2014•2015
FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Are miRNAs a link between air pollution exposure and cardiovascular changes?

Promotor :
Prof. dr. Tim NAWROT

Promotor :
Prof. dr. ir. PATRICK DE BOEVER

Caroline Vuegen
Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

De transnationale Universiteit Limburg is een uniek samenwerkingsverband van twee universiteiten in twee landen: de Universiteit Hasselt en Maastricht University.

universiteit hasselt
KNOWLEDGE IN ACTION

Universiteit Hasselt | Campus Hasselt | Martelarenlaan 42 | BE-3500 Hasselt
Universiteit Hasselt | Campus Diepenbeek | Agoralaan Gebouw D | BE-3590 Diepenbeek

universiteit hasselt | Maastricht University

2014•2015
FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Are miRNAs a link between air pollution exposure and cardiovascular changes?

Promotor :
Prof. dr. Tim NAWROT

Promotor :
Prof. dr. ir. PATRICK DE BOEVER

Caroline Vuegen

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

universiteit hasselt | Maastricht University

Table of content

List of abbreviations

BC:	Black carbon
BH_4:	Tetrahydrobiopterin
CRAE:	Central retinal arteriolar equivalent
CRP:	C-reactive protein
CRVE:	Central retinal venular equivalent
Ct values:	Cycle threshold values
DBP:	Diastolic blood pressure
DNA:	Deoxyribonucleic acid
eNOS:	Endothelial nitric oxide synthase
ET-1:	Endotheline-1
GTP:	Guanosine-5'-triphosphate
H_2O_2:	Hydrogen peroxide
IL-1β:	Interleukin-1β
IRCs:	Inter-run calibrators
mRNA:	messengerRNA
miRNA:	MicroRNA
NADPH-oxidases:	Nicotinamide adenine dinucleotide phosphate-oxidases
$NO^{\cdot}$:	Nitric oxide
NO_2:	Nitrogen dioxide
NTCs:	Non template controls
O_2:	Oxygen
O_3:	Ozone
$OH^{\cdot}$:	Hydroxyl
$ONOO^{-}$:	Peroxynitrite
PM:	Particulate matter
$PM_{2.5}$:	Particulate matter with an aerodynamic diameter smaller than or equal to 2.5 µm
PM_{10}:	Particulate matter with an aerodynamic diameter smaller than or equal to 10 µm
PP	Pulse pressure
qRT-PCR:	Quantitative reverse transcription polymerase chain reaction
RISC:	RNA-induced silencing complex
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
RT:	Reverse transcription
SBP:	Systolic blood pressure
SO_2:	Sulphur oxide
SOD:	Superoxide dismutase
TNF-α:	Tumor necrosis factor-α
UFPs:	Particulate matter with an aerodynamic diameter smaller than or equal to 100 nm also named as ultrafine particles
WHO:	World Health Organization

Acknowledgements

Thomas Edison said once: "Science is 2% genius and 98% hard work" a remarkable but truthful quote that I fully experienced this last months. Getting answers and meaningful results are the main goal of scientists no matter how many hours/days or months they have to spend. This year started as an internship, an intensive practical training, but became a journey full of knowledge, strugglings and new friendships. Trying to deliver an interesting and fully-fledged master thesis requires a lot of work of yourself, but also from those around you. Without the guidance of my supervisors, colleagues and support of my friends and family, this challenge had never become a reality.

Foremost, I would like to thank my daily supervisor Drs. Tijs Louwies for the opportunity to perform this internship. During these months his guidance, patience and critical view have helped me in the research and writing of this thesis. Both my theoretical skills as working independently are widened through his knowledge.

Besides my supervisor, I would like to thank my external supervisor Prof. Dr. Patrick De Boever for his monitoring during this internship. By asking the right question at the right time, he made sure I revised the data and got the opportunity to learn new techniques.

I would also like to thank all the persons who participated in this study, whereby I got the tremendous opportunity to perform my experiments. Furthermore, Karen Hollanders for her assistance during the entire practical work, without her expertise I wasn't able to perform this study. Next, I need to thank postdoc Dr. Sabine Langie, who was always willing to help me and give her best suggestions.

Thanks to Roos Leemans a fellow student at VITO for working with me and making lab work more fun. Secondly, I could always rely on her when I had some questions or discuss things about the project. I thank my fellow students at Hasselt University: Jirka Cops, Stephanie Augustynen, Yörg Dillen, Wim Jacobs and Robby Louis for all the discussions, working moments to catch deadlines and for all the fun we have had in the last five years.

My deepest gratitude goes to my parents for given me this chance to follow my dream to go to college and study Biomedical science even though it was sometimes very tough for the both of us in the last couple of years. I would also like to thank them, together with my aunt and my grandmother, who always were very supportive and encouraged me with their best wishes.

Finally, I would like to thank my best friend, Sarina Lambrechts. She was always there, cheering me up, helping me but most of all she stood by me through the good and bad times.

Abstract

Background: Air pollution is an independent risk factor for cardiovascular morbidity and mortality. Retinal arteriolar narrowing and retinal venular widening are risk factors for cardiovascular disease. Increases in inflammation, oxidative stress and endothelial dysfunction are processes, present in the development of cardiovascular disease that could initiate microvascular changes. The underlying molecular mechanisms behind these microvascular changes remain unclear. Recently, the analysis of miRNA expression patterns revealed that miRNAs are associated with air pollution exposure. We examined the effect of both acute and short-term air pollution exposure on miR-21, miR-222 and miR-146a expression patterns and the relationship with microvascular changes.

Material & methods: Blood samples and fundus photos were gathered at five different time points of fifty healthy adults (50% females), 23 to 58 years of age, during December 2014 and April 2015. PM_{10} and $PM_{2.5}$ was measured at the site of examination and at nearby monitoring stations. Retinal blood vessels were represented as the central retinal arteriolar/venular equivalent (CRAE/CRVE). miRNA was extracted from venous blood samples and the expression patterns were analyzed using Taqman qRT-PCR. The analysis of the three target miRNAs were analyzed in qBase and normalized with two controls RNU44 and RNU48. Mixed-effects models were used in the statistical analysis to examine all associations.

Results: First, CRAE was associated with acute $PM_{2.5}$ and PM_{10} exposure levels as well as short-term PM_{10} exposure levels 48hrs and one week before examination. Each 10 µg/m³ increase in acute $PM_{2.5}$ and PM_{10} was associated with a 1.00 µm (95%CI:-1.60;-0.39) and a 0.30 µm (95%CI:-0.58;-0.02) decrease in CRAE after adding pulse pressure (PP), age, gender, BMI, alcohol- and caffeine consumption, location and day of the week. A 0.82 µm (95%CI:-1.67;-0.03) and a 0.83 µm (95%CI:-1.46;-0.20) decrease in CRAE was associated with each 10 µg/m³ increase in short-term PM_{10} during the previous 48hrs and previous week after control for the same covariates. Acute $PM_{2.5}$ and PM_{10} as well as short-term PM_{10} exposure levels showed a trend towards an increase in CRVE. Second, short-term PM_{10} is associated with miR-21 and miR-222. Each 10 µg/m³ increase in PM_{10} during the previous 24hrs, 48hrs and previous week was associated with respectively a 6.62% (95%CI:-11.07;-2.12), a 8.27% (95%CI:-14.14;-2.40) and a 6.19% (95%CI:-10.89;-1.49) lower miR-21 expression level after control for PP, age, gender, BMI, alcohol consumption, day of the week, caffeine consumption and location. Furthermore, each 10 µg/m³ increase in short-term PM_{10} during the previous 24hrs, 48hrs and previous week were associated with a 6.71% (95%CI:-10.68;-2.75), a 10.93% (95%CI:-15.87;-5.98) and a 10.74% (95%CI:-14.41;-7.07) lower miR-222 expression level after control for the aforementioned covariates. Third, CRVE is associated with miR-222 and miR-146a. Each 10% increase in miR-222 and miR-146a is associated with respectively a 0.16 µm (95%CI:0.31;-0.02) and a 0.26 µm (95%CI:-0.43;-0.09) decrease in CRVE.

Conclusions: These findings demonstrated that miRNAs may be a molecular trigger through which PM particles can cause changes in the microcirculation and induce cardiovascular disease.

Samenvatting

Luchtvervuiling is een onafhankelijke risicofactor van cardiovasculaire ziekte en sterfte. Retinale arteriolaire vernauwing en retinale venulaire verbreding zijn risicofactoren van cardiovasculaire ziekte. De toename in inflammatie, oxidatieve stress en endotheel dysfunctie zijn allemaal processen, aanwezig in de ontwikkeling van hartziekten die microvasculaire veranderingen kunnen veroorzaken. De achterliggende moleculaire mechanismen van deze microvasculaire veranderingen zijn nog niet echt opgehelderd. Recent is aangetoond dat expressieniveaus van miRNAs geassocieerd zijn met luchtvervuiling. Deze studie wil zowel het acute als korte termijn effect van luchtvervuiling bestuderen op de expressie van miR-21, miR-222 en miR-146a en zijn relatie met microvasculaire veranderingen.

Om dit te onderzoeken, werd er op vijf verschillende tijdstippen bloedstalen en retinafoto's verzameld bij vijftig gezonde volwassen (50% vrouwen), 23-58 jaar, in de periode van december 2014 en april 2015. De retinale bloedvaten werden voorgesteld als de centrale retinale arteriolaire equivalent en de centrale retinale venulaire equivalent (CRAE/CRVE). $PM_{2.5}$ en PM_{10} werd gemeten op de plaats van de staalname en door vaste meetstation. Het miRNA werd geëxtraheerd uit tempus tubes waarna het expressie patroon van miR-21, miR-222 en miR-146a werd geanalyseerd met de Taqman qRT-PCR techniek. De analyse van de target miRNAs werd verricht in qBase en genormaliseerd met twee doelgerichte controles, RNU44 en RNU48. De gehele statistische analyse werd uitgevoerd m.b.v. mixed models.

Na analyse zijn er drie belangrijke associaties gevonden. Eerst is er niet alleen een associatie aangetoond tussen CRAE en acute $PM_{2.5}$/PM_{10} concentraties, maar ook met korte termijn PM_{10} concentraties. Elke 10 µg/m³ stijging in acute $PM_{2.5}$ en PM_{10} is geassocieerd met een 1.0 µm (95%BI;-1.60;-0.39) en een 0.30 µm (95%BI;-0.58;-0.02) daling in CRAE na toevoeging van polsdruk, individuele kenmerken zoals leeftijd, geslacht en BMI, alcohol gebruik, locatie, cafeïne gebruik en dag van de week. Een 0.82 µm (95%BI:-1.67;-0.03) en een 0.83 µm (95%BI:-1.46 ;-0.20) daling in CRAE zijn geassocieerd bij elke 10 µg/m³ stijging in korte termijn PM_{10} gedurende de voorbije 48hrs/week na controle met dezelfde variabelen. Acute $PM_{2.5}$ en PM_{10} zowel als korte termijn PM_{10} blootstelling vertoonde een trend richting een associatie met een verhoogde CRVE. Vervolgens werd de korte termijn PM_{10} blootstelling geassocieerd met miR-21 en miR-222. Elke 10 µg/m³ stijging in PM_{10} gedurende de voorbije 24h/48h/ week is geassocieerd met een 6.62% (95%BI:-11.07;-2.12), 8.27% (95%BI:-14.14;-2.40) en een 6.19% (95%BI:-10.89;-1.49) lagere miR-21 expressie na controle met verschillende variabelen. Verder is elke 10 µg/m³ stijging in PM_{10} gedurende de voorbije 24h/48h/week geassocieerd met een 6.71% (95%BI:-10.68;-2.75), 10.93% (95%BI:-15.87;-5.98) en een 10.74% (95%BI:-14.41;-7.07) lagere miR-222 expressie. Als laatste werd aangetoond dat CRVE geassocieerd is met miR-222 en miR-146a. Elke 10% stijging in miR-222 en miR-146a is geassocieerd met een 0.16 µm (95%BI:0.31;-0.02) en een 0.26 µm (95%BI:-0.43;-0.09) daling in CRVE na controle van de variabelen.

Deze bevindingen tonen aan dat miRNAs de moleculaire mechanismen kunnen zijn waarbij luchtvervuiling veranderingen veroorzaken in de microcirculatie, die kunnen leiden tot hartziekten.

1 Introduction

1.1 Air pollution

1.1.1 Effect of air pollution on human health

Air pollution is an indispensable, but alarming part of our modern society. [1, 2] In 2012, the World Health Organization (WHO) stated that 12.5% of all global deaths (ca. 7 million people) was attributable to air pollution exposure. [3] Researchers are focusing on the health effects on the respiratory tract, the primary entering of the ambient air pollutants (AAP) in the lungs and cardiovascular endpoints, such as stroke, hypertension, myocardial infarcts and arrhythmias.[2, 4-6]

According to the WHO, approximately 80% of premature deaths, related with outdoor air pollution, were referable to stroke and ischaemic heart disease, whereas only 14% of the deaths were caused by chronic obstructive pulmonary disease or acute lower respiratory infections. [3] Therefore, research has shifted more towards the effects of air pollution on cardiovascular events. [5, 7, 8]

1.1.2 Air pollution composition

Air pollution is a mixture of solid and gaseous pollutants. The main contributors to the solid pollutants are particulate matter (PM) and traffic related air pollution. [9, 10] PM contributes to 40% of urban PM and consists of primary and secondary particles with a varying aerodynamic diameter. [11] Primary particles, derived from fossil-fuel combustion are directly emitted into the environment. The secondary particles are produced via chemical reactions between gases present in the atmosphere, such as oxygen (O_2), reactive species (e.g. ozone (O_3)), free radicals (e.g. hydroxyl ($OH^{\cdot}$)), pollutants (e.g. NO_x) and organic gases. [2, 4, 12]

PM is classified by size (diameter): coarse particles ≤ 10 µm (PM_{10}), fine particles ≤ 2.5 µm ($PM_{2.5}$) and ultrafine particles (UFPs), which are of nanoscale size (<100 nm). Both PM_{10} and traffic-related air pollution are mixtures of solid components and gaseous compounds, such as sulphur dioxide (SO_2), nitrogen dioxide (NO_2), O_3, transition metals and black carbon (BC), a carboneous traffic-related particle. [13, 14]

1.2 Effects of air pollution on cardiovascular health

1.2.1 Long-term effects

Air pollution exposure is an independent risk factor for cardiovascular morbidity and mortality. [15, 16] Pope III *et al.* (2009) investigated the effect of air pollution exposure on the life expectancy in fifty-one metropolitan areas in the United States between the 1980s and the 2000s. In 1986, a 10 µg/m³ decrease in $PM_{2.5}$ resulted in an increase in mean life expectancy of 0.61 ± 0.20 years. Over a period of twenty years, as $PM_{2.5}$ exposure was reduced in time, life expectancy increased with 2.72 years for these areas. [17] Dockery *et al.* confirmed these findings in a prospective cohort study in U.S. cities: air pollution exposure increased mortality-rate ratio with 1.26, when the most polluted and the least polluted U.S. cities were compared after adjustment for other risk factors such as smoking, education, BMI and etc. Furthermore, they also found a positive association between air pollution and death from cardiopulmonary diseases. [18]

1.2.2 Short- term effects

An acute PM exposure is associated with a significant increase in blood pressure. In healthy volunteers, an increase of 10.5 µg/m³ in $PM_{2.5}$ levels over the past five days was associated with a 2.8 mm Hg increase in systolic (SBP) and a 2.7 mm Hg increase in diastolic blood pressure (DBP). [19] Further, according to Dockery *et al.*, 29% of the subjects in his study showed a faster heartbeat (e.g. 5-10 bpm), when they encounter a 100 µg/m³ increase in PM_{10} on the previous day. [7]

Peretz *et al*. demonstrated that microvascular dysfunction was associated with short-term PM exposure in a human controlled study. An acute vasoconstriction of the brachial artery was found, when adults were exposed to diesel exhaust for two hours. This dysfunction was also related with an increase in plasma endotheline-1 (ET-1) levels. [20] Impaired endothelial vessel response has been demonstrated in a controlled exposure study of six hours to diluted diesel exhaust on healthy adults. They showed an impaired peripheral resistance vessel dilatory response to different vasodilators, such as acetylcholine and bradykinin. [21]

