electron micrographs of the resuspended exosome pellet, presence of CD63 (i.e. an exosome-specific marker) on vesicular structures was demonstrated (Figure 6). However, the size of the vesicles containing the CD63 was variable and also vesicles larger than 100 nm (up to 200 nm) were shown to be CD63 positive. However, determination of exosomes via an exosome-specific ELISA (CD9-specific; ExoTEST, Gentaur) failed to detected exosomes in all samples as the absorbance of samples did not fall in the range of the calibration curve which could quantify up to 500 ng of exosomes per µL.

3.1.2. RNA purification methods

In order to ensure optimal RNA quality of the samples, different extraction methods (i.e TRIzol-based extraction, phenol-chloroform and a column-based extraction) were evaluated. Extraction protocols were evaluated based on RNA yield as well as purity ratios A260/A280 and A260/A230 which were assessed by means of spectrometry. RNA purification using TRIzol (Ambion) resulted in high yields, though samples were highly contaminated with solvents as indicated by low (< 1,00) A260/A230 ratios. The absorption spectra of RNA samples purified using TRIzol deviated from normal patterns by the presence of absorbance peaks at 230 nm and 270 nm, which indicate the contamination of samples by phenol residues. Furthermore, high yields achieved using this extraction method could be explained by the presence of the 270 nm peak as it could elevate the absorbance at 280 nm. Phenol-chloroform extraction of salivary RNA resulted in low yield (40 ng/µL ± 34) (mean ± standard error) and lower purity ratios A260/A280 (1.49 ± 0.09) and A260/A230 (0.12 ± 0.10). Column-based purification using miRNeasy Mini (Qiagen) obtained a RNA yield of 215 ng/µL ± 61, though yield was sample-dependent. In the samples with low RNA concentrations (i.e. <100 ng/µL), the A260/A230 ratios were low (0.76 ± 0.11). When RNA concentrations were higher (>300 ng/µL) the A260/A230 ratios were 2.10 ± 0.11, indicating lesser solvent contamination, however the contamination could also be masked by the high absorbance at 260 nm. Purity ratios obtained by spectrometry showed none too little protein contamination of the samples, since all A260/A280 ratios ranged from 1.80 to 2 (1.94 ± 0.04).

3.1.3. RNA quantification methods

Quantification of nucleic acids can be carried out using different spectrometry techniques. The quantification based on spectrometry using Nanodrop was compared to measuring fluorescence of the Qubit fluorometer.

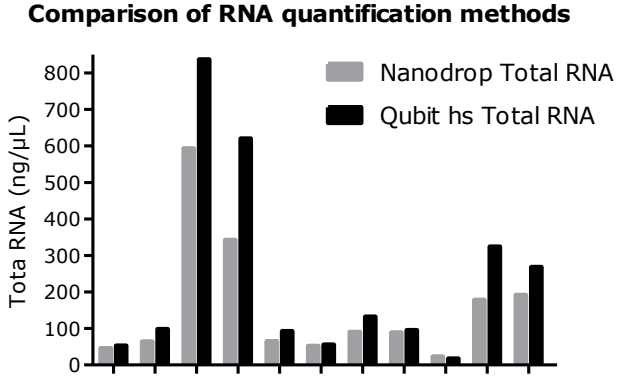

Figure 5: The RNA yield of the samples was determined using both Nanodrop spectrometry and Qubit fluorimetric measurements for independent samples (n=11). RNA concentration measured with the Qubit RNA hs assay show to be higher compared to the Nanodrop measurements.

Total RNA concentrations measured using the Qubit RNA hs Assay showed to be systematically higher compared to Nanodrop measurements of the samples (Figure 7). Concentrations according to the Qubit RNA hs assay were 47 % (± 6 %) higher compared to concentrations measured by Nanodrop.

3.1.4. Determination of miRNA content and quality in the RNA samples

RNA was extracted using the miRNeasy Mini kit, which enables the co-purification of small RNAs with total RNA. The amount of miRNAs in the RNA samples was evaluated using the Qubit miRNA assay and the presence of miRNAs with Small RNA Bioanalyzer. Firstly, as indicated by fluorimetric analysis using the Qubit miRNA assay, all samples contained miRNAs (14% ± 1% of the total RNA) as represented in Figure 8.

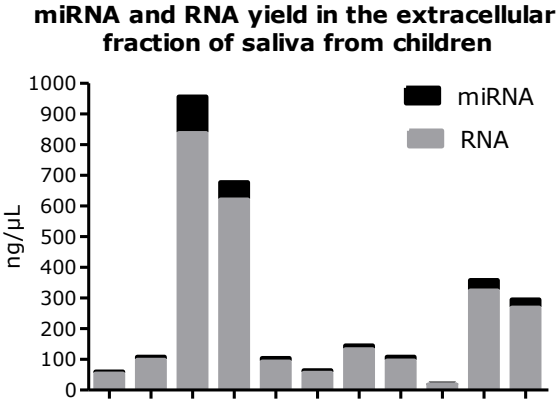

Figure 8: Representation of the amount of total RNA and miRNA purified from salivary exosomes using the miRNeasy mini kit (Qiagen), as measured using Qubit RNA hs and miRNA assays. In independent samples (n=11) miRNAs make up a small fraction (13.77% ± 1.03%) of the total RNA.

Secondly, the presence and quality of miRNAs in the samples was assessed by the small RNA Agilent 2100 Bioanalyzer platform. Bioanalyzer results of the small RNA kit showed the presence of nucleotides in the miRNA range (Figure 8) on the electropherogram.

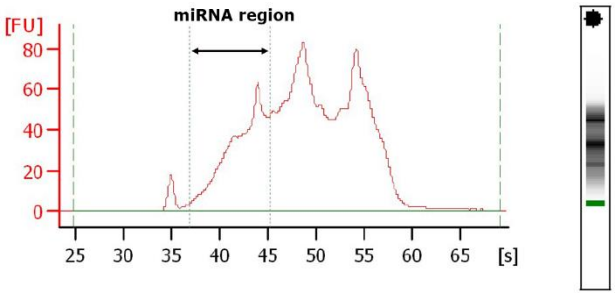

Figure 8: Bioanalyzer analysis of small RNAs from salivary exosomes. The electropherogram plots the migration time (in seconds) at which the different fragment lengths show up in the electropherogram versus the associated fluorescence intensity (in FU). The densitometry plot shows the gel electrophoresis of the samples, indicating the RNA of different size orders is present in the sample.

3.1.5. Efficiencies of Taqman microRNA assays

In order to assure precise measuring of the miRNAs using the Taqman microRNA assays, efficiencies of the PCR reactions were tested using a serial dilution series of five samples (Supplemental information). Efficiencies were compared between cDNA synthesized using solely the Megaplex Human pool A (Life Technologies) primers as well as addition of the *cel-miR39* RT primers to the Megaplex RT reaction (Supplemental information) in order to control for differences in PCR efficiency of the assays. For correct normalization, efficiencies of all assays must between 90% and 110%.

qPCR reactions were found to be more accurate with high cDNA input and more variation between triplicates was found above 30 Ct. Variation between triplicates was shown to be influenced by the Ct. Maximum tolerated variation between triplicates was 0.5 Ct. When the fluorescence threshold was exceeded at lower cycles, the differences between triplicate Ct values were predominantly <0.5

Ct. However with high Ct values (i.e. > 30 Ct) variation between the triplicates was often too high (i.e. > 0.5 Ct) and samples must be excluded. Additional optimizations for the qPCR reactions (i.e. for the reaction volume and the mastermix composition) are described in the supplemental information section. Average PCR amplification efficiencies were calculated based on the PCR efficiency of five samples (Table 6; see Supplemental information for further details on PCR efficiencies).

Table 3: PCR amplification efficiencies of Taqman microRNA assays with salivary exosomal cDNA (Megaplex RT and *cel-miR39* RT).

miRNA	Efficiency	Detection range (Ct)
cel-miR-39	102 %	11-18
hsa-miR-146a-5p	101 %	23-32
hsa-miR-128a-3p	90 %	26-33
hsa-miR-222-3p	108 %	11-18
hsa-miR-21-5p	125 %	26-33
hsa-miR-9-5p	87 %	25-32
hsa-miR155-5p	116 %	26-30

The detection range represents the range of Ct values for cDNA samples diluted 10 to 270 fold for five independent samples.

3.2. Normalization strategy

Since there are no established saliva reference genes in the context of extracellular miRNA research, normalization of the qPCR results is not straightforward. Reference genes tend to be housekeeping genes, necessary for cellular maintenance and are as such not stable in extracellular matrices. In this study, we evaluate different normalization strategies. Normalization of the qPCR experiments was derived by an external spike-in miRNA control during the phenolic phase in the extraction procedure as technical reference and compared to normalization to protein content of the exosomal pellet and RNA and miRNA yield from this fraction.

3.2.1. Comparison between different normalizers

The stability of the normalizers was evaluated using Genorm software (qBase+, Biogazelle). The software allows to estimate the variation of each normalizer, which is reflected in the Genorm M-score. Since these are not reference genes, we want to evaluate whether large deviations are present between different levels of quantification of the start material. Thirty random samples were selected for each qPCR plate in order to test the stability of the normalizers. The Genorm M-score was never smaller than 0.5 (Figure 9). For proper normalization of the qPCR data, the normalizer should be stable and abundance has to be consistent in order to normalize data in an accurate manner. Variation of the total RNA yield is smaller compared to other normalizers.

