Faculty of Sciences

DOCTORAL DISSERTATION

Turning an eye towards cardiovascular health: The retina in public health research

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If I have seen further it is by standing on the shoulders of giants.

Sir Isaac Newton (1643 – 1727)
Bernard of Chartes (12th century)

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SUMMARY

Air pollution exposure and physical inactivity are risk factors for cardiovascular morbidity and mortality. The vascular effects are the result of complex molecular and physiological changes that also occur in the microcirculation, which is the network of smallest blood vessels. These vessels make up the bulk of the cardiovascular system and ensure delivery of nutrients, removal of metabolites and gas exchange. The microcirculation is the primary site of resistance and plays an important role in blood pressure regulation. Cardiovascular risk factors such as air pollution and physical inactivity can induce microvascular functional and structural changes that can play a role in cardiovascular disease development.

The retina offers an unique possibility to visualize the microvasculature in vivo using fundus photography and quantify effects using image analysis. The retinal blood vessels share anatomical, physiological and pathological features with cerebral and coronary blood vessels. In epidemiological studies, retinal arteriolar narrowing and venular widening are associated with cardio- and cerebrovascular risk factors and outcomes. However, the evidence on the impact of air pollution or physical (in)activity on the retinal blood vessels is scarce and not much is known about the short-term impact of these risk factors.

The question whether repeated fundus photography can be used to investigate the association between ambient air pollution exposure or physical (in)activity and retinal blood vessel changes is addressed in this PhD project. The specific objectives of the project were to investigate:

The association between personal or short-term air pollution exposure and retinal arteriolar narrowing and venular widening in healthy adults.

The effects of bedrest, an experimental model for physical inactivity, on the retinal microvasculature.

The retinal microvascular responses after acute exercise in cardiac patients and investigate whether these responses were changed after completion of a cardiac rehabilitation program.

The association between short-term air pollution exposure and retinal microvascular changes were studied in three panel studies in Flanders. In the first panel study (n=84, mean age= 37±9, 60% females; January 2012-May 2012; 3 repeated measurements), each 10 µg/m³ increase in PM_{10} or each 1 µg/m³ increase in black carbon was associated with retinal arteriolar narrowing of respectively 0.93 µm (95%CI: -1.42 to -0.45) and 1.84 µm (95%CI: -3.18 to -0.15). These changes in retinal arteriolar diameter were equivalent to a 1.5 year increase in age (Chapter 3). These observations are in line with experimental controlled exposure studies in which brachial artery endothelial dysfunction or vasoconstriction was observed after air pollution exposure. In the second panel study (n=50, mean age=32±8, 50% females; December 2014-April 2015; 5 repeated measurements), each 10 µg/m³ increase in PM_{10} was associated with a decrease in CRAE of 0.72 µm (95%CI: -1.38 to -0.06), an increase in CRVE of 0.99 µm (95%CI: 0.18 to 1.80) and a downregulation of 6.62% (95%CI: -11.07 to -2.17) and 6.71% (95%CI: -10.68 to -2.75) in respectively miR-21 and miR-222 expression. Changes in microRNA expression were associated with air pollution exposure and retinal microvascular changes. These microRNAs are involved in inflammatory reactions and endothelial dysfunction, suggesting their potential role in mediating the effects of air pollution on the retinal blood vessels (Chapter 4). Short-term air pollution exposure can induce endothelial dysfunction. In this process, the potent vasodilator nitric oxide is lost and inflammatory reactions are upregulated. This may lead to arteriolar vasoconstriction and

venular widening. In the third panel (n=56, mean age=41±10; 93% females; April 2013-May 2013; 4 repeated measurements during one week), each 425 ng/m³ increase in subchronic (long-term) traffic-related air pollution exposure was associated with increases in systolic and diastolic blood pressure and venular widening of respectively 2.77 mm Hg (95% CI: 0.39 to 5.15), 2.35 mm Hg (95% CI: 0.52 to 4.19) and 4.76 µm (95% CI: 0.27 to 9.24). Since the exposure levels and variation in personal exposure between study subjects were very limited during the study period, we were unable to reproduce our associations between short-term traffic-related air pollution exposure and retinal microvascular changes (Chapter 5).