1.3 Air pollution is targeting the microvasculature

1.3.1 The microcirculation

The microcirculation consists of the smallest blood vessels of the human body, vessels with a diameter smaller than 150 µm. These vessels are characterized by myogenic autoregulation (e.g. the change in vessel diameter of the arterioles to maintain the local blood flow) and are the largest contributor to vascular resistance. The major functions of the microcirculation are the exchange of O_2, nutrients and waste products at tissue level and regulation of the blood pressure. [22] Vascular resistance is influenced by many factors, including circulating substances (e.g. hormones), local metabolic factors, autonomic nerves, endothelium derived substances and local myogenic responses. [23, 24]

1.3.2 Practical approach: fundus photography and its relation with air pollution

Fundus photography offers a non-invasive way to study the retinal microcirculation in vivo *(see Figure 1)*. The retinal blood vessels share anatomical, physiological and pathological features with cerebral and coronary blood vessels. Retinal vessels caliber is an independent predictor of cardiovascular diseases. Narrowing of the arteriolar vessels indicates arteriolar damage and predicts hypertension. Venular widening has been associated with inflammation, endothelial dysfunction and atherosclerosis. [16]

In 2010, Adar *et al.* found an association between retinal arteriolar narrowing and long-term air pollution exposure. A 3 $\mu g/m^3$ increase in $PM_{2.5}$ was associated with a 0.8 µm decrease in central retinal arteriolar equivalent (CRAE). [15] In 2013, Louwies *et al.* found an association between short-term exposure and CRAE. Their data showed that each 10 $\mu g/m^3$ increase of PM_{10} was accompanied by a 0.93 µm decrease in CRAE and that each 1 $\mu g/m^3$ increase of BC was associated with a 1.84 µm decrease in CRAE. [16]

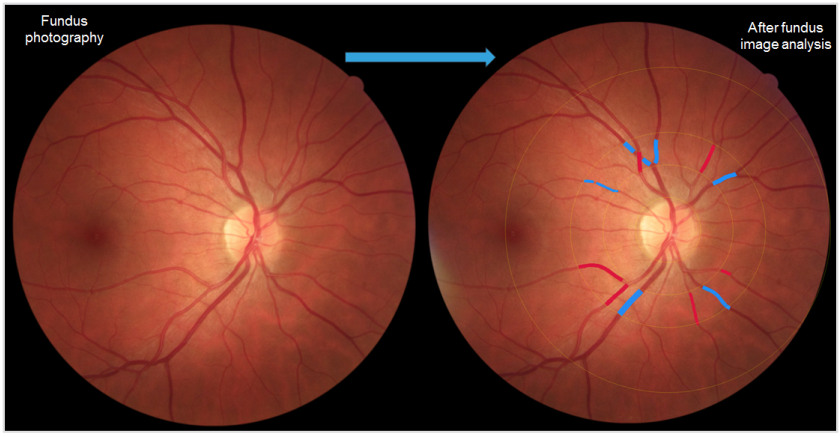

Figure 1: Retinal photography and grading
During the study visit, the fundus of the right and left eye was photographed twice. Afterwards, fundus image analysis software was used to calculate the central retinal arteriolar equivalent (CRAE) and the central retinal venular equivalent (CRVE). These parameters represent the thickness of the blood vessels in the blind spot (white/yellow spot in the middle of the vessels).

1.3.3 How air pollution affects the cardiovascular system

PM can cause direct/acute effects as well as indirect/chronic effects on cardiovascular function. [4, 25, 26] PM can induce direct cardiovascular effects within a few hours after air pollution exposure. Inhaled PM can activate pulmonary neuronal reflexes, which could initiate the autonomic nervous system. This might change heart rate rhythm and cause arrhythmia *(see Figure 2)*. [4, 12]

A population based cohort study in Lausanne showed that each 10 µg/m³ increase in PM_{10} is associated with a 1.00 mm Hg increase for nighttime SBP and 0.5 for nighttime DBP. In addition, each 10 µg/m³ increase in PM_{10} was also associated with a higher nighttime heart rate. [27]

Small particles (PM_{10}, $PM_{2.5}$ and UFPs) can enter the body via the respiratory tract and induce indirect health effects. First, fine and ultrafine particles ($PM_{2.5}$ or UFPs) can cross the pulmonary epithelium and translocate to the blood stream. Here, they can directly damage the organs or induce a local inflammatory response. [4, 25, 26] Coarse and fine particles ($PM_{2.5}$ or PM_{10}) can be inhaled deeply into the lungs and deposited in the alveoli. At this site, particles can cause pulmonary inflammation, which may lead to systemic inflammation, characterized by oxidative stress, endothelial dysfunction and the activation of leukocytes and platelets *(see Figure 2)*. [4, 12, 25]

1.3.4 Role of inflammation, oxidative stress and endothelial dysfunction

The cellular inflammatory and oxidative stress mechanisms are already well investigated in people exposed to air pollution. [28] Chuang *et al.* observed an increase in inflammatory markers, such as fibrinogen and C-reactive protein (CRP) in young people after short-term exposure to PM. [29] Tumor necrosis factor-α (TNF-α), CRP and interleukin-1β (IL-1β) were also elevated in children after a higher exposure to PM. [30] Araujo *et al.* and others have already showed that inflammatory effects, caused by air pollution exposure, are linked to the production of reactive oxygen species (ROS) via the detection of oxygen radicals in macrophages, lung microsomes and bronchial epithelial cells. [12, 31-33] Furthermore, diesel exhaust particles induce oxidative stress in both human and rat epithelial cells. [12, 34, 35] ROS production could activate additional redox-sensitive signaling pathways such as MAP kinases or NF-κB, which was demonstrated by Takizawa *et al.* in 1999 and by Li *et al.* in 2002. [36, 37]

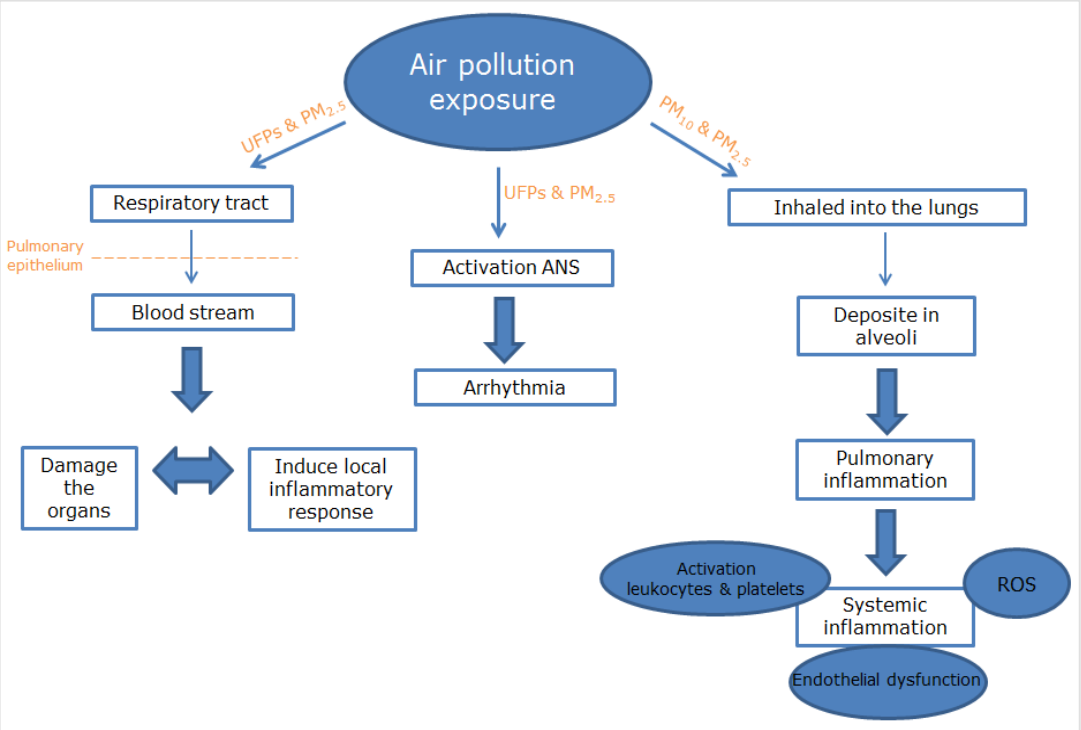

Figure 2: Possible biological pathways linking ambient air pollution with cardiovascular mortality and morbidity
The main particles responsible for the increased cardiovascular mortality and morbidity are UFPs, $PM_{2.5}$ and PM_{10}. The fine and ultrafine particles can react via two different pathways. First, they can enter the body via the respiratory tract crossing the pulmonary epithelium due to their small size and translocate to the blood stream. Here, they can cause direct damage to the organs or they can induce a local inflammatory response. Second, they can also cause a reaction through the activation of the pulmonary neuronal reflexes, including the activation of the autonomic nervous system, leading to changes in heart rate rhythm. The larger particles on the other hand, PM_{10} and $PM_{2.5}$, can be inhaled deeply into the lungs and deposited in the alveoli. At this site, they can cause a pulmonary inflammation, which could lead to a systemic inflammation, characterized by the production of ROS, activation of leukocytes and platelets and endothelial dysfunction.
(UFPs = ultrafine particles; $PM_{2.5}$ = particulate matter smaller than or equal to 2.5 µm; PM_{10} = particulate matter smaller than or equal to 10 µm; ROS = reactive oxygen species)

$PM_{2.5}$ can also induce systemic microvascular dysfunction. [1, 25] Microvascular dysfunction is defined as a reduction in the bioavailability of nitric oxide (NO) (one of the most important endothelial vasodilators). [38, 39] In normal conditions, the endothelial nitric oxide synthase (eNOS) in the epithelium constantly produces and releases NO in the blood, when the substrate L-arginine and cofactor tetrahydrobiopterin (BH_4) are sufficiently present. NO migrates to the smooth muscle cells surrounding the endothelium and induces relaxation of these muscles, leading to a permanent state of vasodilation. [38]

Air pollution induced inflammation and oxidative stress, could decrease this NO bioavailability. [39] The reduction in NO can be explained due to the uncoupling of eNOS *(see appendix A for the function of eNOS)* and/or NO scavenging by ROS. The uncoupling of eNOS prevents NO synthesis, but also leads to increased production of ROS. The increased ROS and decreased NO production is harmful to the microcirculation. [22] Endothelial dysfunction is a risk factor for several cardiovascular diseases. The described processes above are tightly controlled. A minor shift could result in dysfunction and induce the progression of cardiovascular disease.

1.4 MicroRNAs (miRNAs)

1.4.1 Their role in gene regulation and biological processes

A highly conserved novel class of gene regulators, microRNAs (miRNAs) was discovered in 2000. [40] MiRNAs are short, single-stranded ribonucleic acid (RNAs) of twenty to twenty-five nucleotides *(see appendix B for the biogenesis of miRNAs)* transcribed from deoxyribonucleic acid (DNA), which controls gene expression at the posttranscriptional level. [41-43] miRNAs can suppress the synthesis of proteins by inhibiting their translation by binding to the messenger RNA (mRNA), causing silencing of gene expressions. In addition, they can also provoke the degradation or cleavage of the mRNA. [44, 45] In contrast, these miRNAs can ensure an upregulation of the translation of mRNAs under certain conditions. [45]

Already one thousand different miRNAs genes were revealed in humans, regulating one third of the total human genome. [40] Each individual miRNA could regulate hundreds of target genes, but one gene could also be regulated by several miRNAs. [42] Furthermore, research demonstrates that miRNAs are involved in the regulation of several biological processes, such as immunomodulation, apoptosis, cell differentiation, cell proliferation and inflammation. [40, 42, 44, 46] This evidence indicates that miRNAs might play an essential role in several biological systems, such as cardiovascular, circulatory, respiratory and nervous system *(see appendix C for the essential role of miRNA maturation)*.

1.4.2 miRNAs and air pollution exposure

Researchers have investigated the influence of air pollution exposure on miRNA expression patterns. Yamamoto *et al.* have showed that the expression levels of miR-21, miR-30e, miR-215 and miR-144 were altered after diesel exhaust exposure. [47] These findings were confirmed in human air way epithelial cells by Jardim *et al.* [48] Investigation of miRNAs involved in oxidative stress, inflammation and endothelial dysfunction could provide more insights in the association between air pollution and cardiovascular disease.

1.4.3 Role of miRNAs in biological pathways altered by air pollution

Epidemiological studies revealed that air pollution was associated with altered expression of miRNAs related in processes, such as inflammation (e.g. miR-222 and miR-146a), oxidative stress (e.g. miR-21) and endothelial function (e.g. miR-222). [49, 50] Bollati *et al.* (2010) and Jiang *et al.* (2014) have showed that miR-21 is involved in oxidative stress and inflammatory responses. [49, 51] Suarez *et al.* demonstrated in 2007 that miR-222 is important for both endothelial cell gene expression and function by regulation of the endothelial nitric oxide synthase protein level in human endothelial cells. [52] Elevated expression levels of miR-146a are found in human suffering from psoriasis or rheumatoid arthritis, both chronic inflammatory diseases. This indicates that miR-146a plays an important role in the regulation of inflammation probably by inducing the infiltration of inflammatory cells. [53]

1.5 The objectives, goals and aims of this master thesis

Cardiovascular disease development and progression is associated with increases in inflammation and oxidative stress production, which may be due to air pollution exposure. miR-222, miR-21 and miR-146a provide an interesting field to examine and might elucidate the underlying mechanisms of this association. We hypothesize that miR-222, miR-146a and miR-21 are potential molecular pathways, which link air pollution exposure to changes in retinal microcirculation. The main objectives are: i) is air pollution exposure associated with changes in retinal microcirculation (e.g. CRAE and CRVE), ii) are miR-21, miR-222 and miR-146 associated with air pollution exposure, and iii) are miR-222, miR-21 and miR-146a associated with changes in retinal microcirculation (e.g. CRAE and CRVE).

A study with repeated measurements will be conducted to investigate both acute and short-term effects of air pollutions exposure on miRNA expression (e.g. miR-222, miR-21 and miR-146a) and retinal microcirculation in three different areas Mol, Hasselt and Lommel. Fifty healthy participants will be followed up for five months, with one study visit each month. At every visit, a non-invasive exploration of the retinal blood vessels using fundus photography will take place and blood will be collected to investigate the aforementioned miRNAs to verify if these miRNAs could be a potential molecular pathway that can link air pollution with changes in retinal microcirculation.

2 Materials and methods

2.1 Study subjects

The study was conducted in Flanders, Belgium during December 2014 and April 2015. We recruited fifty participants ranged from 23 to 58 years old. The study included twenty employees of the Flemish Institute for Technological Research (VITO), twenty employees of the Hasselt University and ten volunteers from the region of Lommel. Participants were free of cardiovascular diseases and diabetes before and during the study period. All participants participated in six study visits. These visits were one month apart and were scheduled on the same time of day. At each study visit, two questionnaires were filled in, blood pressure was measured, fundus photos were taken and a venous blood sample was collected *(see appendix D and E for full questionnaires in Dutch)*.

At the end of the study, all filled in a detailed questionnaire about their physical activity, time spend in traffic and lifestyle. Written informed consent was provided by all study participants in accordance with procedures approved by the Ethical Committee of the University Hospital of Antwerp.

2.2 Acquisition of data

2.2.1 Retinal photography and grading

By using a Canon 45° 6.3-megapixel digital non-mydriatic retinal camera (Hospithera, Brussels, Belgium), the fundus of the right and left eye of each participant were photographed twice. Analysis software was used to calculate the central retinal arteriolar equivalent (CRAE) and the central retinal venular equivalent (CRVE). The equivalent represents a summary of vessel diameters within an area equal to 0.5-1.0 disc diameters from the optic disc margin. CRAE and CRVE parameters were calculated and averaged over the four pictures.

2.2.2 Blood pressure

Both systolic and diastolic blood pressure were measured with an automatic device (Stabilograph, Stolberg, Germany) according to the guidelines of the European Society of Hypertension. [54] In short, participants rested five minutes before the first measurement. Five measurements were collected and only the last four measurements were averaged and used for data analyses.