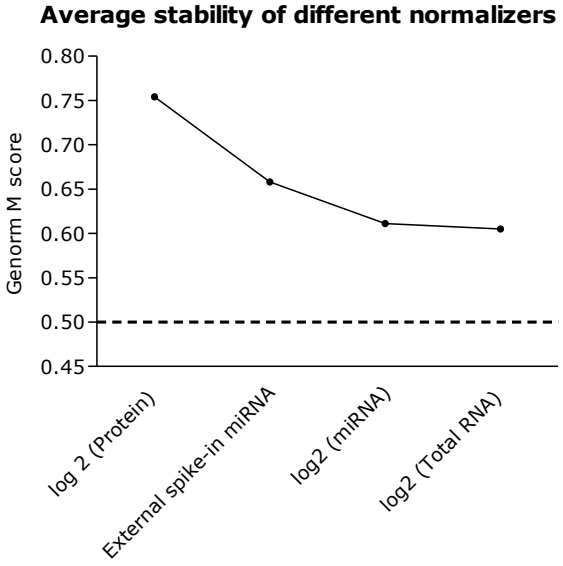

Figure 9: Average stability of the different normalizers. Analysis using Genorm software is carried out in order to evaluate the stability, which can here be defined as the variation between samples, of the normalizers in our study population.

Furthermore, the normalizers were plotted against each other and correlation coefficients were determined in order to estimate differences in the normalization using these normalizers (Figure 10 and 11).

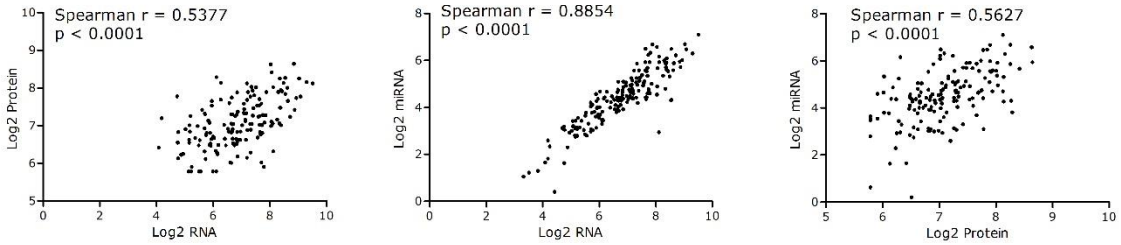

Figure 10: The correlation between sample-dependent candidate normalizers. All sample-dependent normalizers (protein content, total RNA and miRNA yield) are significantly correlated indicating a positive association between all variables. Since there was no Gaussian distribution of the variables, Spearman correlation was carried out.

Firstly, a significant positive correlation is found between the sample-dependent normalizers indicating an association between the protein content and the RNA yield from this fraction as well as the miRNA species within the total RNA (Figure 10). Secondly, a strong negative correlation was found between the external spike-in miRNA and the protein content (r =-0.45); total RNA content (r = -0.77) and the miRNA content (r = -0.65) (Figure 11). A significant positive association was found between protein content and RNA yield (P<0.0001) using a univariate mixed model.

The COGNAC samples are equalised by means of the RNA content before reverse transcriptase, since there is a relatively small variability over the different levels between RNA, miRNA and protein content combined with a high positive correlation.

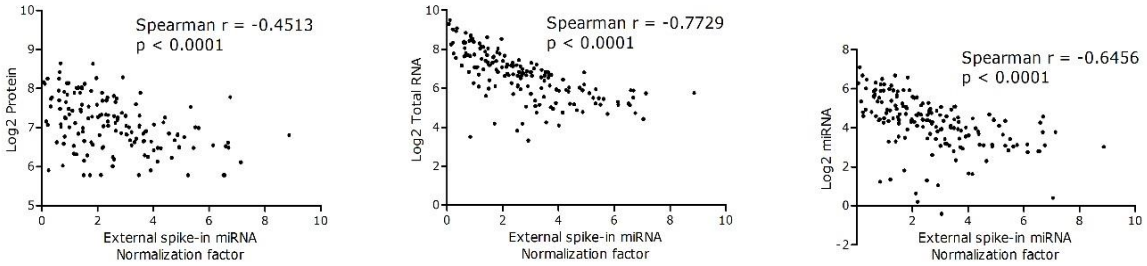

Figure 11: The Spearman correlation between the external spike-in miRNA and sample-dependent candidate normalizers. A significant negative correlation between the spike-in miRNA and sample-dependent variables is found as a consequence of dilution of the samples according to the RNA concentrations.

3.2.2. Effect of air pollution exposure on the different normalizers

To determine the effect of PM exposure on the used normalizers, a univariate linear regression using mixed models was applied for PM exposure parameters. Total RNA yield as well as protein content are both positively associated with PM exposure. For example, indoor UFP concentrations are significantly associated with protein content of the saliva (P=0.0437) and indoor PM_{10} exposure is associated with increased RNA content of the extracellular fraction of the saliva (P=0.0288). Since total RNA and protein levels might be influenced upon short-term exposure to PM, these can be described as experimental variables, which are positively associated with each other (P<0.0001).

Though the synthetic miRNA was induced during sample processing, a negative association between indoor UFP and the normalization factor (NF) for this spike-in miRNA was observed (P=0.0013). The negative association between exposure and the external spike-in miRNA can be caused by the influence of exposure to RNA content. RNA content was found to be positively associated with PM exposure as such high exposure might result in higher RNA content of the sample. Since the samples were diluted to a certain input concentration for RT, the external spike-in miRNA can be influenced by this. Indeed a significantly negative association between the RNA concentration and NF of the spike-in miRNA is found (P<0.0001).

3.3. miRNA expression analysis in the COGNAC study

All subjects participated in two study visits between November 2012 and January 2014. The characteristics of the study population are described in Table 3.

Table 3: Population characteristics (n=90)

Characteristic	Subcharacteristic	No.	Percent (%)
School			
	Kiewit	44	48.9
	Zonhoven	46	51.1
Gender			
	Female	46	51.1
	Male	44	48.9
Age (year)			
	7	1	1.1
	8	6	6.7
	9	20	22.2
	10	35	38.9
	11	26	28.9
Educational level mother			
	Primary education	0	0.0
	Secundary education	28	31.1
	Higher education	59	65.6
	Not known	3	3.3
Educational level father			
	Primary education	2	2.2
	Secundary education	29	32.2
	Higher education	57	63.3
	Not known	2	2.2
Occupation mother			
	Labourer/ Unoccupied	11	12.2
	Employee/ Education	42	46.7
	Entrepeneur/ Executive position	34	37.8
	Not known	3	3.3
Occupation father			
	Labourer/ Unoccupied	10	11.1
	Employee/ Education	38	42.2
	Entrepeneur/ Executive position	41	45.6
	Not known	1	1.1
Passive smoking			
	Yes	10	11.1
	No	79	87.8
	Not known	1	1.1

The interaction between PM_{10} and UFP concentrations was evaluated (Figure 12). An inverse association between PM_{10} and UFP concentrations is found, however, not significant (*P*=0.07) for indoor concentrations. Additionally, a significant inverse correlation between both is found (Spearman r = -0.2552; *P*=0.0005).

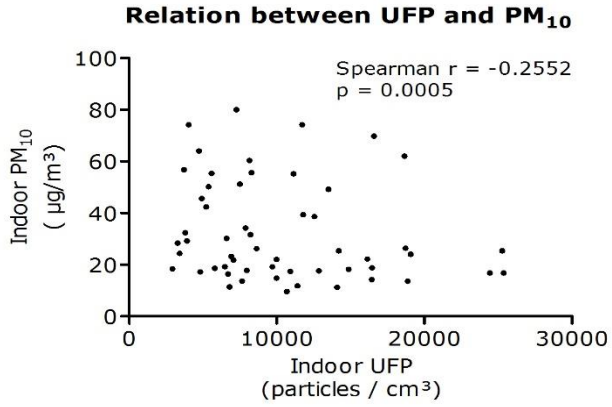

Figure 12: The relation between indoor UFP particles an PM10 concentrations during the examination. A negative association between UFP and PM10 was found (Spearman r = 0.2552; p = 0.0005).

PM exposure was measured during the fieldwork using portable measuring tools. Mean PM concentrations are listed in Table 4. Residential traffic exposure at the current address is determined represented in Table 12 in the supplemental information.