The retinal microvascular responses to physical inactivity were addressed in 14 healthy adult males during a 21-day bedrest cross-over study in normoxic and hypoxic conditions. Normoxic bedrest caused retinal arteriolar vasoconstriction that remained for the whole study period. The maximal decrease in retinal arteriolar diameter was 7.47 µm (95% CI: -10.78 to -4.15). Hypoxic bedrest caused an initial increase in retinal arteriolar diameter of 4.49 µm (95% CI: 1.23 to 7.75) that was attenuated during the study period. These responses were probably due to the autoregulatory properties of the retinal vessels. Under hypoxic conditions, myogenic vasoconstriction attenuated metabolic vasodilation in the retinal arterioles. This mechanism might contribute to the predisposition of physically inactive individuals for cardiovascular disease development or progression (Chapter 6).

The retinal microvascular responses to physical activity were also addressed in 53 cardiac rehabilitation patients. These individuals participated in a rehabilitation program in which they conducted two maximal endurance tests. We observed that retinal microvascular reactivity was preserved in these patients as the blood vessels dilated after exercise and remained dilated for up to 30 minutes after exercise cessation. The microvascular reactivity was assessed again after a 6-week rehabilitation program, but there were no indications that retinal vascular responses were improved (Chapter 7).

Our results suggest that short-term effects exposure to air pollution and physical (in)activity are associated with retinal microvascular changes. The studies on air pollution performed as part of this PhD project contribute to international research investigating the cardiovascular effects of environmental pollutants at the current exposure levels. The experimental studies on physical (in)activity add to our understanding of the detrimental vascular effects of a sedentary lifestyle and the need for regular physical exercise to maintain vascular health.

In conclusion, retinal imaging is a convenient tool to study the microvasculature in epidemiological and (pre-)clinical settings. Dedicated image analysis software is able to quantify functional and structural retinal blood vessel changes that are important on the trajectory of cardiovascular disease development.

SAMENVATTING

Blootstelling aan luchtvervuiling en fysieke inactiviteit zijn risicofactoren voor cardiovasculaire morbiditeit en mortaliteit. De vasculaire effecten zijn het resultaat van complexe moleculaire en fysiologische veranderingen die ook plaatsvinden in de microcirculatie, het netwerk van de kleinste bloedvaten. Deze bloedvaten staan in voor de gasuitwisseling, het afleveren van nutriënten en het verwijderen van restproducten uit de weefsels. De grote vasculaire weerstand van microcirculatie is een belangrijke regulator van bloeddruk. Cardiovasculaire risicofactoren, zoals luchtvervuiling en fysieke inactiviteit, kunnen veranderingen in de functionaliteit en structuur van de microcirculatie veroorzaken die een rol spelen in de ontwikkeling van cardiovasculaire ziekten.
De retina biedt een unieke mogelijkheid om de microcirculatie in vivo te visualiseren met behulp van fundusfotografie en effecten te kwantificeren met beeldanalyse. De anatomie, fysiologie en pathologie van de bloedvaten in de retina vertoont sterke gelijkenissen met de bloedvaten in het hart en de hersenen. In epidemiologische studies is het vernauwen van de retinale arteriolen en/of het verwijden van de retinale venules geassocieerd met cardio- en cerebrovasculaire risicofactoren en eindpunten. Het bewijs van de impact van luchtvervuiling of fysieke (in)activiteit op deze bloedvaten is beperkt en er is weinig geweten van de korte-termijn impact van deze risicofactoren.
De vraag of herhaalde fundusfotografie gebruikt kan worden om de associatie tussen luchtvervuiling of fysieke (in)activiteit en veranderingen in de bloedvaten van de retina, wordt voorgelegd in dit doctoraat. Er werd specifiek onderzoek gedaan naar:

De associatie tussen persoonlijke en korte-termijnblootstelling aan luchtvervuiling en vernauwing van de retinale arteriolen en verwijding van de retinale venules in gezonde volwassenen.
De effecten van bedrust, een experimenteel model voor fysieke inactiviteit, op de retinale microcirculatie.
De retinale microvasculaire veranderingen na inspanning bij hartpatiënten en of dit patroon veranderd was na het afwerken van een trainingsprogramma.