2.2.3 Blood samples

Venous blood samples were collected at every study visit. One tempus tube (Blood RNA Tubes, Applied Biosystems, Diegem, Belgium), and one EDTA-tube (BD Vacutainer, New Jersey, United States) were collected.

2.3 Air pollution levels

Acute exposure to particulate air pollution (e.g. UFPs, PM_{10} and $PM_{2.5}$) was measured at the locations of the study visits. The acute exposure concentrations of PM_{10} and $PM_{2.5}$ (in $\mu g/m^3$) were measured with an Aerocet device (Met One Instrument Inc., US). Outside particulate air pollution concentrations were measured every hour. The Aerocet device sampled PM_{10} and $PM_{2.5}$ concentrations in the air every two minutes. The measurements were conducted each hour for

15 minutes. The average concentrations for PM_{10} and $PM_{2.5}$ were calculated every hour and used in the statistical analysis for the analysis of acute air pollution exposure effects.

Short-term air pollution exposure data was obtained from nearby official monitoring stations (Dessel and Hasselt), operated by the Flemish Environmental Agency (Aalst, Belgium). Here, PM_{10} and $PM_{2.5}$ concentration levels are measured with beta-absorption every 30 minutes. The average exposure was calculated 24hrs, 48hrs and one week before the study visit and used in the statistical analysis for the analysis of short-term air pollution exposure effects.

2.4 miRNA/RNA collection and qualification

2.4.1 Collection of miRNA/RNA from Tempus tubes

Total RNA was purified using total RNA purification kit according to the manufacturer's protocol (Norgen, Thorold, Canada) *(see appendix F for full protocol)*. In brief, lysis of the blood cells released all DNA and RNA. The solution was centrifuged (Eppendorf, centrifuge 5810R, Hamburg, Germany) at 4500 rpm for 30 minutes at 4°C to create a RNA/DNA pellet. Ethanol (Merck Millipore, Emsure, Darmstadt, Germany) was added to the lysate to remove the lysis buffer. DNAse I treatment was performed to eliminate the remaining DNA. Subsequently, different washing steps together with several centrifugation steps (Eppendorf, centrifuge 5415R, Hamburg, Germany) were introduced to gather pure RNA. Total RNA was eluted in 50 µl elution solution.

2.5 Control of RNA/miRNA

The extracted RNA was assessed by quantitative measuring of the RNA yield (ng/µl) and purity (A260/280 & A260/230) using the nanodrop spectrophotometer (ND-1000, Isogen Life Science, De Meern, the Netherlands). All the samples were ran on the bioanalyzer (Agilent Technologies, Diegem, Belgium) to ensure the presence of miRNA *(see appendix G for full protocol)*.

2.6 cDNA synthesis

cDNA synthesis was performed with the TaqMan microRNA reverse transcription kit (Applied Biosystems, Diegem, Belgium) *(see appendix H for full protocol)*. The input concentration of RNA ranged between 1-10 ng, according to the manufacturers protocol. A total of 15 µl solution was made: 3 µl RT primer (5X), 5 µl sample of 10 ng, 7 µl Real-time master mix containing dNTPs with dTTP (100mM), MultiScribe Reverse Transcriptase (50U/µl), RT buffer (10X), RNAse inhibitor (20U/µl) and nuclease free water. This mixture was incubated for five minutes on ice before the reverse transcription (RT) reaction was started. The RT reaction was performed in four steps: two incubation steps for 30 minutes at 16°C followed by 30 minutes at 42°C, reverse transcription step for 5 minutes at 85°C and an inactivation step at 4°C.

2.7 miRNA expression analysis

2.7.1 miRNA selection

For the optimal selection of miRNAs, a literature search was performed as well as the knowledge of a previous master thesis. [41, 42, 46, 49, 50, 55, 56] Eventually, based on their association with air pollution, inflammation, oxidative stress and/or endothelial function, three miRNAs were elected: 1) hsa-miR-222, 2) hsa-miR-21 and 3) hsa-miR-146a, illustrated in *Figure 3*.

According to these studies, miR-21 is involved in several processes. [41, 49, 50] Until now, only one human study mentioned the association of miR-21 with oxidative stress and air pollution. [29] miR-222 is involved in both inflammation and endothelial function and is one of the miRNAs, which always showed an effect after air pollution exposure. [42, 49, 50, 56] Finally, miR-146a, only involved in inflammation, was chosen based on the previous studies performed by Bollati and Fossati. [49, 50] They also used a combination of these three miRNAs, mentioning the involvement in all these processes related to our study.

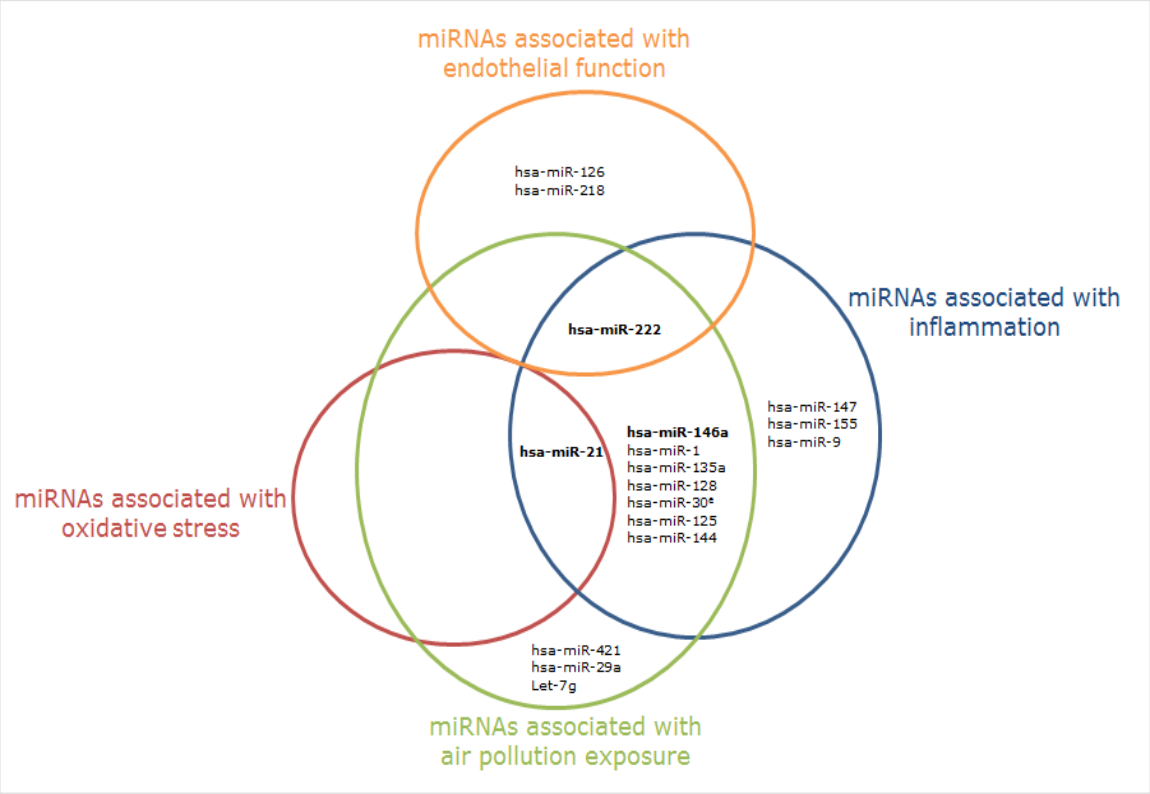

Figure 3: Selection of miRNAs based on literature search and a previous master thesis
Based on their association with air pollution, inflammation, oxidative stress and/or endothelial function, three miRNAs were elected: 1) hsa-miR-222, 2) hsa-miR-21 and 3) hsa-miR-146a.

2.7.2 Validation of control miRNAs

To investigate the most suitable and optimal controls for miRNA expression measurements, a qRT-PCR was performed to test three controls miRNAs RNU6, RNU44 and RNU48 (e.g. whose election was based on literature) in four test samples with a cDNA concentration of 10 ng. Each sample was measured in triplicate. The validation consist of i) the appearance of the control miRNA in the test samples, ii) the time of a detectable fluorescence signal, as such how lower the cycle threshold (Ct)

values, the better the control will be and iii) the Ct of the control need to be situated lower or between the Ct values of the target miRNAs.

The qRT-PCR program for this validation was the same as the qRT-PCR for the validation of the three target miRNAs in the samples collected in the five months. After the qRT-PCR run, an abs quant/2nd derivative max analysis was performed to validate the Ct values.

2.7.3 Primer efficiency test

Before miRNA analysis could be performed, the primer efficiency of the control miRNAs RNU44 and RNU48 were determined. Three different test samples measured in triplicate were used to run a standard curve using a quantitative real-time polymerase chain reaction (qRT-PCR). The qRT-PCR program for these efficiency testing are the same as the qRT-PCR for miRNA analysis in all the collected samples, see section 2.8.

After the qRT-PCR run, an abs quant/2nd derivative max analysis was performed to validate the Ct values. The standard curve contained an initiate concentration of 10 ng together with a ½ dilution performed five times for each sample until a final concentration of 0.3125 ng was reached. The efficiency corresponds to the slope of the standard curve (e.g. concentration versus the mean of the Ct-values), whereas a slope of -3.321928 is seen as the most optimal one. The efficiency of every primer was determined by using the following equation E: ((10^(-1/slope))—1)*100.

2.8 Quantitative real-time polymerase chain reaction (qRT-PCR)

TaqMan qRT-PCR using the TaqMan microRNA assays (Applied Biosystems, Diegem, Belgium) was performed in triplicate to validate the defined target miRNAs *(see appendix I for full protocol)*. For each TaqMan assay reaction, 1.33 µl cDNA was mixed with 10 µl TaqMan master mix, 1.00 µl TaqMan small RNA assay (20X) and 7.67 µl RNAse free water. To remove the inter run variation when data from different experiments are combined, three inter-run calibrators (IRCs) (e.g. the same on each plate) are inserted. Furthermore, three non-template controls (NTCs), consisting of water and master mix, were included for every miRNA in each plate, hereby excluding possible contamination of the master mix. The program consist of three steps: an activation step for ten minutes at 95°C, a denaturation step of 40 cycles whereof five seconds at 95°C and one minute at 60°C and finally a standard cool down step.

2.9 Statistical analysis

We performed pollutant-specific, exposure-response analysis using mixed models (version 9.2, SAS Institute Inc, Cary, NC). A random effect for each participant was used across the five clinical examinations. This method allows each subject to serve as his or her own control over time and controls for potential confounding from within-subject covariates that do not change over time. The acute and short-term exposure data ($PM_{2.5}$ and PM_{10}) were used to investigate the association between air pollution and retinal blood vessels.

The miRNA expression data was normalized with a natural log-transformation prior to data analysis. The normalized miRNA data were used to investigate the associations with retinal blood vessels and air pollution exposure in mixed effect models. All analyses were corrected for gender, age, body mass index (BMI), alcohol and coffee consumption 24 hours prior to the examination (where applicable), day of the week, location, participant's blood pressure (SBP, DBP) and fellow vessel diameter (where applicable).

3 Results

3.1 Study characteristics

The characteristics of our study population (n=50) are summarized in Table 1. The mean age in the population was 32 ± 8 years, and 50% of the participants were women. Only two participants were active smokers. Of the fifty adults who participated in our study, eleven persons did not complete all clinical study visits (e.g. five in total) and three samples of the remaining 239 were not included in the miRNA analysis due to poor sampling and insufficient RNA content, but were used in the other analyses.

Table 1: Characteristics of the study subjects (n=50)

CHARACTERISTICS	SUBCHARACTERISTICS	MEAN ± SD OR PERCENT (%)
Age (years)	/	32 ± 8
Sex (%)		
	Females	50
General health conditions	BMI (kg/m^2)	23 ± 3
	SBP (mmHg)	122 ± 8
	DBP (mmHg)	72 ± 9
	HR (bpm)	71 ± 12
Alcohol Consumption (glasses/24h)	/	2.0 ± 4.0
Smoking (%)	Yes	4
	No	96
Caffeine consumption (glasses/24h)	/	2.0 ± 2.0

All participants have a university or college degree and were free of any clinically diagnosed cardiovascular diseases, diabetes or any inflammatory disease that could influence the results. During the five month study period, $PM_{2.5}$ concentrations ranged from 1 to 61 µg/m^3 with an average concentration of 13.19 ± 12.54 µg/m^3. The PM_{10} concentrations ranged from 3.5 to 161.75 µg/m^3 with an average concentration of 31.36 ± 26.24 µg/m^3.

3.2 Associations between microvascular changes and acute exposures levels

3.2.1 Central retinal arteriolar equivalent (CRAE)

The average CRAE and CRVE in this study population were 152.79 ± 13.86 µm and 222.14 ± 21.59 µm. CRAE tended to be 5.05 µm larger in men then woman, however this association wasn't significant (p=0.3885). Age (p=0.0305), alcohol consumption (p=0.0437) and CRVE (p<0.0001) were significant predictors of CRAE. For each one-unit increase, CRAE decreased respectively with 0.55 µm, 0.32 µm and 0.48 µm. BMI (p=0.8118), pulse pressure (p=0.7785), day of the week (p=0.4343), caffeine (p=0.4108) and location (p=0.6952) were not significant predictors of CRAE.

In order to test our associations, we used mixed-effects models. We constructed three models with an increasing number of included covariates. Model 1 is the most basic and includes gender, age, BMI and pulse pressure as covariates. Model 2 also includes these covariates, but is expanded with alcohol and caffeine consumption in the 24 hours before the examination, the day of the week of the examination and the location of the examination. Model 3 is the full model. In this model all aforementioned covariates and fellow vessel diameter were included. When the association for CRAE was testes, CRVE was included as fellow vessel diameter and vice versa for the associations for CRVE.

In our mixed-effect models, CRAE was negatively associated with acute $PM_{2.5}$ exposure levels (Table 2 and Figure 4). Each 10 μg/m^3 increase in $PM_{2.5}$ was associated with a 1.01 μm (95%CI: -1.59;-0.43;*p=0.0014*) decrease in CRAE (Table 2, model 1). A similar result, a 1.00 μm (95%CI: -1.60;0.39;*p=0.0024*) decrease in CRAE, was found in model 2 (Table 2, model 2). In our final model, where CRVE was added, each 10 μg/m^3 increase in $PM_{2.5}$ was associated with a 0.56 μm (95%CI:-1.04;-0.08;p=0.0270) decrease in CRAE (Table 2, model 3).

Table 2: The association between the central retinal arterial equivalent (CRAE) and acute particulate matter ($PM_{2.5}$ and PM_{10}) exposure. Estimated change is represented in mean CRAE (95% CI).

CENTRAL RETINAL ARTERIAL EQUIVALENT (CRAE)

	Model 1	***Model 2***	***Model 3***
PM2.5 (for each 10 μg/m^3)	-1.01 (-1.59; -0.43)**	-1.00 (-1.60; -0.39)**	-0.56 (-1.04; -0.08)*
PM10 (for each 10 μg/m^3)	-0.36 (-0.63; -0.10)*	-0.30 (-0.58; -0.02)*	-0.16 (-0.38; 0.05)

Model 1 was adjusted for pulse pressure and individual characteristics such as gender, age and BMI. Model 2 includes model 1 plus the covariates location, alcohol consumption, caffeine consumption and day of the week. Model 3 includes model 2 plus the covariate CRVE. Statistical significance is expressed as follows: * p<0.05. ** p<0.01.

Subsequently, the association between PM_{10} and CRAE were examined using the same models (Table 2). Each 10 μg/m^3 increase in PM_{10} was associated with a 0.36 μm (95%CI: -0.63;-0.10;*p=0.0106*) decrease in CRAE in the basic model (Table 2, model 1). The association remained in model 2, as each 10 μg/m^3 increase in PM_{10} was associated with a 0.30 μm decrease (95%CI:-0.58;-0.02;*p=0.0388*) in CRAE (Table 2, model 2). In model 3, when CRVE was added, the association became non-significant (p=0.1459) (Table 2, model 3).