Table 4: Short-term exposure characteristics

School	Measurement	Pollution indicator		Mean ± SD	25th percentile	75th percentile
Zonhoven	**1**	**UFP**	**Indoor**	8338 ± 4010	4907	11715
			Outdoor	29982 ± 23071	15622	32408
			Morning	28707 ± 15989	15992	42773
		PM_{10}	**Indoor**	57 ± 14	46	70
			Outdoor	119 ± 127	55	127
			Morning	135 ± 85	49	177
	2	**UFP**	**Indoor**	12905 ± 5591	7637	17007
			Outdoor	39759 ± 21852	17853	51295
			Morning	35681 ± 17233	22889	56151
		PM_{10}	**Indoor**	17 ± 5	12	19
			Outdoor	32 ± 17	20	51
			Morning	35 ± 17	26	44
Kiewit	**1**	**UFP**	**Indoor**	7176 ± 2639	5382	8208
			Outdoor	19694 ± 7460	14307	26723
			Morning	18095 ± 6009	14544	21099
		PM_{10}	**Indoor**	27 ± 12	18	33
			Outdoor	59 ± 43	25	86
			Morning	55 ± 30	25	80
	2	**UFP**	**Indoor**	12183 ± 7588	6481	17742
			Outdoor	44570 ± 28217	17310	69864
			Morning	36268 ± 20647	17505	53045
		PM_{10}	**Indoor**	24 ± 7	18	26
			Outdoor	29 ± 14	21	33
			Morning	30 ± 12	27	31

UFP concentrations are represented in particles/cm³ and PM_{10} concentrations in µg/m³

3.3.1. The effect of particulate air pollution on miRNA expression

In a multivariate-adjusted model, the association between exposure and miRNA expression is corrected for possible confounding factors such as individual risk factors, spatio-temporal factors, socioeconomic factors as well as a personal exposure to tobacco smoke and the experimental variable RNA content of the extracellular fraction of the saliva. All statistical analysis are carried out using mixed models, taking in account the repeated measurements by subject and school. Normality of the residuals was evaluated using QQ-plots. The statistical analysis is only carried out in case of complete availability of the covariate data, in case of missing data, subjects were excluded resulting in n <180. The number of included individuals in the analysis is shown in Table 5, in which n represents the number of included subjects.

Table 5: Effect of short-term exposure to air pollution on miRNA expression

		miR222				miR146a				miR155			
		n	β	p-value	FDR ŧ	n	β	p-value	FDR ŧ	n	β	p-value	FDR ŧ
UFP	**Indoor**	149	0.000037	**0.0125**	**0.025**	123	6.036E-6	0.5903	0.9621	157	1.8E-5	0.1134	0.2041
	Outdoor	149	0.000015	**0.0014**	**0.0054**	123	2.508E-6	0.4579	0.9621	157	7.315E-6	**0.0276**	0.1242
	Morning	149	0.000011	0.0519	0.0779	123	1.89E-6	0.6414	0.9621	157	9.825E-6	**0.0152**	0.1242
$PM_{2.5}$	**Indoor**	149	6.2068	0.8336	0.8336	121	19.1621	0.388	0.9621	154	5.8781	0.8011	0.8011
	Outdoor	149	-5.0942	0.4045	0.4551	123	-2.3597	0.5917	0.9621	157	-2.1155	0.5878	0.6613
	Morning	149	-7.1958	0.2865	0.3684	123	-1.1494	0.8223	0.9788	157	-4.2411	0.2964	0.3822
PM_{10}	**Indoor**	149	-17.8599	**0.0018**	**0.0054**	121	2.9481	0.4737	0.9621	154	-6.8776	0.0676	0.1879
	Outdoor	149	-7.8763	**0.0104**	**0.0225**	123	-0.05876	0.9788	0.9788	157	-2.1284	0.2973	0.3822
	Morning	149	-11.1741	**0.0002**	**0.0018**	123	0.375	0.8755	0.9788	157	-2.7435	0.0835	0.1879

Covariates considered in the multivariate analysis are age, gender and school of the subject, passive smoking and education and occupation of both parents as an indicator of social-economic status as well as the day and time of the examination and total RNA content.

Indoor and outdoor concentrations represent concentrations at the moment of the examination whereas morning concentration represents an average outdoor concentration between 9 a.m. and 12 p.m. based on an interpolation of the measurements within this time window.

p-values < 0.05 are considered to be significant. n = number of subjects included, β = regression coefficient which represents the estimated effect ŧ p-values adjusted for multiple testing within each miRNA using the Benjamini and Hochberg FDR test.

An interquartile range (IQR) increase in UFP is associated with a 20.8% (± 7.17%) increase in *miR222* expression for indoor exposure during the study visit (*P*=0.025) and 33.8% (± 8.82%) for outdoor concentrations (*P*=0.0054). However, an IQR increase in PM_{10} concentration is associated with a decrease in the *miR222* abundance with 29.24% (± 11.27%) for indoor PM_{10} (*P*=0.0054); 22.57% (± 10.22%) for outdoor PM_{10} (*P*=0.0225) and a 35.85% (± 12.02%) decrease in the abundance for the average PM_{10} concentration during the morning of the study visit (*P*=0.0018) (Figure 13). A significant association was found between outdoor UFP and average of the UFP concentration in the morning and *miR155* expression. An IQR increase in outdoor UFP concentrations is associated with 15.25% (± 6.51%) increase in *miR155* abundance and 18.30% (± 6.99%) for an IQR increase in the average outdoor UFP concentration during the morning of the study visit. However, FDR testing abolished the effects of UFP exposure on *miR155* expression. Additionally, results indicate that *miR146a* is not affected by short-term exposure to PM air pollution (Table 5).

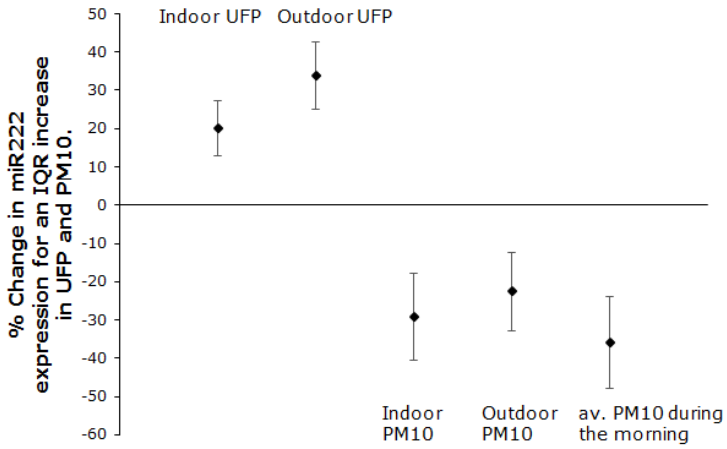

Figure 13: Estimated percentage changes and standard errors in miR222 expression for an interquartile range (IQR) increase in indoor, outdoor UFP concentrations (n=149) and indoor, outdoor and morning (i.e. 9a.m. until 12p.m) outdoor PM10 concentrations. Models are adjusted for age, gender, school, day of the examination, time of the examination, exposure to passive smoking, total RNA content of the extracellular fraction of the saliva and socioeconomic factors (i.e. education and occupation of both parents).

3.3.2. Effects of miR222 and miR146a expression on cardiovascular parameters

The association between miRNA expression and cardiovascular parameters such as blood pressure and heart rate was evaluated (Table 6). Multivariate mixed model analysis took in account the repeated measurements by subject and school as well as following covariates: age, gender, BMI, passive smoking status and school of the subject and the time of the examination.

Table 6: The effect of miRNA expression on cardiovascular parameters

	miR222			miR146a			miR155	
	n	**β**	**p-value**	**n**	**β**	**p-value**	**n**	**β**	**p-value**
Diastolic BP	160	-0.65	0.05	133	-0.075	0.89	169	-0.51	0.28
Systolic BP	160	-0.647	0.07	133	-0.077	0.9	169	-0.512	0.29
Heart rate	160	-0.541	0.13	133	0.28	0.64	169	-0.37	0.45

BP = blood pressure; n = number of subjects included; β = regression coefficient which represents the estimated effect.

Covariates considered for entrance in the model are age, gender, BMI and school of the subject, exposure to passive smoking, the time of the examination and RNA yield from saliva.

p-values < 0.05 are considered significant.

No significant associations are found for the cardiovascular parameters and *miR146a* and *miR155* expression. No association was found for *miR222*, though the association was borderline non-significant (*P*=0.05 for diastolic BP and *P*=0.07 for systolic BP).

However, in a univariate model, which is not adjusted for covariates, a significant association between *miR222* and blood pressure could be found (Table 7). However, stepwise addition of covariates to the model showed that it was the addition of RNA yield, which is significantly associated with miRNA expression, was responsible for the abolishment of the statistical significance.

Table 7: The effect of miR222 expression on cardiovascular parameters without multivariate-adjustment.

	n	**β**	**p-value**
Diastolic BP	165	-0.5452	**0.0326**
Systolic BP	165	-0.5899	**0.0297**
Heart rate	165	-0.4663	0.08

BP = blood pressure; n = included subjects, β = regression coefficient which represents the estimated effect; p-values < 0.05 are considered significant.

3.3.3. Target prediction of the miRNAs

The targets for *miR222*, *miR146a* and *miR155* were determined (Table 8) using online prediction tools as described in section 2.9.

Table 8: Predicted genes of *miR222*, *miR146a* and *miR155* according to online prediction tools

miRNA	**Predicted targets * (TargetScan)**	**Predicted targets (Tarbase)**
miR-222	RIMS3, RFX7, CDKN1B, MIDN, ZNF652, ATXN1, WNK3, BEND4, UBN2, BCL2L11	CDKN1B, KIT, PPP2R2A, p27kip1, CDKN1C, ERα, p27, NM_004064, CDKN1B/p27/Kip1, MMP1
miR-146a	TLN2, TRAF6, SLC38A1, NOTCH2, MPPE1, KPNA6, ABL2, ALX4, MYO5A, SEC23IP	TRAF6, ALP, EGFR, TLR2, ROCK1, IRAK2, CFH, BRCA1, OPN, BRCA2
miR155	ZNF652, FGF7, SMAD2, TRPS1, KLF3, TSHZ3, JARID2, KPNA1, RBMS3, BRWD3	SMAD2, ETS-1, JARID2, POLE3, PICALM, ARL6IP5, NF-κB, C3orf18, FAM135A, E2F2

** Top 10 target genes based on aggregate P CT score using TargetScan 6.2*

4. Discussion

4.1. Optimization phase

miRNAs and RNA originating from salivary exosomes are validated for biomarker research in a diseased populations.[48, 49] Additionally, the miRNA content of salivary exosomes is reported to be sufficient for qPCR analysis.[48, 50] Within this study, the exosomal miRNA expression upon PM exposure will be evaluated. However, up to now there are no reports on the exosome isolation from saliva samples collected in the Oragene® RNA containers.