De associatie tussen korte-termijn blootstelling aan luchtvervuiling en veranderingen in de microcirculatie van de retina werden onderzocht in drie panelstudies. In de eerste panelstudie (n=84, leeftijd= 37±9, 60% vrouwen; januari 2012-Mei 2012; 3 herhaalde metingen) was iedere toename van 10 µg/m³ in PM10 of iedere 1 µg/m³ toename in black carbon geassocieerd met het vernauwen van de retinale arteriolen van respectievelijk 0.93 µm (95%BI: -1.42 tot -0.45) en 1.84 µm (95%BI: -3.18 tot -0.15). Deze veranderingen waren het equivalent van een leeftijdstoename van 1,5 jaar (Hoofdstuk 3). Deze observaties stemmen overeen met experimenteel onderzoek waarin endotheeldysfunctie of vasoconstrictie van de brachiale slagader was waargenomen na blootstelling. In de tweede panelstudie (n=50, leeftijd=32±8, 50% vrouwen; december 2014-april 2015; 5 herhaalde metingen) was iedere 10 µg/m³ toename in PM10 geassocieerd met een daling van CRAE van 0.72 µm (95%BI: -1.38 tot -0.06), een toename in CRVE van 0.99 µm (95%BI: 0.18 tot 1.80) en een downregulatie van 6.62% (95%BI: -11.07 tot -2.17) en 6.71% (95%BI: -10.68 tot -2.75) van respectievelijk miR-21 en miR-222 expressie. Deze microRNA expressie was ook geassocieerd met veranderingen in de retinale bloedvaten. Daar deze microRNA's een rol spelen in inflammatoire reacties en endotheeldysfunctie, zou dit mogelijk kunnen

wijzen op hun potentiële onderliggende rol in de effecten van luchtvervuiling op de retinale microcirculatie (Hoofdstuk 4). Korte-termijn blootstelling aan luchtvervuiling kan endotheeldysfunctie veroorzaken. Hierdoor verliezen bloedvaten de vasodilator NO en worden inflammatoire reacties versterkt. Deze processen kunnen leiden tot het vernauwen van arteriolen en het verwijden van de venules. In de derde panelstudie (n=56, leeftijd=41±10; 93% vrouwen; april 2013-mei 2013; 4 herhaalde metingen gedurende een week), was iedere toename van 425 ng/m³ in subchronische black carbon blootstelling geassocieerd met toenames in systolische en diastolische bloeddruk en retinale venulaire diameter van respectievelijk 2.77 mm Hg (95%BI: 0.39 tot 5.15), 2.35 mm Hg (95%BI: 0.52 tot 4.19) en 4.76 µm (95%BI: 0.27 tot 9.24). Aangezien de blootstellingsconcentratie en –variatie tussen studiedeelnemers zeer beperkt was, konden we geen associaties tussen korte-termijn blootstelling en veranderingen in de retinale microvasculatuur vinden (Hoofdstuk 5).

De retinale microvasculaire veranderingen ten gevolge van fysieke inactiviteit werden onderzocht in 14 gezonde mannen tijdens een 21-dagen durende bedrust cross-over studie in normoxische en hypoxische omstandigheden. Normoxische bedrust veroorzaakte retinale arteriolaire vasoconstrictie gedurende de volledige studieperiode. De maximale afname in arteriolaire diameter was 7.47 µm (95% BI: -10.78 tot -4.15). Hypoxische bedrust veroorzaakte een initiële toename in arteriolaire diameter van 4.49 µm (95%BI: 1.23 tot 7.75) die geleidelijk teniet gedaan werd tijdens de studieperiode. Deze veranderingen werden waarschijnlijk veroorzaakt door de autoregulatie van de retinale bloedvaten. Tijdens bedrust onder hypoxische omstandigheden werd de metabole vasodilatie verkleind door myogene vasoconstrictie. Dit mechanisme kan een bijdrage leveren aan de vatbaarheid van fysiek inactieve individuen voor de ontwikkeling en progressie van cardiovasculaire ziekten (Hoofdstuk 6).

De microvasculaire veranderingen in de retina ten gevolge van fysieke activiteit werden onderzocht in 53 revaliderende hartpatiënten. Tijdens dit revalidatieprogramma werden legden de patiënten twee maximale inspanningstesten af. In deze patiënten was de retinale microvasculaire responsiviteit bewaard: de bloedvaten dilateerden en bleven gedilateerd tot 30 minuten na het beëindigen van de inspanningstest. De microvasculaire reactiviteit werd na 6 weken in het revalidatieprogramma opnieuw getest, maar er waren geen indicaties dat deze verbeterd zou zijn (Hoofdstuk 7).