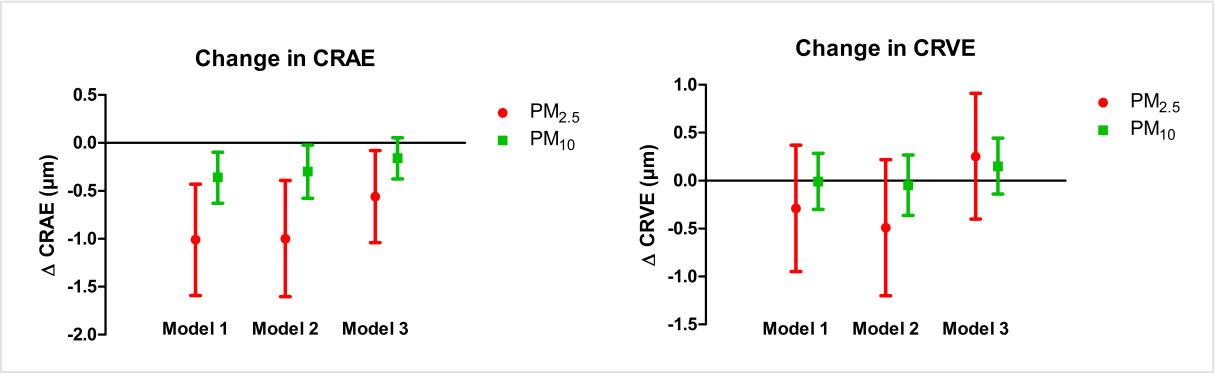

Figure 4: The estimate change in CRAE (95% CI) and CRVE after a 10 μg/m^3 increase in acute $PM_{2.5}$ or PM_{10} represented in each of the three models.

Model 1 was adjusted for pulse pressure and individual characteristics such as gender, age and BMI. Model 2 includes model 1 plus the covariates location, alcohol consumption, caffeine consumption and day of the week. Model 3 includes model 2 plus the covariate CRVE.

3.2.2 Central retinal venular equivalent (CRVE)

We examined the association between air pollution exposure and CRVE using the models described above. We did not find any significant associations between acute air pollution exposure to $PM_{2.5}$ or PM_{10} and CRVE (Table 3). However a trend was observed towards an association between acute $PM_{2.5}$ and PM $_{10}$ exposure levels and venular widening.

Table 3: The association between the central retinal venular equivalent (CRVE) and acute particulate matter ($PM_{2.5}$ and PM_{10}) exposure. Estimated change is represented in mean CRVE (95% CI).

	CENTRAL RETINAL VENULAR EQUIVALENT (CRVE)		
	Model 1	***Model 2***	***Model 3***
PM2.5 **(for each 10 µg/m^3)**	-0.29 (-0.95; 0.37)	-0.49 (-1.20;0.22)	0.25 (-0.40; 0.91)
PM10 **(for each 10 µg/m^3)**	-0.01 (-0.30; 0.29)	-0.05 (-0.36; 0.27)	0.15 (-0.14; 0.44)

Model 1 was adjusted for pulse pressure and individual characteristics such as gender, age and BMI. Model 2 includes model 1 plus the covariates location, alcohol consumption, caffeine consumption and day of the week. Model 3 includes model 2 plus the covariate CRAE.

3.3 Associations between microvascular changes and short-term air pollution exposure

We used air quality measurements from a nearby monitoring station (operated by the Flemish Environmental Agency) in order to investigate short-term effects of air pollution exposure on CRAE and CRVE.

3.3.1 Central retinal arteriolar equivalent (CRAE)

The association between short-term air pollution exposure and CRAE was examined using the same mixed models as previously described. We did not find any significant association between short-term $PM_{2.5}$ exposure (e.g. during the previous 24hr, 48hr or previous week) and CRAE, *see Table 4 and Figure 5*.

CRAE was not associated with short-term PM_{10} exposure levels during the previous 24hrs in either of our models (Table 4, model 1 till 3 and Figure 5). However, each 10 µg/m^3 increase in PM_{10} during the previous 48hrs was associated with a 0.83 µm (95%CI:-1.56;-0.09;*p=0.0327*) decrease in CRAE (Table 4, model 1 and Figure 5). This association became borderline significant in model 2 (p=0.0637) (Table 4, model 2 and Figure 5). When CRVE was added to the model, each 10 µg/m^3 increase in PM_{10} during the previous 48hrs was associated with a 0.67 µm (95%:-1.28; -0.06;*p=0.0367*) decrease in CRAE (Table 4, model 3 and Figure 5).

An association was found between short-term PM_{10} exposure levels during the previous week and CRAE (Table 4 and Figure 5). Each 10 μg/m^3 increase in PM_{10} was associated with a 0.85 μm (95%CI:-1.44;-0.27;*p=0.0063*) decrease in CRAE (Table 4, model 1). The association remained in model 2, as each 10 μg/m^3 increase in PM_{10} was associated with a 0.83 μm (95%CI: -1.46;0.20;*p=0.013*) decrease in CRAE (Table 4, model 2). In our final model, the association became non-significant (Table 4, model 3).

Table 4: The association between the central retinal arteriolar equivalent (CRAE) and short-term particulate matter ($PM_{2.5}$ and PM_{10}) exposure. Estimated change is represented in mean CRAE (95% CI).

		CENTRAL RETINAL ARTERIAL EQUIVALENT (CRAE)		
		Model 1	***Model 2***	***Model 3***
PM2.5	*Lag 24*	-0.27 (-1.03; 0.50)	-0.36 (-1.23; 0.51)	-0.32 (-0.97; 0.33)
	Lag 48	-0.59 (-1.57; 0.40)	-0.65 (-1.75; 0.46)	-0.48 (-1.31; 0.34)
	Lag week	-0.48 (-1.17; 0.21)	-0.52 (-1.25; 0.22)	-0.20 (-0.76; 0.37)
PM10	*Lag 24*	-0.42 (-0.99; 0.15)	-0.55 (-1.22; 0.12)	-0.45 (-0.93; 0.04)
	Lag 48	-0.83 (-1.56; -0.09)*	-0.82 (-1.67; 0.03)	-0.67 (-1.28; -0.06)*
	Lag week	-0.85 (-1.44; -0.27)**	-0.83 (-1.46; -0.20)*	-0.41 (-0.88; 0.07)

*Model 1 was adjusted for pulse pressure and individual characteristics such as gender, age and BMI. Model 2 includes model 1 plus the covariates location, alcohol consumption, caffeine consumption and day of the week. Model 3 includes model 2 plus the covariate CRVE. Statistical significance is expressed as follows: * p<0.05. ** p<0.01.*

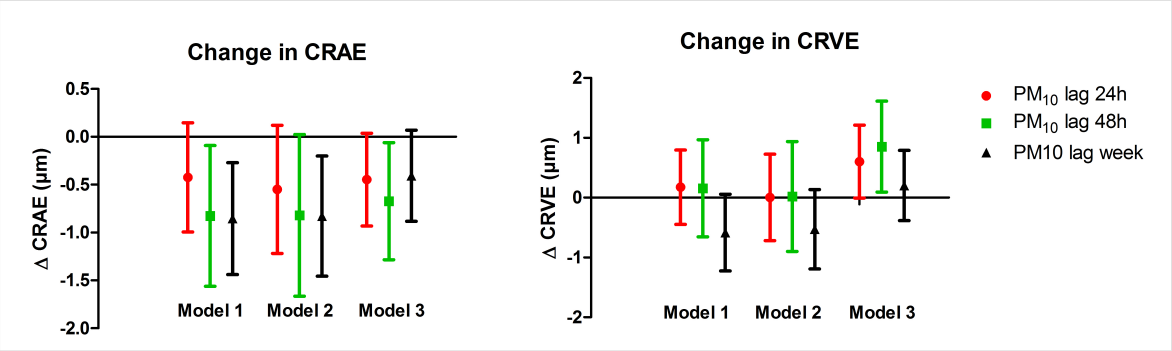

Figure 5: The estimate change in CRAE (95% CI) and CRVE after a 10 μg/m^3 increase in short-term $PM_{2.5}$ or PM_{10} represented in each of the three models.
Model 1 was adjusted for pulse pressure and individual characteristics such as gender, age and BMI. Model 2 includes model 1 plus the covariates location, alcohol consumption, caffeine consumption and day of the week. Model 3 includes model 2 plus the covariate CRVE.

3.3.2 Central retinal venular equivalent (CRVE)

Subsequently, we examined the associations between short-term air pollution exposure and CRVE using the same mixed models. We did not find any significant associations between short-term air pollution exposure of $PM_{2.5}$ CRVE based on these models except (Table 5). However, we did found a trend towards an association between short-term PM_{10} exposure levels and venular widening. All the results showed a borderline statistical significance except for PM_{10} during the previous 48hrs. In our final model, each 10 μg/m^3 increase in PM_{10} during the previous 48hrs was associated with a 0.85 μm (95%CI:0.09;1.61;*p=0.034*) increase in CRVE (Table 5, model 3).

Table 5: The association between the central retinal venular equivalent (CRVE) and short-term particulate matter ($PM_{2.5}$ and PM_{10}) exposure. Estimated change is represented in mean CRVE (95% CI).

		CENTRAL RETINAL VENULAR EQUIVALENT (CRVE)		
		Model 1	***Model 2***	***Model 3***
PM2.5	*Lag 24*	0.27 (-0.56; 1.10)	-0.03 (-0.95; 0.90)	0.56 (-0.26; 1.39)
	Lag 48	0.12 (-0.96; 1.20)	-0.24 (-1.40; 0.93)	0.69 (-0.35; 1.73)
	Lag week	-0.43 (-1.17; 0.32)	-0.39 (-1.16; 0.37)	0.15 (-0.53; 0.83)
PM10	*Lag 24*	0.17 (-0.45; 0.80)	0.00 (-0.72; 0.73)	0.60 (-0.01; 1.21)
	Lag 48	0.16 (-0.66; 0.97)	0.02 (-0.90; 0.94)	0.85 (0.09; 1.61)*
	Lag week	-0.59 (-1.23; 0.06)	-0.53 (-1.19; 0.14)	0.20 (-0.39; 0.79)

Model 1 was adjusted for pulse pressure and individual characteristics such as gender, age and BMI. Model 2 includes model 1 plus the covariates location, alcohol consumption, caffeine consumption and day of the week. Model 3 includes model 2 plus the covariate CRAE.

3.4 miRNA/RNA quantification

RNA quantification needed to be assessed before miRNA analysis could be performed in order to obtain meaningful expression results. The purity and integrity of RNA was measured using the Nanodrop spectrophotometer. Almost all RNA samples showed a sufficient amount of RNA (> 50 ng/µl) to apply these in the miRNA analysis except for three samples with a concentration below 20 ng/µl. All the remaining RNA samples showed a high quality and intact RNA based on the 260/280 ratio. They were all located within a range of 2.0 and 2.3. Figure 6 represents a) the RNA concentration of ten independent samples chosen over the whole period of the study and b) the quality of these ten RNA samples to illustrate the preceding.

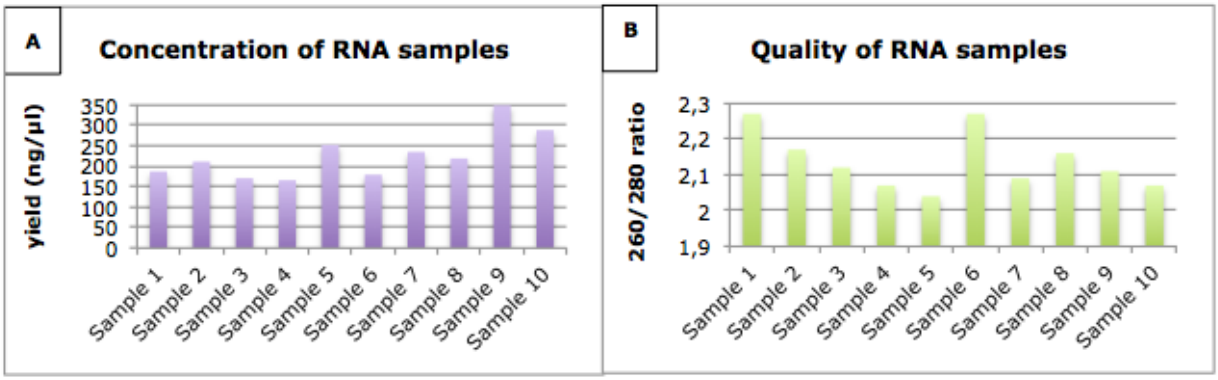

Figure 6: Panel A) *Illustrates the RNA concentration/yield of ten independent samples chosen over the whole period of the study. The yield was determined using the Nanodrop spectrophotometer. All RNA samples show a higher concentration then 50 ng/µl.* ***Panel B)*** *Shows the quality of the ten RNA samples. All the samples have a higher 260/280 ratio then 2.00 indicating a very high and intact RNA quality, necessary to continue for miRNA analysis.*

3.4.1 Detection of miRNA content and quality in RNA samples

The amount and quality of miRNA in the RNA samples was measured with the Agilent 2100 Bioanalyzer. All bioanalyzer results, using the small RNA kit, showed the presence of miRNAs in the samples on the electropherogram (see Figure 7 panel A). The x-axis represents the migration time in seconds and the y-axis the fluorescence intensity (FU). Furthermore, the densitometry plots, indicates the presence of miRNA *(see Figure 7 panel B)*.

The bioanalyzer also measured the amount of miRNA present in the purified RNA. According to these data, miRNA only count for 18.58 ± 13.16% of the total RNA, *see Figure 7 panel C*. Besides the percentage, an estimation of the miRNA concentration was given by the Bioanalyzer. An example is given in Figure 7 panel D of eight independent RNA samples chosen over the first four sampling periods.

3.5 Validation using qRT-PCR

3.5.1 Determination of internal control miRNAs

miRNA analysis requires proper internal controls to become accurate and reliable end results. A first qRT-PCR plate with four test samples was carried out. These data showed that RNU6 appeared between a Ct value of 28.50 and 32.50, RNU44 between 24 and 28 and RNU48 between 24 and 26.50. The target miRNAs appeared between a Ct value of 23.50 and 31. Based on these parameters, all the subsequent analyses were performed with RNU44 and RNU48 as internal controls.

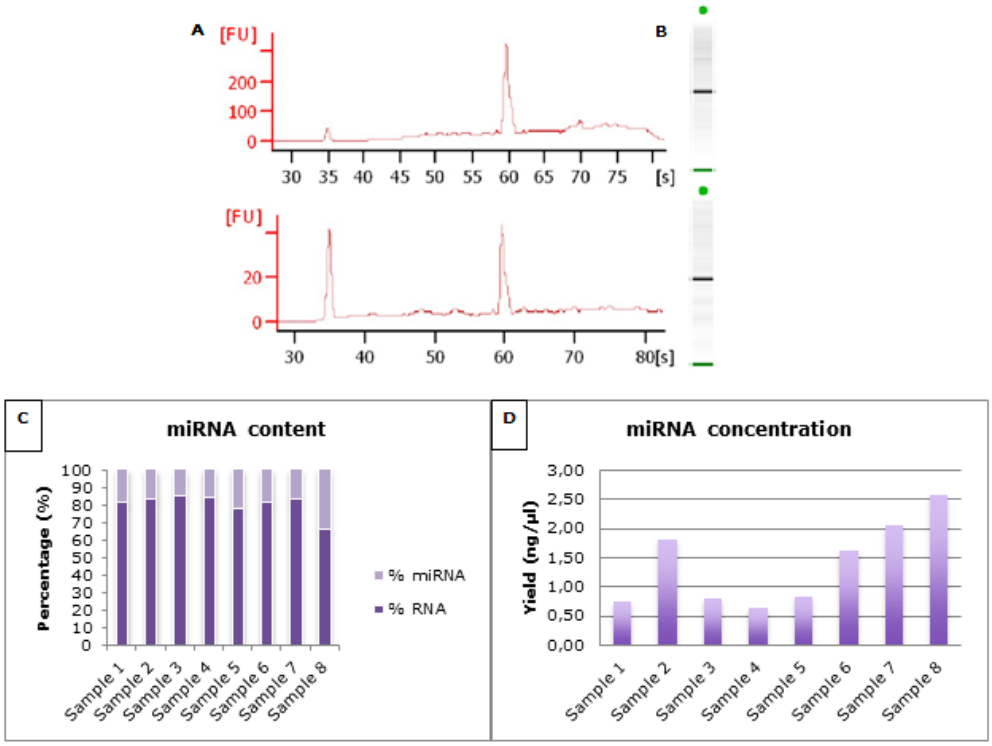

Figure 7: ***Determination of the amount and quality of miRNA in the RNA samples purified from tempus tubes.*** ***Panel A)*** *represents the electropherogram. On the X-axis the migration time in seconds and on the y-axis the fluorescence intensity (FU). The peak represents the time at which a fragment with a certain length showed up.* ***Panel B)*** *is the densitometry plot. It represents the gel electrophoresis of the RNA sample, indicating that miRNA is present in the samples.* ***Panel C)*** *shows an estimation of the amount of miRNA and RNA extracted from the tempus tubes using the total RNA purification kit (Norgen). Finally* ***panel D)*** *shows the miRNA concentration present in eight independent RNA samples.*

3.5.2 Efficiency validation of RNU44 and RNU48

The efficiency of the two internal control miRNAs (RNU44 and RNU48) was measured before miRNA analysis could be performed. RNU44 has an efficiency of 81% and RNU48 has an efficiency of 70%, listed in *Table 6*. According to these values, only RNU44 could be used as a valid internal control miRNA. However, only a small difference was set-up between the start and end concentration due

to the selected protocol. As a result, it is not recommended to fully rely on these results. Therefore, both internal control miRNAs were measured in the miRNA analysis. qBase and statistical analysis will confirm, which of these two controls or even both will be used as internal control(s) to validate all the samples.