4.1.1. Exosome characterisation

Using electron microscopy (EM), the vesicles achieved by repeated centrifugation of saliva at different centrifugation forces were visualized. Vesicles of different size orders, including in the exosome size range of <100 nm in diameter, were present (Figure 5). Identification of CD63 on the surface of exosomes using DAB immunohistochemistry revealed also the presence of vesicles >100 nm in diameter with the CD63 marker. However, this is larger than would be expected for exosomes as described in literature.[33] The use of DAB for immunohistochemical purposes is rather robust, the use of a CD63-immunogold labeling might be carried out in order to strengthen the results using DAB staining. The identification of exosomes based on a CD9-specific ELISA failed to show the presence of exosomes in our samples. The low absorbance of the samples could be due either to the little presence of the CD9 antibody on salivary exosomes, or too little exosomes according to the sensitivity range (i.e. up to 500 ng/µL) of the ExoTEST or that the CD9-antibody is not present at all on salivary exosomes. Furthermore, no previous reports on the use of the ExoTEST were found and there was no information present on the use of the test with saliva samples. This could indicate that the ExoTEST, which is specifically developed for the identification of exosomes, is not compatible with the use of salivary exosomal samples. It is difficult to distinguish between exosomes and microvesicles based on size or markers and use of a more broader term such as extracellular vesicles (EV) might be at this point more appropriate, which is also stated in a report of the international society for extracellular vesicles (ISEV).[51] EV isolation can be carried out by both differential centrifugation and gel filtrations.[51] Differential centrifugation will eliminate the cells and food debris as well as bacteria before pelleting of the vesicles. Differential centrifugation steps, in which the centrifugation force is gradually increased, were already successfully applied for exosomal isolation from saliva.[48] Here, the exosomes were characterized using TSG101-specific Western Blotting.[48]

Exosomal isolation based on ultracentrifugation results in the co-purification of other vesicles as well as protein complexes such as Argonaut 2 (Ago2) (personal communication prof. dr. Olivier De Wever (Ghent University)). Ago2 is a protein which complexes with miRNAs. The co-purification of these protein-miRNA complexes with the exosomes, might lead to a higher yield of RNA and miRNAs. Density gradient centrifugation using OptiPrep will lead to a more pure isolation of exosomes without the co-purification of other vesicle types or protein complexes (personal communication prof. dr. Olivier De Wever). In the future, the composition of our samples will be evaluated using Nanosight at Ghent University, which allows determining the size distribution and concentration of the particles.

4.1.2. Extracellular RNA isolation with inclusion of small RNA species

Three different methods for isolation of RNA from the extracellular fraction were compared: TRIzol-based extraction, phenol-chloroform extraction and column-based extraction using the miRNeasy Minit kit (Qiagen). These techniques allow the co-purification of small RNA species such as miRNAs with the total RNA. Phenol-based extraction methods resulted in high quality of the RNA, though contamination of the RNA samples using these methods is commonly reported when RNA yield is low.[52] The use of acid guanidium phenol, such as TRIzol and QIAzol, is known to cause peaks at 270 nm and 230 nm in the ultraviolet (UV) spectra. Column-based extraction methods can reduce the carry-over of these products.[52] The column-based extraction using QIAzol (miRNeasy mini kit) showed to outperform the TRIzol-based phenol-chloroform extractions in our comparative analysis, since we found less contamination. However, in samples with low RNA yield, an absorbance peak at 230 nm is still present and contributes to low A260/A230 ratios in these samples. This could interfere with PCR reactions, therefore before quantifying miRNAs with specific assays on our samples, we tested the efficiencies of the assays.

The presence of small RNA species in the purified RNA was evaluated using Agilent Small RNA Bioanalyzer. In this analysis, fluorescent peaks in the region of the miRNAs (Figure 8) suggest the presence of miRNA in RNA purified from salivary exosomes. Presumably due to low concentrations we cannot obtain an electropherogram showing excellent quality of miRNA. Quantification of the miRNAs using Qubit measurements also strengthens this observation. Previous reports also report the abundant presence of miRNAs and RNAs from exosomes and other extracellular vesicles.[50]

4.1.3. Amplification efficiency of Taqman microRNA assays

We tested the compatibility between our samples and the Taqman assays (Life Technologies), in terms of efficiencies. The ideal primer amplification efficiency is 1 (100%), which means that the PCR product will double with each cycle in the exponential phase of the reaction. However, deviations from the ideal efficiency can be accepted in a certain range (i.e. 90%-110%) since primers rarely have an ideal amplification efficiency.[53, 54] Since miRNAs have a low abundance in the COGNAC samples (27ng/µL ± 2 ng/µL), the highest diluted samples in our serial dilution series fail to reach the fluorescence threshold within 40 cycles or have a Ct > 30, which results in high variation. This implies that these highly diluted samples have insufficient miRNA for efficient qPCR reactions. Therefore, we chose to include the assays with 1) an acceptable PCR amplification efficiency and 2) Ct scores lower than 32. Only *miR222*, *miR146a* and *miR155* as well as the spike-in *cel-miR39* for normalization were quantified.

4.2. Normalization strategy

The protein content and RNA and miRNA yield were compared to an external spike-in miRNA for normalization of qPCR data. A moderate correlation (i.e. 54.16%) was found between protein content and RNA yield, however RNA and miRNA yield were highly correlated. Protein content and total RNA yield were both associated by PM concentrations. Increased levels of proteins and RNA might be a result of increased intercellular communication. Exosomal transport is increased in cases of cancer by an increase of exosomes[55], however there is no information available on the associated RNA load. As such, protein content and RNA content might be identified as experimental variables, which indicates that these should be confounded for in the multivariate model. Though variability of the external spike-in miRNA among the samples is relatively high (M-score is approximately 0.65; Figure 9) for normalization of expression data, this might be explained by the variation induced by dilution of the samples according to their RNA concentration and the heterogeneity of the samples. Furthermore, this could also explain the effect of PM on the external spike-in NF. Since protein level and RNA yield are experimental variables which 1) correlated highly with the candidate normalizer miRNA content and 2) might influence the variability of the external spike-in and explain effects of PM on the spike-in NF via RNA-based dilutions, normalization to the external spike-in miRNA is shown to be the most reliable normalization strategy. Addition of either protein and RNA levels in the statistical model for the estimation of the effect of PM on miRNA expression is reasonable since both might be interdependent confounding variables.

Exosome-research is still growing and reports on exosomal miRNA expression analysis are already published, however there is no consistency in the use of reference genes. Several small RNA species provide possible reference genes for miRNA normalization (i.e. small nuclear RNA, small nucleolar RNA (snoRNA), etc). The use of an external spike-in RNA is already reported in extracellular miRNA research using the comparative Ct method ($2^{-\Delta\Delta Ct}$).[47] Since a synthetic spike-in miRNA is added in an equal amount to each sample, it cannot correct for biological variation between samples, which is desired for a reference gene. Though, it can correct for technical variation induced by manipulation of the samples throughout processing. Recently, the use of an external spike-in miRNA for qPCR normalization was compared to other common techniques such as normalization to the average Cq and the use of candidate reference miRNAs in an extracellular context.[56] Results concluded the importance of adequate normalization when effect sizes are smaller or miRNA is less abundant – as it is in our samples. Furthermore, results from this study are consistent with ours, where miRNA en RNA content can be affected by study variables and spike-in miRNA showed to be the best normalizer as candidate reference miRNAs might also be affected by experimental conditions.[56] Additionally, when miRNA and RNA content are affected by study variables, normalization to a endogenous candidate reference miRNA may not take this into account and erroneous conclusions could be made.[56] The use of *cel-miR39* spiked-in during RNA isolation as a normalizer corrects for the global RNA content, which is yielded upon an equal amount of saliva.

4.3. miRNA expression analysis in the COGNAC Study

In this project, the effect of air pollution on extracellular miRNAs in saliva samples from children was investigated. miRNA expression was evaluated in a miRNA-specific manner, as such *miR222*, *miR146a* and *miR155* were selected for estimating the effects of UFP, PM_{10} and cardiovascular outcomes.

4.3.1. Effect of air pollution on miRNA expression

A negative association between PM_{10} concentrations and *miR222* was observed whereas UFP concentrations showed to be positively associated with *miR222*. These associations were consistent for both indoor and outdoor concentrations of the air pollutants. Effects of indoor pollutants tend to have larger estimated effects on *miR222* expression though the concentrations are higher outdoors and result in larger changes in *miR222* expression. Controversially, opposite trends of the associations between UFP and PM_{10} and *miR222* are observed (Figure 12). Indoor PM_{10} and UFP concentrations showed to be significantly correlated in an inverse manner. This can explain the opposite association for *miR222* with UFP and PM_{10}, as an increase in PM_{10} is associated with both a decrease in *miR222* expression and in UFP concentrations. The parameters for particulate air pollution exposure reflect short-term exposure with measurements as early as one hour before the study visits for outdoor concentrations and acute exposure indoors during the study examinations. No associations with PM exposure and *miR146a* and *miR155* expression were found after FDR correction. However, without FDR testing *miR155* was positively associated with UFP exposure, this might indicate that the study population was too small in order to detect changes in *miR155* expression.