Table 6: The detection range (Ct values) of both target and control miRNAs present in the qRT-PCR plate for the validation of the internal control miRNA.

Type of miRNAs	Detection range (Ct value)	Efficiency
Target miRNAs		
miR-21	23.50 - 28.50	-
miR-222	24 – 30.50	-
miR-146	26 - 31	-
Control miRNAs		
RNU6	28.50 – 32.50	-
RNU44	24 - 28	81 %
RNU48	24.50 – 26.50	70 %

3.6 miRNA analysis

3.6.1 Association between miRNAs and short-term PM_{10}/ $PM_{2.5}$ exposure levels

First, we examined the association between miR-21, miR-222 and miR-146a and short-term $PM_{2.5}$ or PM_{10} exposure. All our models were corrected for pulse pressure, age, gender, BMI, location, alcohol consumption, day of the week and caffeine consumption. No associations were found between acute $PM_{2.5}$ or PM_{10} exposure levels and miRNAs (Table 7 and Figure 8). Furthermore, we did not find any association between short-term $PM_{2.5}$ exposure levels during the previous 24hrs, 48hrs or the previous week and the three target miRNAs, except for miR-222, which was negatively associated with short-term $PM_{2.5}$ exposure levels during the previous week. Each 10 μg/m^3 increase in $PM_{2.5}$ was associated with a 5.43% (95%CI:-10.07;-0.79;*p=0.0214*) lower expression of miR-222 (Table 7).

Table 7: The association between miRNA expression and short-term particulate matter ($PM_{2.5}$ and PM_{10}). Estimated change is represented in mean percentage (95% CI).

		Change in miRNA expression (%)		
		miR-21	***miR-222***	***miR-146a***
PM2.5	*Lag 24*	-0.90 (-6.35; 4.56)	1.76 (-2.90; 6.43)	1.89 (-2.76; 6.54)
	Lag 48	0.49 (-6.52; 7.50)	2.35 (-3.53; 8.22)	2.88 (-3.02; 8.78)
	Lag week	-1.94 (-7.42; 3.54)	-5.43 (-10.07; -0.79)*	-0.19 (-5.27; 4.90)
PM10	*Lag 24*	-6.62(-11.07; -2.17)**	-6.71 (-10.68; -2.75)**	-1.36 (-5.79; 3.07)
	Lag 48	-8.27 (-14.14; -2.40)**	-10.93 (-15.87; -5.98)***	-2.56 (-8.32; 3.20)
	Lag week	-6.19 (-10.89; -1.49)**	-10.74 (-14.41; -7.07)***	-3.48 (-8.07; 1.11)

*The associations are corrected for pulse pressure, age, gender, BMI, location, alcohol consumption, caffeine consumption and day of the week. Statistical significance is expressed as follows: *p<0,05; **p<0,01; ***P<0,001.*

Subsequently, a negative association was found between miR-21,miR-222 and short-term PM_{10} exposure levels during the previous 24hrs, 48hrs and previous week. (Table 7 and Figure 8). Each 10 μg/m^3 increase in PM_{10} during the previous 24hrs was associated with a 6.62% (95%CI: -11.07;-2.17;*p=0.0038*) and a 6.71% (95%CI:-10.68;-2.75;*p=0.0012*) lower expression in respectively miR-21 and miR-222 (Table 7). Each 10 μg/m^3 increase in PM_{10} in the previous 48hrs, was associated with a 8.27% (95%CI:-14.14;-2.40;*p=0.0054*) and a 10.93% (95%CI:-15.87; -5.98;*p<0.0001*) lower expression in respectively miR-21 and miR-222 (Table 7). Each 10 μg/m^3

increase in PM_{10} levels from the previous week was associated with a 6.19% (95%CI:-10.89; -1.49;*p=0.0099*) and a 10.74% (95%CI:-14.41;-7.07;*p<0.0001*) lower expression in respectively miR-21 and miR-222 (Table 7). Finally, the same trend was observed between short-term PM_{10} exposure levels during the previous 24hrs, 48hrs and previous week and miR-146a, although no significant association was found (Table 7).

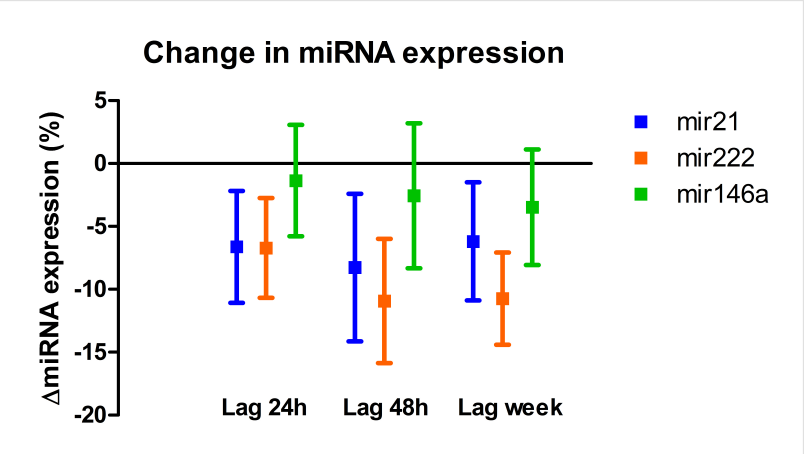

Figure 8: The estimate percentage change in miRNA expression (95% CI) after a 10 µg/m³ increase in short-term PM_{10} during the previous 24hrs, 48hrs and previous week.
Model adjusted for pulse pressure, age, gender, BMI, alcohol consumption location, caffeine consumption and day of the week.

3.6.2 Associations between miRNAs and retinal blood vessels CRAE and CRVE.

Mixed-effect models were used to examine the association between CRAE and miRNA expression levels (Table 8 and Figure 9). Model 1 was corrected for pulse pressure, age, gender, BMI, alcohol consumption, location, caffeine consumption and day of the week. Model 2 includes all aforementioned covariates and fellow vessel diameter. We did not find any significant association between CRAE and miRNA expression, except for miR-21 (Table 8, model 2). Each 10% increase in miR-21 was associated with a 0.12 µm (95%CI:0.01;0.23;*p=0.0446*) increase in CRAE (Table 8, model 2). However, a trend was observed between miR-222, miR-21 and miR-146 and retinal arteriolar widening.

Table 8: The association between the central retinal arteriolar equivalent (CRAE) and miRNA expression. Estimated change is represented in mean CRAE (95% CI)

	CENTRAL RETINAL ARTERIAL EQUIVALENT (CRAE)	
	Model 1	***Model 2***
miR-21	0.09 (-0.05; 0.23)	0.12 (0.01; 0.23)*
miR-222	-0.03 (-0.14; 0.09)	0.05 (-0.04; 0.14)
miR-146a	0.02 (-0.14; 0.18)	0.09 (-0.04; 0.21)

*Model 1 includes following covariates: pulse pressure, age, gender, BMI, location, alcohol consumption, caffeine consumption and day of the week. Model 2 includes also fellow vessel diameter. Statistical significance is expressed as follows: *p<0,05; **p<0,01.*

Subsequently, the same models were used to examine the association between CRVE and miRNA expression (Table 9 and Figure 9). We did not found a significant association between miR-21 expression and CRVE (Table 9), although a trend towards the same association between miR-22 and miR-146a and CRVE can be noticed. The expression of miR-222 was negative associated with CRVE. A 0.16 µm (95%CI:-0.31;-0.02;*p=0.0324*) decrease in CRVE was observed after each 10 %

increase in miR-222 (Table 9). When CRAE was added to the model the association became non-significant. miR-146a expression was associated with CRVE. A 0.26 µm (95%CI:-0.43; -0.09;*p=0.0042*) decrease in CRVE was associated with each 10% increase in miR-146a (Table 9). This association remained significant after adding CRAE to the model with a 0.23 µm (95%CI: -0.39;-0.07;*p=0.0085*) decrease in CRVE after each 10% increase in miR-146a.

Table 9: The association between the central retinal venular equivalent (CRVE) and miRNA expression. Estimated change is represented in mean CRVE (95% CI)

	CENTRAL RETINAL VENULAR EQUIVALENT (CRVE)	
	Model 1	***Model 2***
miR-21	-0.072 (-0.231; 0.087	-0.072 (-0.221; 0.077)
miR-222	-0.163 (-0.307; -0.018)*	-0.110 (-0.236; 0.015)
miR-146a	-0.263 (-0.434; -0.092)**	-0.226 (-0.388; -0.065)**

*Model 1 includes following covariates: pulse pressure, age, gender, BMI, location, alcohol consumption, caffeine consumption and day of the week. Model 2 includes also fellow vessel diameter. Statistical significance is expressed as follows: *p<0,05; **p<0,01.*

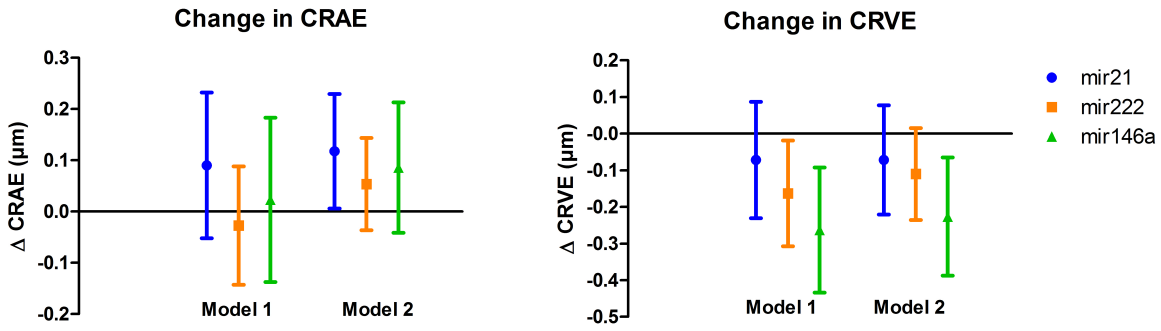

Figure 9: The estimate change in CRAE/CRVE (95% CI) after each 10% increase in miRNA expression.
Model 1 includes following covariates: pulse pressure, age, gender, BMI, location, alcohol consumption, caffeine consumption and day of the week. Model 2 includes also fellow vessel diameter.

4 Discussion

4.1 Retinal blood vessels and air pollution exposure

4.1.1 General outcomes

Epidemiological studies have indicated that ambient air pollution levels are associated with several adverse health outcomes, of which a compromised cardiovascular health might be the most important. [4, 5] A study, conducted in Atlanta over a period of seven years, reported an association between cardiovascular disease emergency department visits and peak concentrations of $PM_{2.5}$. [57] The microcirculation can be non-invasively studied and measured in the retinal blood vessels. Retinal arterial narrowing is an independent risk factor of cardiovascular disease, indicating arteriolar damage that could predict hypertension. [58, 59] Chew *et al.* demonstrated that each 10 mm Hg increase in mean arteriolar blood pressure was associated with a 3 µm decrease in CRAE. [60] Retinal venular widening has been associated with inflammation and endothelial dysfunction. [58, 59]

The first objective of this study was to examine the effect of acute and short-term air pollution exposure of $PM_{2.5}$ and PM_{10} on retinal blood vessels. In this study, we found an association between acute $PM_{2.5}$ and PM_{10} exposure levels and retinal arteriolar narrowing. However, we did not found any significant association between acute air pollution exposure of $PM_{2.5}$ or PM_{10} and retinal venular widening. Subsequently, we observed a negative association between short-term PM_{10} exposure levels in several exposure windows and CRAE. Moreover, PM_{10} exposure showed a trend towards an association with increases in CRVE. We did not find an association between short-term $PM_{2.5}$ exposure levels and CRAE or CRVE.

4.1.2 The association between CRAE and air pollution exposure

Acute $PM_{2.5}$ and PM_{10} exposure concentrations were associated with narrower arteriolar diameters. Each 10 µg/m³ increase in $PM_{2.5}$ or PM_{10} were associated with respectively a 1.0 µm (95%CI: -1.60;-0.39) or a 0.30 µm (95%CI:-0.58;-0.02) decrease in CRAE. This negative association between acute $PM_{2.5}$ or PM_{10} exposure levels and CRAE was in accordance with previous studies performed by Adar *et al.* and Louwies *et al.* [15, 16]

Our acute and short-term PM_{10} exposure results were smaller, but consistent with the associations between CRAE and PM_{10} reported by Louwies *et al.* [16] We can only compare our acute results with the shorter time windows reported by Louwies *et al.* They found a 0.62 µm decrease in CRAE after each 10 µg/m³ increase in PM_{10} during the previous 2hr, whereas our acute exposure was associated with a 0.30 µm (95%CI:-0.58;-0.02) decrease in CRAE. Louwies *et al.* reported a 0.93 µm (95%CI:-1.42;-0.45) decrease in CRAE after each 10 µg/m³ increase in PM_{10} during the previous 24hrs in healthy subjects, but did not report a significant association for 48hrs. [16] We found a 0.67 µm (95%CI:-1.28;-0.06) decrease in CRAE after each 10 µg/m³ increase in PM_{10} during the previous 48hrs, but could not find an association between CRAE and PM_{10} levels during the previous 24hrs. Despite the difference in exposure window, these results are comparable.

Adar *et al.* found a 0.4 µm (95%CI:-0.80;0.10) decrease in CRAE after a 9 µg/m³ increase in $PM_{2.5}$ on the previous day. [15] The effect size, reported in the MESA cohort, is smaller than the result mentioned in our study. The participants encountered $PM_{2.5}$ exposure concentration on the concurrent day between a range of 1.4 and 19.7 as opposed to 1 to 61 µg/m^3 in our study. However, it is difficult to compare the $PM_{2.5}$ exposure levels between our studies, because the MESA study used modelled air pollution data based on nearby official monitoring stations, whereas we measured air pollution levels at the same time the sampling occurred. Exposure misclassification is a smaller problem in our study, when compared to Adar's.