Bollati and colleagues[28] found an effect of PM on miRNA expression in workers of a steel plant, who are subjected to PM_{10} and heavy metal exposure during work. Expression of miRNAs was compared between a baseline and after three days of work, hereby evaluating an effect of short-term exposure. They found an increased *miR222* expression after occupational exposure compared with the baseline, though no effects were found on *miR146a* expression. *miR222* was significant positively associated with occupational exposure yet individual exposure levels only indicated an effect of lead exposure on the expression levels. Furthermore, *miR146a* expression was negatively associated with cadmium and lead exposure and occupational exposure did not result in a changed expression compared to the baseline, which implies responsiveness of the miRNA to exposure on long-term.[28] Within the same study population and same study set-up, Motta reported a differential expression of *miR146a* after occupational exposure.[31] In a gene-specific manner, changes in *miR222* could be found with occupational metal-rich PM exposure, though associations are due to the heavy metals.[28] Evaluating samples of the same study cohort using microarray, no effects could be found for *miR222* yet *miR146a* shows to be up-regulated with metal-rich exposure. If data would be available on the exposure characteristics[28, 31] or on the association of the miRNA expression with PM concentrations[31], these data could be more straightforwardly compared with data from our study. Firstly, since for the microarray data no associations with PM or metal components are reported, changes in *miR146a*

expression cannot be attributed with certainty to PM.[31] Additionally, an association between *miR146a* and lead and cadmium was described earlier as well as an association between lead exposure and *miR222* expression.[28] Secondly, the occupational exposure in this cohort is more extreme compared to the exposure in our study cohort. Although, no exposure characteristics are available to compare PM_{10} exposure between both cohorts, *miR222* expression follows the same trend in expression as our study does for increased PM_{10} exposure. Recently, Bollati also observed changed miRNA expression in extracellular vesicles in blood within the same study set-up of the steel factory study.[29] As previous reports within this cohort were on the miRNA expression in leukocytes, this study can be more adequately compared with our study results. Whereas in leukocytes associations in *miR222* [28] and *miR146a* [31] expression with metal-rich exposure could be found, expression of these miRNAs is not affected in microvesicles in blood.[29] However, an up-regulation of *miR128* and *miR302c* was found in blood microvesicles.[29] *miR128a* was not highly abundant in the salivary extracellular fraction of our study population (i.e. Ct >35 due to which the miRNA was not quantified in our population). This could be explained by the dose-dependency of increased *miR128* expression which is associated with PM exposure in A549 pulmonary cell lines.[29] PM_{10} concentrations to which our study population is exposed to are lower compared to the occupational exposed study group, therefore it could be possible that a certain threshold of PM_{10} exposure in order to affect *miR128a* expression is not yet reached within our study population.

Fossati and colleagues found a negative association between $PM_{2.5}$ exposure (as well as other pollutants) and the expression of eight miRNAs, including *miR146a*, *miR155* an *miR222*.[30] They found that an IQR increase in $PM_{2.5}$ (i.e. 3,38 µg/m³, 7-day) was associated with a change of -20% (-34% to -8%) in *miR222* expression. The $PM_{2.5}$ exposure in this study population is lower compared to outdoor $PM_{2.5}$ concentrations in our study population (IQR = 21,79 µg/m³; average morning of the examination). Based on the IQR, indoor $PM_{2.5}$ concentrations are similar to $PM_{2.5}$ concentration reported by Fossati et al.[30] However, the changes in *miR222* expression are comparable to the changes we find in association with UFP. It should be kept in mind that the findings of this study are in leukocytes and not on extracellular vesicles. Still, a change in cellular miRNA expression might also be communicated via extracellular miRNA (i.e. in complex with proteins or packaged in vesicles) transport to other cells, in which case the miRNA expression in the extracellular fraction can also be altered upon PM exposure.

In comparison with existing literature, that finds a negative association between particulate air pollution exposure and *miR146a* expression in leukocytes, we do not find an association between extracellular *miR146a* en PM. Possibly, cellular changes in *miR146a* might not be persistent enough for communication via extracellular transport to other cells. Then this might explain why no changes in the *miR146a* expression are found in association with PM exposure in our study. Furthermore, a positive association between metal-rich PM exposure, which is enriched with PM_{10}, is found for *miR222* [28] though for smaller PM species a negative association was found with *miR222* expression.[30]

This is consistent with our findings as we find a positive association for PM_{10} and a negative association for UFP, no effect for $PM_{2.5}$ was found on *miR222* expression.

Moreover, the negative association between *miR222* expression levels and blood pressure (both systolic and diastolic) was borderline not significant (respectively *P*=0,07 and *P*=0,05). The association is not significant in our study population, a trend towards a decreased blood pressure with increasing *miR222* expression is hypothesized. To our knowledge, there are no studies that associate the *miR222* expression with blood pressure. In subjects with coronary artery disease (CAD) *miR222* expression was increased.[57] However, CAD is associated with plaque formation in the vessels, which would be associated with a narrowing of the vascular lumen.[58] If *miR222* would associated with blood pressure in patients with cardiovascular diseases, such as CAD, a positive relation might be more evident.

4.3.2. Biological interpretation of the associations between exposure and miRNA

We investigated the role of extracellular miRNA in as a mediatior of the effects of PM possibly resulting in inflammation and oxidative stress or on the other hand as a consequence of these processes. We found an association between changed *miR222* expression and PM exposure but not for *miR146a* and *miR155*. *miR222* is found to have an anti-angiogenic[59] as well as an oncogenic[60] function and is involved in redox signaling. *miR222* is also known to target the receptors for angiogenic factors and is involved in several aspects of vascular biology. Additionally, air pollution exposure is associated with an increased incidence of cardiovascular morbidity.[8, 61]

An up-regulation of *miR222* is found to be associated with vascular damage via decreased EPC differentiation via c-Kit targeting and an increased VSMC cycling via p27 targeting. This is associated with narrowing of the vascular lumen[62], which might contribute to increased blood pressure. p27 is an inhibitor of cyclin dependent kinases (Cdk), and will thus induce a cell cycle arrest at the G1 phase. In alveolar epithelial cells, a decreased Cdk activity due to PM-induced oxidative stress resulted in an arrest of the cell cycle progression.[63] Though a role for p27 was not explored in this study, p21, which is also a Cdk inhibitor, was shown to mediate the effects of PM on cell cycle arrest.[63] Targeting of Cdk inhibitors also implies a role for *miR222* in cancer development by the abolishment of a mechanism to halt entrance to the cell cycle.[64] Additionally, *miR222* expression can be induced by factors, which are commonly involved in cancer, such as c-Jun and NF-κB[65] and ETS-1.[66] PM exposure was shown to be associated with an increased risk of cancer development.[5] *miR222* is also involved in angiogenesis by targeting cKit, which results in a decreased production of VEGF.[67] Exposure to whole diesel exhaust, which contains DEP, in mice is associated with an increase in VEGF expression and decrease in endothelial nitric oxide synthase (eNOS) production.[68]

Furthermore, *miR222* is known to regulate eNOS expression, in an indirect way.[64] eNOS is important regulator in endothelial cell biology as well as in angiogenic responses. However, it can be both a protective enzyme and a contributor to the development of oxidative stress, depending on its product.[69] Uncoupling of the eNOS dimer, which can be induced by atherogenic risk factors, is responsible for a switch in NO production to the production of O_2^- and $ONOO^-$.[69, 70] Exposure to ambient UFP is associated with a S-glutathionylation of eNOS, which results in a reduced NO production.[71] Furthermore, vascular ROS production associated with PM exposure can inactivate NO and hereby leading to endothelial dysfunction.[72] Defective eNOS might be a link between endothelial dysfunction and atherosclerosis.[69, 70] PM-associated inflammation might result in endothelial dysfunction, which in turn might contribute to cardiovascular disease development. Atherogenesis can already early in life start and hereby affect cardiovascular health later in life. Recently an increased risk for endothelial dysfunction, by the measurement of tissue factor and thrombomodulin, in association with air pollution in a pediatric population was reported.[73] This indicates the susceptibility of children for detrimental effects of air pollution on cardiovascular health,[73] in which *miR222* also might play a role.

Redox-signaling plays an essential role in vascular biology, as such superoxide dismutase-2 (SOD2) counteracts ROS formation and is involved in the expression of *miR222*.[74] In ischemia, *miR222* expression will be blocked as a consequence of decreased SOD2.[74] Elevated *miR222* transcription might contribute to a blocked angiogenesis and erythropoiesis, low *miR222* levels in ischemia might be a coping mechanism for tissue regeneration and formation of novel vessels. Mice exposed to second-hand tobacco smoke (SHS) early in life showed to have a higher oxidant load as well as decreased SOD2 specific activity.[75] Mice with a decreased activity of SOD2 suffer after environmental tobacco smoke (ETS) exposure, which is high in PM, from increased oxidative stress, measured by the redox-sensitive enzyme aconitase.[76] In human lung epithelial A549 cells, $PM_{2.5}$ exposure resulted in increased ROS generation and reduced activity of antioxidant enzymes, such as SOD and catalase (CAT).[77] As PM exposure is associated with oxidative stress[1], *miR222* levels might be decreased due to low SOD2.

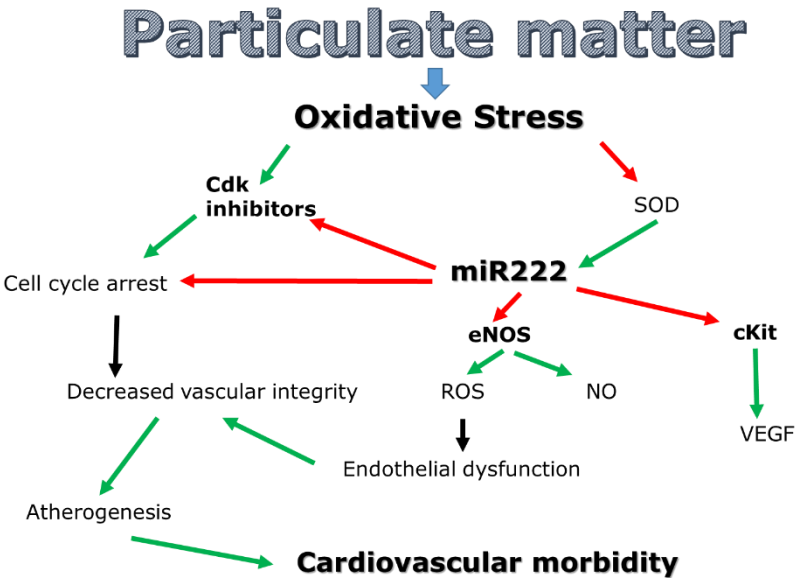

Figure 13: Hypothetically, miR222 might be a central player in oxidative stress-induced cardiovascular morbidity, which is associated with PM exposure.

All together, we speculate on a central role for *miR222* as a mediator between air pollution-induced oxidative stress and cardiovascular disease (Figure 13), already early in life. *miR222* expression was associated with PM on a very short notice in our study (i.e. within an hour), which indicates the fast response to PM exposure. As its expression is oppositely associated with PM_{10} and UFP exposure, rapid changes in *miR222* expression might be necessary for fine-tuning biological processes, rather than disease development. Since the association between blood pressure and *miR222* expression was borderline non-significant, further analysis with higher power should be carried out in order to strengthen or to refute the involvement of *miR222* in the cardiovascular health status in association with PM exposure.

Both *miR146a* and *miR155* are associated with inflammation in an NF-κB-dependent manner. NF-κB is an important pro-inflammatory transcription factor. In response to inflammatory stimuli, *miR146a* and *miR155* can be induced and provide a negative feedback system on the inflammatory response. As such, when PM exposure and *miR146a* and *miR155* expression are negatively associated[30], a negative feedback on NF-κB-dependent inflammation by both miRNAs might be inhibited. This suggests a role for both miRNAs in the PM-induced inflammation. However, these associations were not found in our study, which can be explained by several arguments. Firstly, the miRNA levels are compared with acute PM exposure (i.e. within a few hours). *miR146a* and *miR155* expression might not change rapidly enough within this time window. Secondly, *miR146a* and *miR155* expression are triggered by pro-inflammatory stimuli in a NF-κB dependent manner. It could be possible that PM exposure in our population did not elicit a sufficient pro-inflammatory stimulus for NF-κB activation. This suggests that either there is no rapid response to inflammation by *miR146a* and *miR155* or the exposure did not result in an inflammatory response. However, there are no data on inflammatory state or NF-κB expression to confirm this. In order to confirm this, we could measure NF-κB gene expression in the saliva.

4.4. Strengths and limitations of the study

This study is performed within the scope of the COGNAC study in which 334 children are enrolled. Due to the repeated measurement design of the study, each individual can function as its own control. In this study, two measurements (out of three) of 90 individuals were used for miRNA expression analysis. The availability of multiple measurements at different time points, gives the study more power and as such it was sufficient to include 90 out of 334 individuals to find statistical significant associations for at least one of the quantified miRNAs (i.e. *miR222*). Additionally, a large set of variables which could influence the effects of exposure, such as different factors of socioeconomic status, individual risk factors as well as spatio-termporal factors, are available for each individual. As such the multivariate-adjusted models for estimating the effect of PM exposure on miRNA expression take into account possible confounders and reduce the chance of false-positives.

The use of saliva within molecular epidemiological research is not common. Since saliva is composed out of a variety of molecules, including cellular debris, food debris, enzymes etc., the use a specific saliva collection kit (i.e. the Oragene® RNA collection method) which ensures the stability of the RNA in the sample is most definitely an added value to the project. Furthermore, the effects of air pollution on the miRNA expression in children are never evaluated before. As a whole, the project combines several novel topics into one research question, which makes it an interesting and unique approach for conducting epidemiological research. However, in a novel concept there are also uncertainties that must be tackled.

The exosome isolation from saliva collected in Oragene® RNA containers is not yet reported, therefore the integrity and presence of the exosomes must be evaluated. Additionally, availability of miRNAs in the saliva needed to be confirmed, as well as the origin of the miRNAs. Within this study, the abundant presence of miRNAs yielded by exosome-isolation protocol is already confirmed. However, there was not yet certainty about the exclusive presence of exosomes and as such the origin of the miRNAs. The origin of the miRNAs still must be confirmed by the use of additional techniques such as an Ago2 determination on the samples as well as Nanosight analysis and CD63-immunogold labelling of exosomes under EM. Nanosight analysis might shed more light on the composition of the samples as well as the origin of the RNAs. This will also help to confirm or refute the presence of exosomes. Furthermore, Ago2 determination in the samples can distinguish between protein-RNA complexes and RNA packed in extracellular vesicles. Furthermore, additional clinical parameters for comparison with the *miR222* expression data are eligible. In the future, data can be compared with the retinal arteriolar diameter, which is a measure for microvasculature and cardiovascular health of the subjects. Since FDR testing excluded a significant association between UFP exposure and *miR155* expression, more subjects need to be included to increase the power of the study. Additional, *miR146a* en *miR155* are involved in the same pathway. Therefore it might be possible that some effects are bypassed and are not detected.

5. Conclusion

Within this study, the miRNA expression of three miRNAs in salivary exosomes of primary school children was evaluated in association with PM exposure. Up to now, this study is the first to consider miRNA expression in **saliva** in association with PM exposure. Furthermore, the isolation of exosomes from saliva collected in Oragene® RNA containers was never reported before. Though using EM an abundance of vesicles was shown, as well as an abundance of protein and RNA within the isolated fraction, the presence of exosomes is not yet confirmed using CD9, which is an exosome-specific marker. CD63-positive vesicles were shown on EM, however their size was larger than is accepted for exosomes. In the future, additional techniques will be applied in order to determine the origin of the isolated RNAs and miRNAs. As such specific nomenclature must be avoided and use of a more broad term such as '**the extracellular fraction**' is currently more appropriate.

The use of an **external spike-in miRNA** for normalization of qPCR expression data is shown to outperform sample-dependent variables such as RNA concentration as the latter might be confounding experimental variables during statistical analysis.

The key finding of this study is a significant association between particulate air pollution exposure and ***miR222*** expression in the salivary extracellular fraction in children. The differential expression upon PM exposure might be a **mediator** between **PM-induced oxidative stress** and **cardiovascular disease** development. However, we did find an association between *miR222* and blood pressure, however after taking covariates in account the association showed a statistical significance of $p<0.1$. In the future, data on the microvasculature as measured by the retinal arteriolar diameter will be available since these measurements were carried out during fieldwork. These clinical parameters will be evaluated in association with *miR222* expression data.

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7. Supplemental information

7.1. Optimization of exosomal miRNA isolation from saliva

In the optimization phase of this experiment, different RNA isolation techniques were evaluated as reported in section 3.1.2. of the results. Technical information concerning these techniques is here described.

7.1.1. TRIzol-based RNA extraction

Exosomes were isolated using ultracentrifugation methods and the pellet was resuspended in 200 µL of RNAse-free water. For disruption of the vesicles, TRIzol reagent was added to the samples and briefly incubated at room temperature. For phase separation of the RNA, 1:5 amount of chloroform was added. All reagents were mixed with the sample by vortexing and incubated for 3 minutes at room temperature. After centrifuging at 12 000x g for 15 minutes at 4 °C, the aqueous phase was transferred to a clean tube containing chloroform. Samples were mixed by shaking and centrifuged at maximum speed for 15 minutes at 4 °C. The aqueous phase was transferred into a clean tube. For precipitation of the RNA, 2-propanol (100 %) was added to the samples and incubated for 20 minutes at -20 °C. Pelleting of the RNA was achieved by centrifuging at 12 000x g for 10 minutes at 4 °C. The supernatant was removed and the pellet was resuspended in ethanol (75 %) for washing of the RNA. Samples were briefly vortexed and centrifuged for 5 minutes at maximum speed at 4 °C. The supernatant was removed and after air drying for 5 minutes, the pellet was resuspended in 25 µL of RNAse-free water.