4.1.3 The association between CRVE and air pollution exposure

We did not find any significant association between acute air pollution exposure to $PM_{2.5}$ or PM_{10} and CRVE in this study. This was also confirmed in the study performed by Adar *et al.* between short-term air pollution exposure to $PM_{2.5}$ and CRVE. [15] In contrast with Adar *et al.*, Louwies *et al.* observed a 0.86 µm (95%CI:-1.42;-0.30) decrease in CRVE after each 10 µg/m^3 increase in PM_{10} during the previous 24hrs. [16]

Adar *et al.* also noticed a trend towards a positive association between $PM_{2.5}$ exposure levels and CRVE. They found a 0.40 µm (95%CI:-0.30;1.10) increase in CRVE after each 10 µg/m³ increase in $PM_{2.5}$ during the previous day, although this association was not significant. [15] We observed a non-significant 0.56 µm (95%CI:-0.26;1.39) increase in CRVE after each 10 µg/m³ increase in $PM_{2.5}$ during the previous day. As opposed to Adar et al., Louwies et al. reported a 0.60 µm (95%CI:-1.26;0.07) decrease in CRVE after each 10 µg/m³ increase in PM_{10} during the previous day. [16] This is in contrast with our findings with a non-significant 0.60 µm (95%CI:-0.01;1.21) increase in CRVE after each 10 µg/m³ increase in PM_{10} during the previous 24hrs. In addition, we found a significant association between short-term PM_{10} exposure and CRVE. Each 10 µg/m³ increase in PM_{10} during the previous 48hrs was associated with a 0.85 µm (95%CI:0.09;1.61) increase in CRVE. This is in contrast with the results reported by Louwies *et al*., who reported a 0.84 µm (95%CI:-1.61;-0.08) decrease in CRVE. [16]

4.1.4 Possible biological mechanisms

Generally, our findings are consistent with literature based on controlled air pollution exposure in animal and human studies. [25, 61-64] Nurkiewicz *et al.* demonstrated in 2004, 2006 and 2009 that high short-term air pollution levels are associated with systemic microvascular dysfunction. [25, 62, 63] They demonstrated that local PM deposition and oxidative stress response in rats after pulmonary air pollution exposure caused systemic inflammation. This process caused the uncoupling of eNOS, reducing the bioavailability of NO, which is a key mediator of normal endothelial function.

Peretz et al. also confirmed these toxicological studies in vivo: short-term diesel exhaust exposure is associated with acute endothelial responses and vasoconstriction. [20] Furthermore, an acute arterial vasoconstriction was observed in healthy adults by Brooke *et al.* in 2002. [61] Törnqvist *et al.* demonstrated in 2007 persistent endothelial dysfunction in fifteen healthy men after diesel exhaust exposure. [64]

The pathway responsible for the association between air pollution and narrowing of the retinal blood vessels will most likely involve systemic inflammation and endothelial dysfunction. Inhalation of the PM particles will cause a pulmonary inflammation together with oxidative stress. Secretion of proinflammatory markers in the circulation will induce a systemic inflammatory response. This process is also characterized by oxidative stress, and inflammation (e.g. activation of leukocytes and platelets) and will target the endothelium to induce endothelial dysfunction. [64]

This described mechanism might explain the differences between the study of Louwies *et al.* and ours. Moreover, the differences in exposure concentrations will likely have influenced the results. In the study by Louwies *et al.* the PM_{10} exposure levels was related with a spike in particulate matter in the second visit. This caused a high air pollution exposure for all the participants. This was not the case in our study. The PM_{10} concentrations in our study were rather sustained, resulting in little fluctuations and less larger exposure contrasts. When differences in exposure were present, only a few participants encountered these highest exposure levels. In the short-term study by Louwies *et al.,* higher exposures during the 24-hour time span, might have lead to a larger effect on CRAE. Subsequently, PM particles in our study might not have enough time the time to cause the same adversely effect on vascular function as they can in 24 hrs. Still, we found at even low exposures levels an effect probably explained by the large power input. We used fifty people in our study and five different sampling periodes creating a very large power.

No results were found between short-term $PM_{2.5}$ exposure levels and CRAE or CRVE. Short-term $PM_{2.5}$ levels in the several exposure windows showed little variation compared to our acute $PM_{2.5}$ exposure levels, which might explain the absence of the effect on the retinal blood vessels. $PM_{2.5}$ is very spatial and temporal, which makes it very difficult to measure. When using just monitoring stations, exposure misclassification may occur, whereas this is absent when air pollution was acutely measured on the site of the clinical visit.

We hypothesize that the difference between the study of Adar *et al.* and our study can be attributable to differences in encountered $PM_{2.5}$ concentrations. Adar *et al.* reported lower concentrations, but these were based on modelled databased on a (distant) monitoring station. In addition, our study only included healthy subjects, whereas the MESA study contained older and a more divers study population. In healthy people, arteriolar narrowing may be more pronounced than in older persons as in the MESA cohort. Theoretically, the microvasculature of healthy and young adults should be respond better to changes in air pollution exposure. In addition, older persons or persons with a cardiovascular disease might have an affected microvasculature, which could not response in the same way as the microvasculature of young healthy people.

4.1.5 Differences in CRAE outcomes between $PM_{2.5}$ and PM_{10}

For each 10 µg/m³ increase in $PM_{2.5}$ and PM_{10}, we reported a decrease in CRAE of respectively 1.00 µm and 0.30 µm. $PM_{2.5}$ particles are part of the fine and ultrafine particles, which can cross the pulmonary epithelium, enter the bloodstream and penetrate the human body much further than PM_{10} particles. In addition, $PM_{2.5}$ particles have a smaller aerodynamic diameter than PM_{10}, allowing them to be deposited deeper into the lungs. This enables $PM_{2.5}$ to cause a larger pulmonary inflammation, making fine particles more reactive than PM_{10} or coarse particles.

4.2 Integrative analysis of miR-21, miR-222 and miR-146a

4.2.1 General outcomes

Although epidemiological studies suggest several biological pathways linking PM exposure with cardiovascular disease, the molecular pathways behind oxidative stress, inflammation and endothelial dysfunction remain less clear. Wang *et al.* reported in 2005 the modification of gene expression of inflammatory genes in peripheral blood of individuals who were exposed. [65] In addition, miRNAs can bind to mRNA and control gene expression at a post-transcriptional level. [50] Moreover, an association was found between PM exposure levels and a dysregulation of miRNAs in vitro, indicating that miRNAs can be involved in the PM effects on the cardiovascular system. [48]

The second objective of this study was to examine if miR-21, miR-222 and miR-146 were associated with air pollution exposure. Here, we report a negative association between short-term PM_{10} exposure levels and both miR-21 and miR-222 expression levels. Overall, we did not find any significant association between short-term $PM_{2.5}$ exposure levels and miR21, miR-222 and miR-146a expression.

Subsequently, the third and last objective required the examining of an association between miR-21, miR-222, miR-146a and retinal blood vessels. The results in our study indicate a trend towards a positive association between each 10% increase in miRNA expression and CRAE, although our results remain non-significant. As opposed to CRAE, a negative association was found between miR-222 and miR-146a expression and CRVE. We did not find any significant association between miR-21 expression and CRVE, although the same trend was seen in this miRNA as the other two.

4.2.2 The association between miRNAs and short-term air pollution exposure

An overall negative association was observed between miR-21, miR-222 and short-term PM_{10} exposure levels in our study, with a similar trend for miR-146a. These results are in accordance with Fossati *et al.*, who reported a negative association between miR-21, miR-222, miR-146a and short-term $PM_{2.5}$ exposure levels in a population of foundry workers. [50]

Our study found smaller effects based on low background ambient PM_{10} exposure levels as opposed to Fossati *et al.* who reported a 34% (95%CI:-48;-17) lower miR-146a expression together with a 35% (95%CI:-48;-18) lower miR-21 expression after an increase in $PM_{2.5}$ during the previous week. [50]

We did not find any significant association between miR-21, miR-222 or miR-146a and short-term $PM_{2.5}$ exposure levels in our study. The occupational setting of the study by Fossati *et al.* may induce a larger effect, because these $PM_{2.5}$ particles are much more reactive than our background $PM_{2.5}$ exposure levels measured by the monitoring stations. Furthermore, exposure misclassification can also contribute to the non-significance between $PM_{2.5}$ and the miRNA expression in our study. In addition, only small variations were presents in our short-term $PM_{2.5}$ exposure levels, making our PM exposure relatively stable.

Previous studies mostly compared miRNA expression patters between pathological samples and normal tissues, but only a few studies have evaluated changes in miRNA expression due to PM exposure. In human bronchial epithelial cells, 197 of 313 miRNAs showed a disrupted expression ≥ 1.5 fold after treatment with diesel exhaust particles. [48] The results found by Bollati *et al.* and Motta *et al.* were in contrast with our study. [42, 49] Bollati *et al.* reported an upregulation of miR-21 and miR-222 in peripheral blood leukocytes of foundry workers after short-term occupational exposure to metal rich PM. [49] Again, an occupational setting was used as opposed to our study design together with the differences in particles composition, which can explain the conflicting results. Subsequently, Motta et al. reported that the expression of miR-146a was differentially expressed after meatal rich PM exposure. [42] He only used ten participants with a very high metal rich PM exposure as opposed to our fifty healthy normal exposed subjects.

According to our study and Fossati *et al.* the expression of miR-21, miR-146a and miR-222 are downregulated after air pollution exposure. It has been found that lower levels of these miRNAs are associated with a higher expression pattern of their target mRNA. [42] Processes, such as inflammation, oxidative stress and endothelial dysfunction, which are associated with miR-21, miR-146a and miR-222 could thus be increased.

We did not find any associations between miR-21, miR-222, miR-146a and acute $PM_{2.5}$ or PM_{10} exposure levels. We only found a negative association between miR-21, miR-222 and short-term PM_{10} exposure levels after the previous 24hrs and 48hrs. Changes on miRNA level requires alterations in gene expression and its regulation, processes which demand a certain amount of time before it can be detected.

4.2.3 The association between miRNAs and retinal blood vessels

This study demonstrates that miR-222 and miR-146a are positively associated with retinal venular vessels. miR-146a is associated with inflammation, whereas miR-21 is associated with inflammation, atherosclerosis and endothelial dysfunction. Retinal venular widening is also associated with these processes. Furthermore, short-term PM_{10} exposure levels showed a trend towards retinal widening with a significant association between CRVE and each short-term PM_{10} during the previous 48hrs. It has been found that retinal venular widening is a risk factor of cardiovascular disease, indicating that miRNAs may be the possible pathway linking air pollution exposure with microvascular changes.

High PM_{10} exposure levels cause a downregulation in miRNA expression levels, such as miR-21 and miR-222. These disrupted miRNA levels might be increasing inflammation, oxidative stress production and endothelium dysfunction, which may initiate changes in the microcirculation e.g. venular widening and arteriolar narrowing in the retina.

Conclusion

In conclusion, three major associations were found in our repeated measurement design study of fifty healthy participants. First, acute $PM_{2.5}$ and PM_{10} exposure levels are associated with arteriolar narrowing. Short-term PM_{10} exposure levels are also associated with a decrease in CRAE. Furthermore, acute $PM_{2.5}$ and PM_{10} as well as short-term PM_{10} exposure levels showed a trend towards an association with venular widening. Second, short-term PM_{10} exposure levels are associated with a downregulation of miR-21 and miR-222, more particularly miRNAs involved in inflammation, oxidative stress and endothelial dysfunction. Third, a 10% increase of miR-146a and miR-222 are associated with a decrease in CRVE. This negative association is as expected, because higher levels of miRNAs are associated with lower expression levels with their target mRNA.

A few studies have investigated the effect of PM on miRNAs or the effect of PM on retinal blood vessels. We investigate both directions and found a new possible pathway that could help us understand on a molecular level how PM particles can cause changes to the retinal blood vessels that may lead to the development of cardiovascular disease.

An increase in PM concentration causes a downregulation in miRNAs involved in inflammation, oxidative stress and endothelial dysfunction. These disruptions in miRNAs interfere with gene expression and its regulation and might result in higher expression levels of inflammation and oxidative stress-related genes. These two processes can initiate endothelial dysfunction, which may causes changes in the microcirculation. In addition both arteriolar narrowing and venular widening are risk factors of cardiovascular disease. Narrowing of the retinal blood vessels may predict hypertension and venular widening is associated with atherosclerosis, both are well known causes of heart disease.

Thus, PM exposure can cause little changes in the microcirculation of healthy individuals and influence cardiovascular health. These changes might be mediated by air pollution-induced changes in the expression of miRNAs, which post-transcriptionally control genes involved in inflammatory and atherosclerotic pathways. Although these changes were reversible in our study population, accumulation of these adverse effects of air pollution might eventually result in permanent vascular changes and this can have serious consequences on cardiovascular health.

Bibliography

(1) Barath S, Mills NL, Adelroth E, Olin AC, Blomberg A. Diesel exhaust but not ozone increases fraction of exhaled nitric oxide in a randomized controlled experimental exposure study of healthy human subjects. *Environ Health* 2013;12:36.

(2) Stapleton PA, Minarchick VC, McCawley M, Knuckles TL, Nurkiewicz TR. Xenobiotic particle exposure and microvascular endpoints: a call to arms. *Microcirculation* 2012 February;19(2):126-42.

(3) Organization WH. WHO links 7 million premature deaths annually to air pollution; 12.5% of total global deaths. *WHO* 2014 March 25;Available at: URL: http://www.greencarcongress.com/2014/03/20140325-who.html.

(4) Brook RD, Franklin B, Cascio W et al. Air pollution and cardiovascular disease: a statement for healthcare professionals from the Expert Panel on Population and Prevention Science of the American Heart Association. *Circulation* 2004 June 1;109(21):2655-71.

(5) Brook RD, Rajagopalan S, Pope CA, III et al. Particulate matter air pollution and cardiovascular disease: An update to the scientific statement from the American Heart Association. *Circulation* 2010 June 1;121(21):2331-78.

(6) Grahame TJ, Schlesinger RB. Cardiovascular health and particulate vehicular emissions: a critical evaluation of the evidence. *Air Qual Atmos Health* 2010 March;3(1):3-27.

(7) Dockery DW. Epidemiologic evidence of cardiovascular effects of particulate air pollution. *Environ Health Perspect* 2001 August;109 Suppl 4:483-6.

(8) Kunzli N, Jerrett M, Garcia-Esteban R et al. Ambient air pollution and the progression of atherosclerosis in adults. *PLoS One* 2010;5(2):e9096.

(9) Burnett RT, Cakmak S, Brook JR. The effect of the urban ambient air pollution mix on daily mortality rates in 11 Canadian cities. *Can J Public Health* 1998 May;89(3):152-6.

(10) Laden F, Schwartz J, Speizer FE, Dockery DW. Reduction in fine particulate air pollution and mortality: Extended follow-up of the Harvard Six Cities study. *Am J Respir Crit Care Med* 2006 March 15;173(6):667-72.

(11) Barath S, Mills NL, Lundback M et al. Impaired vascular function after exposure to diesel exhaust generated at urban transient running conditions. *Part Fibre Toxicol* 2010;7:19.

(12) Araujo JA, Nel AE. Particulate matter and atherosclerosis: role of particle size, composition and oxidative stress. *Part Fibre Toxicol* 2009;6:24.

(13) Basu R, Harris M, Sie L, Malig B, Broadwin R, Green R. Effects of fine particulate matter and its constituents on low birth weight among full-term infants in California. *Environ Res* 2014 January;128:42-51.

(14) Savitz DA, Bobb JF, Carr JL et al. Ambient fine particulate matter, nitrogen dioxide, and term birth weight in New York, New York. *Am J Epidemiol* 2014 February 15;179(4):457-66.

(15) Adar SD, Klein R, Klein BE et al. Air Pollution and the microvasculature: a cross-sectional assessment of in vivo retinal images in the population-based multi-ethnic study of atherosclerosis (MESA). *PLoS Med* 2010;7(11):e1000372.

(16) Louwies T, Panis LI, Kicinski M, De BP, Nawrot TS. Retinal microvascular responses to short-term changes in particulate air pollution in healthy adults. *Environ Health Perspect* 2013 September;121(9):1011-6.

(17) Pope CA, III, Ezzati M, Dockery DW. Fine-particulate air pollution and life expectancy in the United States. *N Engl J Med* 2009 January 22;360(4):376-86.

(18) Dockery DW, Pope CA, III, Xu X et al. An association between air pollution and mortality in six U.S. cities. *N Engl J Med* 1993 December 9;329(24):1753-9.

(19) Lee BJ, Kim B, Lee K. Air pollution exposure and cardiovascular disease. *Toxicol Res* 2014 June;30(2):71-5.

(20) Peretz A, Sullivan JH, Leotta DF et al. Diesel exhaust inhalation elicits acute vasoconstriction in vivo. *Environ Health Perspect* 2008 July;116(7):937-42.

(21) Mills NL, Tornqvist H, Robinson SD et al. Diesel exhaust inhalation causes vascular dysfunction and impaired endogenous fibrinolysis. *Circulation* 2005 December 20;112(25):3930-6.
(22) Judy M M-D. The coronary microcirculation in health and disease. *ISRN Physiology* 2013 June 6;2013(Article ID 238979):1-24.
(23) Bill A, Sperber GO. Control of retinal and choroidal blood flow. *Eye (Lond)* 1990;4 (Pt 2):319-25.
(24) Flammer J, Mozaffarieh M. Autoregulation, a balancing act between supply and demand. *Can J Ophthalmol* 2008 June;43(3):317-21.
(25) Nurkiewicz TR, Porter DW, Barger M, Castranova V, Boegehold MA. Particulate matter exposure impairs systemic microvascular endothelium-dependent dilation. *Environ Health Perspect* 2004 September;112(13):1299-306.
(26) Samet JM, Rappold A, Graff D et al. Concentrated ambient ultrafine particle exposure induces cardiac changes in young healthy volunteers. *Am J Respir Crit Care Med* 2009 June 1;179(11):1034-42.
(27) Tsai DH, Riediker M, Wuerzner G et al. Short-term increase in particulate matter blunts nocturnal blood pressure dipping and daytime urinary sodium excretion. *Hypertension* 2012 October;60(4):1061-9.
(28) Khandoga A, Stampfl A, Takenaka S et al. Ultrafine particles exert prothrombotic but not inflammatory effects on the hepatic microcirculation in healthy mice in vivo. *Circulation* 2004 March 16;109(10):1320-5.
(29) Chuang KJ, Chan CC, Su TC, Lee CT, Tang CS. The effect of urban air pollution on inflammation, oxidative stress, coagulation, and autonomic dysfunction in young adults. *Am J Respir Crit Care Med* 2007 August 15;176(4):370-6.
(30) Zeka A, Sullivan JR, Vokonas PS, Sparrow D, Schwartz J. Inflammatory markers and particulate air pollution: characterizing the pathway to disease. *Int J Epidemiol* 2006 October;35(5):1347-54.
(31) Lippmann M, Frampton M, Schwartz J et al. The U.S. Environmental Protection Agency Particulate Matter Health Effects Research Centers Program: a midcourse report of status, progress, and plans. *Environ Health Perspect* 2003 June;111(8):1074-92.
(32) Nel AE, Diaz-Sanchez D, Ng D, Hiura T, Saxon A. Enhancement of allergic inflammation by the interaction between diesel exhaust particles and the immune system. *J Allergy Clin Immunol* 1998 October;102(4 Pt 1):539-54.
(33) Nel AE, Diaz-Sanchez D, Li N. The role of particulate pollutants in pulmonary inflammation and asthma: evidence for the involvement of organic chemicals and oxidative stress. *Curr Opin Pulm Med* 2001 January;7(1):20-6.
(34) Gong KW, Zhao W, Li N et al. Air-pollutant chemicals and oxidized lipids exhibit genome-wide synergistic effects on endothelial cells. *Genome Biol* 2007;8(7):R149.
(35) Hirano S, Furuyama A, Koike E, Kobayashi T. Oxidative-stress potency of organic extracts of diesel exhaust and urban fine particles in rat heart microvessel endothelial cells. *Toxicology* 2003 May 3;187(2-3):161-70.
(36) Li N, Kim S, Wang M, Froines J, Sioutas C, Nel A. Use of a stratified oxidative stress model to study the biological effects of ambient concentrated and diesel exhaust particulate matter. *Inhal Toxicol* 2002 May;14(5):459-86.
(37) Takizawa H, Ohtoshi T, Kawasaki S et al. Diesel exhaust particles induce NF-kappa B activation in human bronchial epithelial cells in vitro: importance in cytokine transcription. *J Immunol* 1999 April 15;162(8):4705-11.
(38) Allen RW, Carlsten C, Karlen B et al. An air filter intervention study of endothelial function among healthy adults in a woodsmoke-impacted community. *Am J Respir Crit Care Med* 2011 May 1;183(9):1222-30.
(39) Delaey C, Van D, V. Regulatory mechanisms in the retinal and choroidal circulation. *Ophthalmic Res* 2000 November;32(6):249-56.
(40) Zhang C. MicroRNAs: role in cardiovascular biology and disease. *Clin Sci (Lond)* 2008 June;114(12):699-706.
(41) Hou L, Wang D, Baccarelli A. Environmental chemicals and microRNAs. *Mutat*

Res 2011 September 1;714(1-2):105-12.
(42) Motta V, Angelici L, Nordio F et al. Integrative Analysis of miRNA and inflammatory gene expression after acute particulate matter exposure. *Toxicol Sci* 2013 April;132(2):307-16.
(43) Wilker EH, Baccarelli A, Suh H, Vokonas P, Wright RO, Schwartz J. Black carbon exposures, blood pressure, and interactions with single nucleotide polymorphisms in MicroRNA processing genes. *Environ Health Perspect* 2010 July;118(7):943-8.
(44) Urbich C, Kuehbacher A, Dimmeler S. Role of microRNAs in vascular diseases, inflammation, and angiogenesis. *Cardiovasc Res* 2008 September 1;79(4):581-8.
(45) Xiao C, Rajewsky K. MicroRNA control in the immune system: basic principles. *Cell* 2009 January 9;136(1):26-36.
(46) Ikeda S, Kong SW, Lu J et al. Altered microRNA expression in human heart disease. *Physiol Genomics* 2007 November 14;31(3):367-73.
(47) Yamamoto M, Singh A, Sava F, Pui M, Tebbutt SJ, Carlsten C. MicroRNA expression in response to controlled exposure to diesel exhaust: attenuation by the antioxidant N-acetylcysteine in a randomized crossover study. *Environ Health Perspect* 2013 June;121(6):670-5.
(48) Jardim MJ, Fry RC, Jaspers I, Dailey L, Diaz-Sanchez D. Disruption of microRNA expression in human airway cells by diesel exhaust particles is linked to tumorigenesis-associated pathways. *Environ Health Perspect* 2009 November;117(11):1745-51.
(49) Bollati V, Marinelli B, Apostoli P et al. Exposure to metal-rich particulate matter modifies the expression of candidate microRNAs in peripheral blood leukocytes. *Environ Health Perspect* 2010 June;118(6):763-8.
(50) Fossati S, Baccarelli A, Zanobetti A et al. Ambient particulate air pollution and microRNAs in elderly men. *Epidemiology* 2014 January;25(1):68-78.
(51) Jiang R, Jones MJ, Sava F, Kobor MS, Carlsten C. Short-term diesel exhaust inhalation in a controlled human crossover study is associated with changes in DNA methylation of circulating mononuclear cells in asthmatics. *Part Fibre Toxicol* 2014 December 9;11(1):71.
(52) Suarez Y, Sessa WC. MicroRNAs as novel regulators of angiogenesis. *Circ Res* 2009 February 27;104(4):442-54.
(53) Williams AE, Perry MM, Moschos SA, Larner-Svensson HM, Lindsay MA. Role of miRNA-146a in the regulation of the innate immune response and cancer. *Biochem Soc Trans* 2008 December;36(Pt 6):1211-5.
(54) Parati G, Stergiou GS, Asmar R et al. European Society of Hypertension guidelines for blood pressure monitoring at home: a summary report of the Second International Consensus Conference on Home Blood Pressure Monitoring. *J Hypertens* 2008 August;26(8):1505-26.
(55) Annette Vriens. Extracellular miRNAs in saliva as a reflection of the body's response to particulate matter exposure in children 2015.
(56) Suarez Y, Fernandez-Hernando C, Pober JS, Sessa WC. Dicer dependent microRNAs regulate gene expression and functions in human endothelial cells. *Circ Res* 2007 April 27;100(8):1164-73.
(57) Metzger KB, Tolbert PE, Klein M et al. Ambient air pollution and cardiovascular emergency department visits. *Epidemiology* 2004 January;15(1):46-56.
(58) Wong TY, Kamineni A, Klein R et al. Quantitative retinal venular caliber and risk of cardiovascular disease in older persons - The Cardiovascular Health Study. *Archives of Internal Medicine* 2006 November 27;166(21):2388-94.
(59) Wong TY, Islam FMA, Klein R et al. Retinal vascular caliber, cardiovascular risk factors, and inflammation: The Multi-Ethnic Study of Atherosclerosis (MESA). *Investigative Ophthalmology & Visual Science* 2006 June;47(6):2341-50.
(60) Chew SKH, Xie J, Wang JJ. Retinal Arteriolar Diameter and the Prevalence and Incidence of Hypertension: A Systematic Review and Meta-analysis of Their Association. *Current Hypertension Reports* 2012 April;14(2):144-51.

(61) Brook RD, Brook JR, Urch B, Vincent R, Rajagopalan S, Silverman F. Inhalation of fine particulate air pollution and ozone causes acute arterial vasoconstriction in healthy adults. *Circulation* 2002 April 2;105(13):1534-6.
(62) Nurkiewicz TR, Porter DW, Barger M et al. Systemic microvascular dysfunction and inflammation after pulmonary particulate matter exposure. *Environmental Health Perspectives* 2006 March;114(3):412-9.
(63) Nurkiewicz TR, Porter DW, Hubbs AF et al. Pulmonary Nanoparticle Exposure Disrupts Systemic Microvascular Nitric Oxide Signaling. *Toxicological Sciences* 2009 July;110(1):191-203.
(64) Tornqvist H, Mills NL, Gonzalez M et al. Persistent endothelial dysfunction in humans after diesel exhaust inhalation. *American Journal of Respiratory and Critical Care Medicine* 2007 August 15;176(4):395-400.
(65) Wang Z, Neuburg D, Li C et al. Global gene expression profiling in whole-blood samples from individuals exposed to metal fumes. *Environ Health Perspect* 2005 February;113(2):233-41.
(66) Yang WJ, Yang DD, Na S, Sandusky GE, Zhang Q, Zhao G. Dicer is required for embryonic angiogenesis during mouse development. *J Biol Chem* 2005 March 11;280(10):9330-5.

Appendix

Appendix A: Function of eNOS in normal and disease state

Normally, eNOS will convert O_2 to NO, *see Figure 10*. When eNOS is uncoupled, it produces $O_2^{\cdot}$ instead of NO. Furthermore, another source of $O_2^{\cdot}$ in the body could be the respiratory chain or the nicotinamide adenine dinucleotide phosphate-oxidases (NADPH oxidases). This high production of $O_2^{\cdot}$ will react with NO producing peroxynitrite ($ONOO^-$). This $ONOO^-$ production could be prevented by the superoxide dismutase (SOD), which convert $O_2^{\cdot}$ to hydrogen peroxide (H_2O_2) eventually resulting in water and oxygen. [22]

Thus SODs ensure that another vasodilator is produced. Lamentably, when transition metals (e.g.Fe^{2+}) are present, as in the case of air pollution exposure, a very high cytotoxic radical $^{\cdot}OH$ will be made. [22]

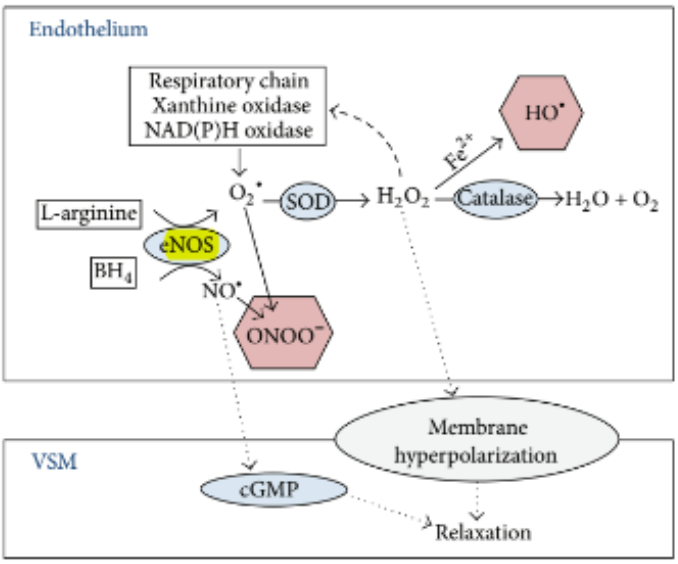

Figure 10: Function of eNOS in normal and disease state [22]
In normal conditions, the endothelial nitric oxide synthase (eNOS) will convert O_2 to NO. Thus, when substrates such as L-arginine and cofactors such as tetrahydrobiopterin (BH_4) are present, a constantly release of NO by the endothelium will occur, in order to maintain vessel relaxation. However, in disease states the eNOS is uncoupled and it will produces $O_2^{\cdot}$ instead of NO. Furthermore, there is another source of $O_2^{\cdot}$ in the body produced by the respiratory chain or the nicotinamide adenine dinucleotide phosphate-oxidases (NADPH oxidases). This high production of $O_2^{\cdot}$ will react with NO producing peroxynitrite ($ONOO^-$). This $ONOO^-$ production could be prevented by the superoxide dismutase (SOD), which convert $O_2^{\cdot}$ to H_2O_2 at the appropriate times resulting in the shift to water and oxygen. Thus SODs ensure that another vasodilator is produced. Unfortunately, when transition metals (e.g.Fe^{2+}) are present, as in the case of air pollution exposure, a very high cytotoxic radical $^{\cdot}OH$ will be made.

Appendix B: The biogenesis of miRNAs

The most part of the miRNAs are present in the human genome, but they can also be located in exons or introns of both coding and non-coding genes. [44, 45] The general biogenesis of miRNAs is represented in *Figure 11*. First, miRNAs are transcribed into pri-miRNA. Then a complex, including DGCR8 and Drosha, an endonuclease III in the nucleus, cleave pri-miRNA into pre-miRNA, a sixty to seventy nucleotides stem loop intermediate. This pre-miRNA is then exported to the cytoplasm by exportin 5, which activation is depending on guanosine-5'-triphosphate (GTP). Second, another endonuclease III in the cytoplasm, named Dicer, cleaves pre-miRNA, producing a double-stranded RNA duplex of twenty to twenty-five nucleotides. [40, 44, 45] This duplex contains the mature miRNA and the antisense strand. [44]

After this maturation step, one strand is incorporated into the RNA-induced silencing complex (RISC). The other strand, also known as the passenger strand, is returned to the nucleus where it can regulate gene transcription. The strand in the RISC can directly target mRNA, leading to repression or degradation. [40, 44, 45] This discrepancy of the negative regulation of miRNAs on gene expression, depend on the complementarity of the miRNA with the target mRNA. An imperfect complementarity causes a translation silencing due to the blocking of the mRNA target, a translational repression. When the binding between the miRNAs and mRNA target is a perfect complementary, they provoke a cleavage of the target mRNA. [40, 44] Still, both will lead to a downregulation of the corresponding protein expression. [45]

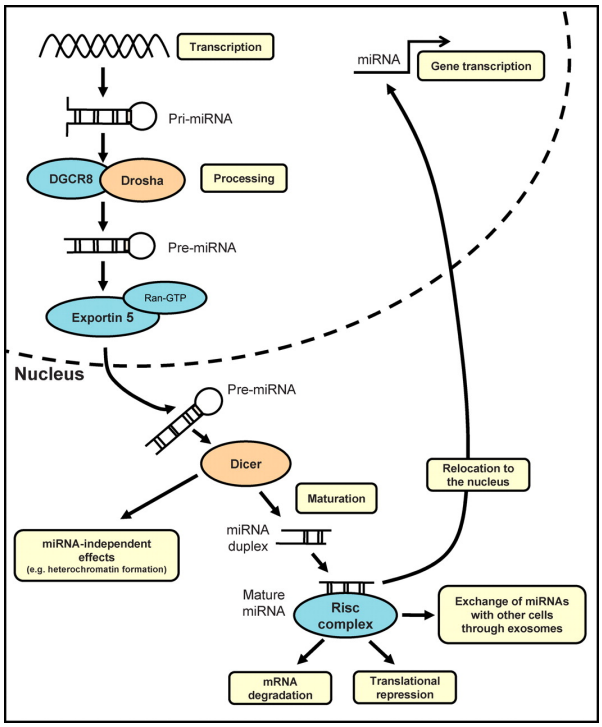

Figure 11: The biogenesis of miRNAs [45]
When a transcript is made, a pri-miRNA is formed. An endonuclease III complex in the nucleus, DGCR8 together with Drosha, will convert pri-miRNA into pre-miRNA. By means of exportin 5, this pre-miRNA a sixty to seventy nucleotides stem loop intermediate, will be exported to the cytoplasm when GTP is present. There, a second endonuclease III, Dicer, will cleave this pre-miRNA into a double-stranded RNA duplex of twenty to twenty-five nucleotides. This last-mentioned is known as the maturation step. After this process, one strand will be incorporated into the RISC targeting mRNA. The other strand will return to the nucleus, regulating gene transcription. When mRNA is targeted, this can result in a degradation or repression of the translation, but both will lead to a downregulation of the corresponding protein expression. (Guanosine-5'-triphosphate (GTP), RNA-induced silencing complex (RISC))