7.1.2. Phenol-chloroform based RNA extraction

Exosomes were isolated using ultracentrifugation methods and pellets containing exosomes were resuspended in RNAse-free water. The resuspended vesicles were disrupted by adding lysis buffer. For appropriating mixing, samples were vortexed. Phenol-chloroform was added to the samples and incubated for 5 minutes at room temperature. Phase separation was achieved by centrifuging the samples at maximum speed for 5 minutes at 4 °C. The aqueous phase was transferred to a tube containing chloroform and mixed by shaking before centrifuging at maximum speed for 2 minutes at 4 °C. The aqueous phase was transferred to a clean tube and ethanol (100 %) and sodium acetate (3M, pH 4.5) were added for RNA precipitation. Samples incubated at -20 °C for 30 minutes and were centrifuged at maximum speed for 15 minutes afterwards. The supernatant was removed and ethanol (80 %) was added to the samples. After centrifuging at maximum speed for 2 minutes, the supernatant was removed. Last three steps, were repeated and all fluid was removed from the pellet. The pelleted RNA was resuspended in 15 µL RNAse-free water.

7.2. Optimization of the microRNA and spike-in miRNA cDNA synthesis

During the phenolic phase of the RNA isolation procedure, samples were spiked-in with a synthetic miRNA in order to correct for technical variation as well as for normalization of the qPCR results. As the synthetic spike-in miRNA is not from human origin, it could not be reverse transcribed using the megaplex cDNA reaction which is carried out for reverse transcription of human miRNAs. However, it is preferred that RT for the spike-in miRNA and human miRNAs are carried out in the same reaction in order to correct for technical variation within this step. Therefore, it was tested whether a combination of megaplex primers and the *cel-miR-39* primers has an effect on downstream applications in qPCR. Primer amplification efficiencies were tested for six Taqman microRNA assays using both cDNA samples with only human miRNA cDNA as well as samples with both human miRNA cDNA and cDNA of the spike-in miRNA.

7.2.1. Human megaplex cDNA synthesis and spike-in miRNA cDNA synthesis

Reverse transcription of the miRNA was carried out both with and without addition of the *cel-miR-39* primers to the megaplex pool. Mastermixes were made for both conditions. In case of absence of the *cel-miR-39*-specific RT primers, RNAse-free water was added. For each reaction, 5 µL of mastermix was added to 2.5 µL of RNA sample. Mastermix contained 0.2 µL of dNTPs (100 mM); 1.5 µL Multiscribe™ Reverse transcriptase (50 U/µL); 0.1 µL of RNAse Inhibitor; 0.8 µL of RT buffer (which are all provided with the TaqMan ® MicroRNA Reverse Transcription Kit (Life Technologies)) and 0.9 µL magnesium chloride (25 mM) and 0.8 µL of Megaplex™ RT primers (10x). Additionally, 0.7 µL of RNAse-free water (i.e. condition in absence of *cel-miR-39* primers) or 0.7 µL of *cel-miR-39* RT primers (5x) was added to the mastermix. cDNA synthesis was carried out in a gradient thermal cycler (Techne TC-5000) using following thermal cycling profile: 40 cycles of two minutes at 16°C for primer annealing, one minute at 42°C for primer extension and one second at 50°C for separation from the primers. The reverse transcriptase enzyme was inactivated by incubation for five minutes at 85°C. Afterwards, the samples were hold at 4°C until the end of the run and stored at -20°C afterwards.

7.2.2. Effect of combinatory cDNA synthesis on primer amplification efficiencies

The efficiencies of the assays were tested for both cDNA samples with and without addition of the *cel-miR-39* primers in the RT step. In order to evaluate the efficiency of the assays, a 4-fold serial dilution series starting from a 10-fold dilution of the cDNA was used. Each reaction was performed in triplicate. Deviation of the triplicates above 0.5 Ct was not tolerated and deviating samples were eliminated for determination of primer efficiencies. qPCR reactions were carried out in 384-well plates in 10 µL reaction volume. Mastermix contained 0.5 µL of Taqman Small RNA assay (20x), 3.835 µL Taqman Fast Advanced Mastermix (2x) and 3.165 µL of RNAse-free water for each reaction together with a 2.5 µL sample input. qPCR was carried out on ABI 7900HT sequence detection system (Applied Biosystems) using following thermal cycling: 10 minutes at 95 °C followed by 40 cycles of 15 seconds at 95 °C and one minute at 60°C. Since efficiencies of the primers showed to be better in the samples with higher cDNA input and with Ct values above 30 variation between triplicates was often too high,

both efficiencies for the entire dilution set and the highest three or four concentrations were calculated (Table 10).

For one serial dilution of a cDNA sample without *cel-miR-39* RT, the Taqman Assay for *cel-miR-39* was carried out. Although the megaplex RT reaction only contains primers for human miRNAs, the assay could still detect the presence of the *C. elegans miR-39*. Compared with the qPCR of the same RNA sample and addition of the *cel-miR-39* RT primers in the cDNA synthesis the threshold was exceeded approximately 11 cycles later (i.e. a difference of 11 Ct).This could indicate a non-specificity of the megaplex primers or the Taqman *cel-miR-39* assay.

7.3. Optimization of qPCR

In order to limit the use of consumables, it was tested whether qPCR reactions in 384-well plates could also be carried out in a 8 µL volume instead of 10 µL, without affecting the primer efficiencies of the assays. Starting from a 10-fold dilution, a serial 3-fold dilution series was used in order to determine PCR amplification efficiencies. This comparison was carried out for the three assays (i.e. *miR-128a*; *miR-132* and *miR-222*). Mastermix contained 0,4 µL of Taqman Assay (20x); 3,07 µL Taqman Fast Advanced Mastermix and 2,53 µL RNAse-free water for each 8 µL reaction with 2 µL cDNA input. Reactions carried out in 10 µL remained the same compositions as previous plates for primer efficiencies. Each reaction was carried out in triplicate using a ABI 7900HT Fast sequence detection system (Applied Biosystems).

7.3.1. The qPCR reaction volume

Primer efficiencies of the reactions carried out in 8 µL reactions were compared with identical reactions in 10 µL volume. In order to compare correctly between both qPCR plates, one interrun calibrator (IRC) was used as 10 µL and 8 µL reactions were carried out on different 384-well plates. The threshold was adjusted for each separately. Primer amplification efficiencies were calculated using equation (1) based on one three-fold serial dilution series starting from a ten-fold dilution of the cDNA (Table 9). The comparison was carried out for three different assays.

Table 9: Primer efficiencies in 8 µL and 10 µL reaction volume

	8 µL	10 µL
miR128	97.43%	96.50%
miR132	97.06%	129.31%
miR222	113.61%	120.27%

The Taqman miRNA assays showed to be more efficient in 8 µL reaction compared to identical reactions (i.e. same sample and mastermix) reactions in 10 µL.

7.3.2. The qPCR mastermix composition

The effect of adaptations in the composition of the mastermix on primer amplification efficiency of the Taqman miR128 assay. The composition of previously used mastermixes was compared to a mastermix containing 0.4 µL Taqman assay (20x), 4 µL Taqman Fast Advanced mastermix (2x) and 1.6 µL RNAse-free water. qPCR reaction was carried out on a 384-well plate in a 8 µL volume for

Taqman assay *miR128*. Primer amplification efficiency was 93.86% compared to 97.43% for the previous mastermix. Though the efficiency was a little lower, qPCR reaction was still efficient using the alternative mastermix.

Table 10: PCR amplification efficiencies based on the slope of the calibration curve designed by plotting the Ct value against the logarithm of the concentration for a serial dilution series.

	cDNA (Megaplex RT)				cDNA (Megaplex RT + *cel-miR-39* RT)			
	All dilutions included		Highest 3/4 concentrations		All dilutions included		Highest 3/4 concentrations	
Sample	**Slope**	**Efficiency**	**Slope**	**Efficiency**	**Slope**	**Efficiency**	**Slope**	**Efficiency**
Taqman Assay *cel-miR-39*								
250					-4,136	0,745	-3,445	0,951
251					-3,647	0,880	-3,376	0,978
252	-3,658	0,877			-3,647	0,880	-3,135	1,084
253					-3,791	0,836	-3,193	1,057
254					-3,924	0,798	-3,270	1,022
					Average	**0,828**	**Average**	**1,018**
Taqman Assay *hsa-miR-146a*								
250	-3,883	0,809	-3,648	0,880	-5,053	*0,577*	-3,745	0,849
251	-3,930	0,797	-3,208	1,050	-4,014	0,775	-3,018	1,144
252	-3,698	0,864	-3,298	1,010	-4,338	0,700	-4,338	*0,700*
253	-3,416	0,962	-3,416	0,962	-3,265	1,024	-3,265	1,024
254	-2,554	*1,463*	-2,554	*1,463*	-3,263	1,025	-3,263	1,025
	Average	**0,858**	**Average**	**0,975**	**Average**	**0,881**	**Average**	**1,011**
Taqman Assay *hsa-miR128a*								
250	-3,766	0,843	-3,605	0,894	-4,566	0,656	-3,668	0,873
251	-4,034	0,770	-3,532	0,919	-4,290	0,710	-3,721	0,857
252	-4,047	0,766	-3,678	0,870	-4,290	0,710	-3,444	0,952
253	-4,076	0,759	-3,682	0,869	-4,220	0,726	-3,613	0,892
254	-3,842	0,821	-3,640	0,883	-4,166	0,738	-3,502	0,930
	Average	**0,792**	**Average**	**0,887**	**Average**	**0,708**	**Average**	**0,901**
TaqMan Assay *hsa-miR-222*								
250	-3,411	0,964	-2,952	1,181	-3,905	0,803	-3,320	1,001
251	-3,964	0,788	-3,184	1,061	-3,702	0,863	-3,328	0,997
252	-3,644	0,881	-3,174	1,065	-3,702	0,863	-3,108	1,098
253	-3,625	0,887	-3,301	1,009	-3,621	0,889	-3,077	1,113
254	-3,433	0,956	-3,320	1,001	-3,583	0,902	-2,942	1,187
	Average	**0,895**	**Average**	**1,06346026**	**Average**	**0,864**	**Average**	**1,079**
Taqman Assay *hsa-miR-132*								
250	-2,904	1,210			-3,146	1,079	-3,265	1,024
251	-2,364	1,648			-3,428	0,958	-3,428	0,958
252	-3,673	0,872			-3,208	1,050	-3,208	1,050
253					-1,917	*2,325*	-1,917	*2,325*
254	-2,549	1,468			-3,719	0,857	-3,719	0,857
	Average	**1,299**			**Average**	**0,986**	**Average**	**0,972**
Taqman Assay *hsa-miR-21*								
250	-4,107	0,752	-2,626	1,404	-3,382	0,975	-3,227	1,041
251	-4,147	0,742	-2,866	1,233	-2,637	1,395	-2,637	1,395
252	-2,825	1,260	-2,294	1,728	-2,637	1,395	-2,637	1,395
253	-3,542	0,916	-3,542	*0,916*	-3,679	*0,870*	-3,679	*0,870*
254	-2,897	1,214	-2,592	1,431	-2,822	1,261	-2,969	1,172
	Average	**0,977**	**Average**	**1,449**	**Average**	**1,257**	**Average**	**1,251**