Appendix C: Maturation of miRNA and its role in disease state

The critical role of miRNAs is already well described in literature. In 2005, Yang *et al.* showed that mice didn't develop a normal and functional heart or even blood vessels when the maturation step of miRNAs was impaired, by reason of a deficient Dicer complex. [66] Further evidence suggests that Dicer is not only involved in cardiovascular development, but is also required for normal skeletal muscle development, embryonic development and angiogenesis. When the function of the Dicer complex is deficient or even inadequate, the maturation step of miRNAs in the blood will be prevented, leading to impaired processes (e.g. cardiovascular, skeletal muscle and embryonic development and angiogenesis). Furthermore, research in humans demonstrated that the expression of miRNAs is altered, when blood samples are compared between healthy people and disease state. [46]

Appendix D: Short questionnaire (Dutch)

VRAGENLIJST

Datum van vandaag: ……/……./……..
Uur van de dag: …. u….
Naam:

GEZONDHEID

1 Hoeveel weegt u? ___kg

2 Hoe groot bent u? ___cm

3 Hoe is uw algemene gezondheidstoestand nu?

Zeer goed goed gaat wel (redelijk) slecht zeer slecht

4 Heeft u de laatste 24 h medicatie genomen? o ja, welke_____
o nee

5 Voelt u zich vermoeid? o ja o nee

6 Hoeveel uur heeft u deze nacht geslapen? ___uren

7 Heeft u last van stress? o ja o nee

8 Bent u de afgelopen maand (zwaar) ziek geweest? o ja, welke_____
Periode?_____

o nee

9 Heeft u de afgelopen maand medicatie genomen? o ja, welke_____
Periode?_____
o nee

BLOOTSTELLING GEDURENDE DE VOORBIJE DAG

10 Heeft u de laatste 24 h alcoholhoudende dranken genuttigd? o ja o nee
___glazen bier
___glazen wijn
___glazen sterk alcoholische drank

11 Heeft u de laatste 24 h cafeïnehoudende dranken genuttigd?
___koppen koffie
___koppen thee
___energiedranken
___andere

12 Hoeveel sigaretten/sigaren heeft u de afgelopen 24 uur gerookt?
___ sigaretten/sigaren

13 Hoeveel uren heeft u de laatste 24 h doorgebracht in een ruimte waar gerookt werd door (u of) anderen?
___uren

14 Heeft u gedurende de laatste 24 h lichamelijke inspanningen verricht waarbij u bezweet geraakte?
o ja: ___minuten
o nee

15 Hoe intens nam u deel aan het verkeer gedurende de voorbije 24 h?

Hoeveel minuten zat u in een auto, autobus of tram (gemiddeld) ___minuten

Hoeveel minuten hiervan zat u in druk verkeer of in de file (gemiddeld) ___minuten

Hoeveel minuten fietste u of ging u te voet langs straten met verkeer (gemiddeld) ___minuten

ENKEL VOOR VROUWEN

16 Wanneer zijn uw laatste maandstonden begonnen? datum_____

STAALNAME

BLOEDDRUK

Meting	SBP	DBP	HR
1			
2			
3			
4			
5			

RETINAFOTO

Meting	Links	Rechts
1		
2		

BLOEDAFNAME

o EDTA
o TEMPUS

Appendix E: Perceived Stress scale questionnaire

Perceived Stress Scale

De vragen in deze vragenlijst zijn gericht op uw gevoelens en gedachten in de afgelopen maand. In elk item zal u gevraagd worden hoe vaak u bepaalde gevoelens en gedachten hebt ervaren. Hoewel sommige vragen op elkaar lijken, zijn er toch verschillen en dient u elke vraag te behandelen als een aparte vraag. De beste manier van benadering is om elke vraag vrij snel te beantwoorden. Dat betekent dat het niet de bedoeling is een optelling te maken van hoe vaak u zich op die manier hebt gevoeld, wel van een aanvaardbare inschatting te maken.

Voor elke vraag kan u uit volgende mogelijke antwoorden een keuze maken:

0=nooit
1=bijna nooit
2= soms
3=regelmatig
4=heel vaak

1	In de loop van de voorbije maand, hoe vaak bent u van streek geweest omdat zich iets onverwachts voordeed?	
2	In de loop van de voorbije maand, hoe vaak voelde u zich onmachtig om de belangrijke zaken in uw leven te controleren?	
3	In de loop van de voorbije maand, hoe vaak voelde u zich nerveus en gestresseerd?	
4	In de loop van de voorbije maand, hoe vaak bent succesvol kunnen omgaan met dagdagelijkse problemen en ergernissen?	
5	In de loop van de voorbije maand, hoe vaak heeft u het gevoel gehad op een effectieve manier om te kunnen gaan met belangrijke veranderingen in het leven?	
6	In de loop van de voorbije maand, hoe vaak heeft u zich zelfverzekerd gevoeld inzake uw bekwaamheid om uw persoonlijke problemen aan te pakken?	
7	In de loop van de voorbije maand, hoe vaak heeft u kunnen ervaren dat de zaken volgens uw wens verliepen?	
8	In de loop van de voorbije maand, hoe vaak heeft u het gevoel gehad dat u niet om kon met alle zaken die u diende te doen?	
9	In de loop van de voorbije maand, hoe vaak voelde u zich in staat uw ergernissen in uw leven te controleren?	
10	In de loop van de voorbije maand, hoe vaak heeft u het gevoel gehad uw zaakjes onder controle te hebben?	
11	In de loop van de voorbije maand, hoe vaak bent u kwaad geworden omwille van dingen die zich buiten uw controle voordeden?	
12	In de loop van de voorbije maand, hoe vaak heeft u zitten nadenken over dingen die u nog moet zien te bereiken?	
13	In de loop van de voorbije maand, hoe vaak heeft u zelf kunnen controleren hoe u uw tijd doorbrengt?	
14	In de loop van de voorbije maand, hoe vaak heeft u gevoeld dat problemen zich zo hoog opstapelden dat u ze niet meer aankon?	

Appendix F: Collection of miRNA/RNA from tempus tubes (full protocol)

Preparation:

- Prepare the working solution before using the Wash solution. Add 50 ml ethanol (95-100%) to the concentrated wash solution bottle until a final solution of 72 ml is reached. Shake a couple of times before using.

Protocol:

1. Decant the entire content of the TEMPUS tube in a 50 ml tube
2. Add 3 ml of the Tempus Blood RNA tube diluent to reach a total volume of 12 ml. Close the 50 ml tubes very good and vortex for 30 seconds
3. Centrifuge the 50 ml tubes at 3000-5000 x g (min. 4500 rpm) at 4°C for 30 minutes.
4. Discard the supernatant. The RNA pellet will be at the bottom of the tube and is transparent and invisible. → be careful
5. Invert the tube and place it on paper for 2 minutes to dry the pellet
6. Add 600 µl lysis solution to the RNA pellet and vortex for 30 seconds to suspend to the entire RNA pellet.
7. Add 300 µl ethanol (100%) and vortex during 10 seconds until foam is present.
8. Prepare the collection tubes with the columns for each sample
9. Add 600 µl of the solution in the 50 ml tubes into the columns and centrifuge for 1 minute.
10. Discard all the flowthrough. Repeat step 9-10 until all the solution in the 50 ml tube is finished.
11. Prepare the DNA incubation mix for every sample (do not vortex!):
    a. 15 µl DNAse I (-20°C)
    b. 100 µl Enzyme incubation mix
12. Add 400 µl of the Wash solution to the column and centrifuge for 2 minutes
13. Discard all the flowthrough
14. Add 100 µl of the DNA incubation mix to the column and centrifuge for 1 minute at 14000 x g
15. Pipet the flowthrough back in the column and incubate for 15 minutes at room temperature. (To achieve the max. DNA activity and to reach the max. amount of RNA and miRNA.)
16. Add again 400 µl Wash solution to the column and centrifuge for 1 minute
17. Discard all the flowthrough
18. Was a third time and add 400 µl Wash solution to the column and centrifuge for 1 minute.
19. Discard the flowthrough.
20. Take a new collection tube and centrifuge again for 2 minutes to dry the membrane.
21. Discard all the flowthrough
22. Place the column on a elution tube provided in the kit
23. Add 50 µl elution solution to the column and centrifuge for 2 minutes at 0.2 x g. When the centrifugation is finished, centrifuge again for 1 minute at 14 000 x g.
24. Gather the samples on ice!
25. The purified RNA will undergo a quality control by the Nanodrop to verify the purity
26. Store the purified RNA at -80°C or -20°C in terms of a short period.

Appendix G: Agilent 2100 Bioanalyzer for miRNA (full protocol)

Staring a new kit:

- Gel matrix:
  - Prepare 650 µl of the RNA 6000 nano gel matrix into a spincolomn.
  - Centrifuge for 15 minutes at 10000x g at room temperature
  - Prepare aliquots of 40 µl of this 650 µl RNA gel matrix in RNAse free microcentrifuge tubes of 0.5 ml
    - Stored for 1 month at 4°C
- Ladder:
  - Minispin the whole tube of 35 µl for 10 seconds
  - Denature for 2 minutes at 70 °C followed by cooling down on ice
  - Prepare aliquots of 3.5 µl of this 35 µl solution
    - Stored for 3 months at -80°C

Preparation:

- Put the reagents on the bench to adjust at room temperature for 30 minutes.
- Start the program on the computer and wash the electrodes:
  a. Pipet 350 µl RNAse free water in the chip
  b. First, wash the electrodes for 5 minutes with the RNAse free chip
  c. Followed with 30 seconds drying (open)
- Dilute all the samples until the concentration is fit in the range between 1-100 ng. After the dilution, the samples need to be denatured for 2 minutes at 70°C. Put the samples immediately back on ice, when the 2 minutes are over.

Protocol:

1. Vortex the RNA 6000 nano dye concentrate (blew) for 10 seconds, minispin very shortly and add 2 µl of the dye to the aliquot of the gel matrix. Suspend very well before centrifugation;
2. Centrifuge for 10 minutes at 13000 x g at room temperature
3. Prepare the chip and priming station:
   a. Open the chip priming station and pull the handle at 1ml
   b. Set the cling in the lowest position.
   c. Inside, the cling is positioned at the C
   d. Place the chip in the conditioned place
4. Pipet 9 µl of the gel matrix on the well-marked with a circled G
5. Close the priming station and push the handle until the cling is reached. Wait 1 minute before releasing the cling. Count till 5 when the handle has stopped and pull the handle back to the original position. Open the priming station. (To take away all the air in the chip)
6. Pipet 9 µl of the gel matrix on the well-marked with a G (2 wells)
7. Pipet 9 µl of the conditioning solution (white) on the well- marked with a CS (positive control)
8. Pipet 5 µl of the green marker (green) in all the wells for the samples and the ladder.
9. Pipet 1 µl of the ladder in the well-marked with a ladder
10. Pipet 1 µl of the samples in the correct well.
11. When all the pipetting is finished, vortex the chip for 1 minute at 2400 rpm
12. Use the chip in 5 minutes in the Bioanalyzer
    a. Select the correct assay
    b. Save at the right place
    c. Press start
13. After the run, was the electrodes for 1 minute with RNAse free water followed by drying for 30 seconds.

Appendix H: cDNA synthesis (full protocol)

Protocol:

1. Calculate the RNA input concentrations (10 ng).
2. Thaw the primer 5X (-20°C) and the samples together with the TaqMan microRNA reverse transcription kit components (-20°C) on ice. Vortex very good before using them.
    a. Followed by a very short minispin
3. Make the following reverse transcription master mix for 1 sample (calculate 3 extra samples):

dNTPs with dTTP (100 mM)	0,15 µl
MultiScribe Reverse Transcriptase (50U/µl)	1,00 µl
10 X RT buffer	1.50 µl
RNase inhibitor (20U/µl)	0,19 µl
Nuclease free water	4.16 µl
Total	**7 µl**

4. Mix the tube and centrifuge very short. (keep it on ice)
5. 3 µl RT primer (for each miRNA) is needed. Calculated the total amount (3 extra samples) and add this to the master mix.
6. Make the dilutions with nuclease free water until a final volume of 5 µl is reached.
7. Pipet 10 µl of this reverse transcription master mix together with the primer in every strip
8. Seal the strips and mix.
9. Centrifuge the strips for 1 min at 700 rpm
10. Incubate for 5 minutes on ice.
11. Running method:
    a. Cover: 105°C
    b. HOLD: for 30 minutes at 16°C
    c. HOLD: for 30 minutes at 42°C
    d. HOLD: for 5 minutes at 85°C
    e. HOLD: infinite at 4°C
12. Load the strip in the machine and push again to the covers of the strips to be sure that these are sealed very tightly
13. Afterwards, the cDNA can be stored for 1 week at -20°C

Appendix I: qRT-PCR (full protocol)

Protocol:

1. Using the correct start concentration (0.89 ng) and plate (96 or 384 wells)
2. Prepare the master mix for each sample:

TaqMan Master mix	10.00
TaqMan Small RNA assay (20X)	1.00
Nuclease free water	7.67
Total	**18.67**

3. Vortex the master mix and minispin very shortly before using
4. Pipet 18.70 µl master mix to each well
5. Add 1.30 µl sample to each well
6. Seal the plate with the correct cover
7. Centrifuge for 2 minutes at 700 rpm
8. Load the plate in the machine and select the correct program:
    a. Activation step:
        i. For 10 minutes at 95 °C
    b. Denaturation step:
        i. 40 cycles:
            1. For 5 seconds at 95°C
            2. For 60 seconds at 60°C
    c. Cool down step:
        i. Standard
9. Start the run
10. Analysis of the plate (Ct-values)

Auteursrechtelijke overeenkomst

Ik/wij verlenen het wereldwijde auteursrecht voor de ingediende eindverhandeling:
Are miRNAs a link between air pollution exposure and cardiovascular changes?

Richting: **master in de biomedische wetenschappen-klinische moleculaire wetenschappen**
Jaar: **2015**

in alle mogelijke mediaformaten, - bestaande en in de toekomst te ontwikkelen - , aan de Universiteit Hasselt.

Niet tegenstaand deze toekenning van het auteursrecht aan de Universiteit Hasselt behoud ik als auteur het recht om de eindverhandeling, - in zijn geheel of gedeeltelijk -, vrij te reproduceren, (her)publiceren of distribueren zonder de toelating te moeten verkrijgen van de Universiteit Hasselt.

Ik bevestig dat de eindverhandeling mijn origineel werk is, en dat ik het recht heb om de rechten te verlenen die in deze overeenkomst worden beschreven. Ik verklaar tevens dat de eindverhandeling, naar mijn weten, het auteursrecht van anderen niet overtreedt.

Ik verklaar tevens dat ik voor het materiaal in de eindverhandeling dat beschermd wordt door het auteursrecht, de nodige toelatingen heb verkregen zodat ik deze ook aan de Universiteit Hasselt kan overdragen en dat dit duidelijk in de tekst en inhoud van de eindverhandeling werd genotificeerd.

Universiteit Hasselt zal mij als auteur(s) van de eindverhandeling identificeren en zal geen wijzigingen aanbrengen aan de eindverhandeling, uitgezonderd deze toegelaten door deze overeenkomst.

Voor akkoord,

Vuegen, Caroline

Datum: **9/06/2015**