Efficiencies depicted in italics were identified as outliers and were not included in the mean PCR amplification efficiency.

7.4. miRNA expression analysis

Saliva from two different time points was used, 1 mL of saliva collected in Oragene RNA containers was used for exosome isolation; subsequent total RNA purification and miRNA quantification.

7.4.1. Total RNA yield and purity of the samples.

Since exosome isolation resulted in pellets of different sizes, large differences in RNA yield were found between samples (Table 11).

Table 11: RNA yield of salivary exosomes.

RNA yield	n (samples)	%
>50 ng/µL	39	21,67
50-100 ng/µL	38	21,11
100-300 ng/µL	81	45,00
300-500 ng/µL	18	10,00
>500 ng/µL	4	2,22

Since samples with higher RNA concentrations tend to have better purity ratios compared to samples with lower RNA yield, the correlation between both purity ratios was evaluated. Spearman correlation coefficients were calculated using SAS software. The correlation coefficient between the A260/A280 ratio and RNA yield (i.e. based on Qubit br measurements) is 64 % (55 % - 72 %; P<0,0001) (95 % confidence interval; p-value) and 73 % (65 % - 79 %; *P*<0,0001) for the A260/A230 ratio and RNA yield. The Spearman correlation coefficient between the measurements using Nanodrop and the Qubit br assay was 95 % (94 % - 96 %; *P*<0,0001).

7.4.2. Statistical analysis of qPCR data

The effect of residential traffic exposure at the current address on miRNA expression was evaluated in a multivariate-adjusted linear regression model, which corrects for multiple measurements. As such, individual risk factors, factors for socio-economic status and spatio-temporal effects, which may be confounding factors for the effect of exposure on miRNA, are taken in account. The effects of PM exposure (section 3.3 of the results) are estimated in the same multivariate-adjusted model.

7.4.3. Choice of covariates

Covariates for entrance in the statistical model were determined based on current knowledge on the effects of air pollution on human health as well as personal interpretation. Variables for age and gender were included as potential individual risk factors of the subjects [78], as well as the school since both are located at different cities in Belgium and there might be differences between the schools. For example, PM_{10} concentrations in the school in Zonhoven are much higher for the first measurements compared with the second measurement and both visits at the schools in Kiewit (Table 3). Furthermore, air pollution can be affected by temporal cycles such as season, day of the week and time of the day.[78] Since time-varying variables might have an effect on the air pollution induced effects on miRNA expression, the day of the week and the time of the examination are included in the model. The latter might also be important since composition of saliva is known to be subject for some components to diurnal variation. Addition of the school, day of the week and time of examination to the model can take into account at least to some extend the spatio-temporal-

dependent effects of PM exposure on miRNA expression. Furthermore, it was found that PM-induced health effects are modified by socioeconomic status indicated by factors such as educational level and occupation. [79] The protein content of the extracellular fraction of the saliva and total RNA yield are both affected by PM exposure and have a significant effect on miRNA expression. Therefore, both are considered as confounding experimental variables and are added to the statistical model. Passive smoking was added to the model since tobacco smoke can contribute to personal exposure to particulate air pollution.

7.4.4. The effect of residential traffic exposure on miRNA expression

Residential traffic exposure was estimated using GIS based on the current address (Table 12). Analysis provided information on the traffic density within different buffer (i.e. within a radius of 50 meters, 100 meters, 200 meters or 500 meters of the residency) as well as the distance-weighted traffic density which takes in to account a larger contribution to exposure of the roads closest to the residency. Furthermore, the distance to big roads was evaluated for both freeways (i.e. N-roads) and highways (i.e. E-roads).

Table 12: Long-term residential traffic exposure characteristics

	Kiewit			Zonhoven		
Pollution indicator	**Geometrical mean**	**25th percentile**	**75th percentile**	**Geometrical mean**	**25th percentile**	**75th percentile**
DWTD	160	78	197	223	79	630
TD 50	62	40	76	88	42	85
TD 100	275	106	334	339	118	860
TD 200	1109	498	3946	1607	527	7655
TD 500	8039	3678	23358	14944	3206	47910
Dis N-roads	476	210	1178	273	96	786
Dis E-roads	3687	3224	4422	1848	1459	2778

DWTD = distance-weighted traffic density; TD = traffic density within a xx m buffer; Dis = distance

Traffic densities are expressed in number of vehicles x km/ day ; distance to roads is expressed in meter.

The effects of different parameters for traffic related exposure on the expression of the miRNAs are estimated in multivariate-adjusted mixed model. Covariates considered for entrance in the model were age, gender and school of the subject, day of the week and time of the measurements as well as education and occupation of both parents and the RNA content of the extracellular fraction.

Table 13: Long-term effects of residential traffic exposure at the current address on miRNA expression.

	miR222			miR146a			miR155		
	n	**β**	**p-value**	**n**	**β**	**p-value**	**n**	**β**	**p-value**
DWTD	147	-0.00008	0.33	122	-0.00009	0.23	155	0.000037	0.62
TD 50m	147	-0.00018	0.15	122	-0.00018	0.099	155	0.000071	0.52
TD 100m	147	-0.00004	0.41	122	-0.00004	0.29	155	0.000028	0.49
TD 200m	147	-0.00001	0.56	122	-8.96E-06	0.59	155	4.49E-06	0.78
TD 500m	147	-5.76E-06	0.28	122	-6.94E-06	0.15	155	-2.49E-06	0.60
Dis E road	147	-0.00001	0.88	122	0.000078	0.34	155	-0.00004	0.66
Dis N road	147	0.000053	0.74	122	0.000179	0.20	155	-0.00009	0.50

Dis = distance; DWTD = distance-weighted traffic density; TD = traffic density (within a xx m buffer); β = regression coefficient which represents the estimated effect; n = number of subjects included.

Covariates considered in the multivariate analysis are age, gender and school of the subject, passive smoking, smoking during pregnancy and education and ocupation of both parents as an indicator of social-economic status as well as the day and time of the examination and total RNA content.

p-values < 0,05 were considered to be significant. ł p-values adjusted for multiple testing within each miRNA using the Benjamini Hochberg FDR test.

No associations could be found for residential traffic exposure at the current home address of the study volunteers and miRNA (i.e. *miR222*, *miR146a* and *miR155*) expression (Table 13).

7.4.5. The effect of different normalization strategies on study outcomes

In order to evaluate if the findings of this study will be affected by the choice of normalization strategy, the effect of indoor exposure to PM_1 on the expression levels will be estimated for each normalization strategy separately. Indoor UFP exposure was selected because exposure to PM_1 at the moment of the examination, did not affect one of the normalizers in a univariate mixed model analysis. Applying the model will estimate the contribution of each covariate and possible significant contribution to the effect (Table 14).

Though the p-values are different, the key findings from the normalization by RNA, miRNA or protein content remain the same. However, when normalizing the qPCR for the spike-in miRNA the result changes and a significant effect of indoor PM_1 exposure on *miR222* expression can be found (*P*=0,0275). Furthermore, the age of the child showed to be a significant determinant of the effects of PM on *miR222* expression.

Table 14: Contribution of covariates in a mixed model estimating the effect of indoor UFP concentration on miR222 expression normalized by different strategies.

		Normalizer for miR222 expression			
		RNA (n=159)	**miRNA (n=162)**	**Protein (n=144)**	**Spike-in miRNA (n=152)**
Covariates	**Indoor UFP**	0.478	0.84	0.66	**0.0204**
	Gender	0.64	0.37	0.38	0.16
	Age	0.42	0.37	0.6	0.3
	School	0.11	0.55	**0.0282**	0.45
	Day	0.31	0.99	0.99	0.91
	Time	0.14	0.8	0.15	0.75
	Education mother	0.53	0.73	0.5	0.96
	Education father	0.09	0.16	0.19	**0.0053**
	Occupation mother	0.52	0.13	0.67	0.11
	Occupation father	0.17	0.58	0.12	**0.0031**
	Passive smoking	0.64	0.9	0.46	0.34

p-values < 0.05 were considered to be significant; n = numbers of included subjects.

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Vriens, Annette

Datum: **10/06/2014**