Onze resultaten tonen aan dat korte-termijn blootstelling aan luchtvervuiling en fysieke (in)activiteit geassocieerd zijn met retinale microvasculaire veranderingen. De studies omtrent luchtvervuiling dragen bij tot internationaal onderzoek dat aangeeft dat cardiovasculaire gezondheidseffecten nog steeds opduiken bij de huidige concentraties van luchtvervuiling. De experimentele studies rond fysieke (in)activiteit dragen bij tot onze kennis van de schadelijke effecten van een sedentaire levensstijl en de nood aan regelmatige fysieke activiteit om de vasculaire gezondheid op peil te houden.

Uit dit doctoraat blijkt dat fundusfotografie een geschikte techniek is om de microvasculatuur te onderzoeken in een epidemiologische en (pre-)klinische setting. Beeldverwerkingssoftware laat toe om functionele en structurele veranderingen, die belangrijk zijn in de ontwikkeling van cardiovasculaire ziekten, in de bloedvaten van de retina op te sporen.

LIST OF ABBREVIATIONS

Ang-II	Angiotensin II
ARIC	Atherosclerosis Risc in Communities Study
AVR	ArterioVenous Ratio
BC	Black Carbon
BDES	Beaver Dam Eye Study
BH4	Tetrahydrobiopterin
BMI	Body Mass Index
CHS	Cardiovascular Health Study
CRAE	Central Retinal Arteriolar Equivalent
CRVE	Central Retinal Venular Equivalent
DBP	Diastolic Blood Pressure
eNOS	Endothelial Nitric Oxide Synthase
ET-1	Endothelin-1
FMD	Flow-Mediated Dilation
HAMB	Hypoxic Ambulation
HBR	Hypoxic Bedrest
HDL	High-Density Lipoprotein
HMGB1/RAGE	High Mobility Group Box 1/Receptor for Advanced Glycation Endproducts
LDL	Low-Density Lipoprotein
miR	microRNA
miRNA	microRNA
NADPH	Nicotineamide Adenine Dinucleotide Phosphate
MESA	Multi-Ethnic Study of Atherosclerosis
NF-κB	Nuclear Factor Kappa Beta
MPO	Myeoloperoxidase
NBR	Normoxic Bedrest
NO	Nitric Oxide
OECD	Organisation for Economic Cooperation and Development
OCT	Optical Coherence Tomography
PM	Particulate Matter
PM10	Particles with an aerodynamic diameter smaller than or equal to 10 micrometer
PM2.5	Particles with an aerodynamic diameter smaller than or equal to 2.5 micrometer
PM0.1	Particles with an aerodynamic diameter smaller than or equal to 100 nanometer
PP	Pulse Pressure
PTEN	Phosphatase and Tensin Homolog
RES	Rotterdam Eye Study
ROS	Reactive Oxygen Species
SBP	Systolic Blood Pressure
SOD	Superoxide Dismutase
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

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Chapter 1

Problem Statement

PROBLEM STATEMENT

Cardiovascular diseases are the main cause of death throughout the world. The World Health Organization (WHO) estimated that 17.5 million deaths each year are attributable to cardiovascular diseases of which 4 million deaths are counted in Europe.[1, 2] In 2012, 14.290 men and 17.156 women died from cardiovascular diseases in Belgium. Cardiovascular disease aetiology is a complex process and the interplay between many risk factors can tip the scales in favour of disease development. Lifestyle factors and environmental exposures play a crucial role in cardiovascular health. Epidemiological and experimental research for example has indicated that air pollution exposure and physical inactivity are cardiovascular risk factors.[3, 4]

Ambient air pollution is a complex mixture of many different gases, particles and aerosols that originates from numerous natural sources (erosion, forest fires, volcanic activity,…) and human processes such as agriculture, wood smoke, (industrial) burning of fossil fuels, and traffic exhaust.[5] The most important compound known to be linked with health effects is particulate matter (PM),[6] which consists of a complex mixture of solid and liquid particles of an organic or inorganic nature. These particles are classified according to their diameter as coarse (PM_{10}, particles with an aerodynamic diameter smaller than or equal to 10 micrometer), fine ($PM_{2.5}$ particles with an aerodynamic diameter smaller than or equal to 2.5 micrometer) and ultrafine particles ($PM_{0.1}$ particles with an aerodynamic diameter smaller than or equal to 100 nanometer). An important traffic-related contributor to the $PM_{2.5}$ fraction is black carbon (BC). This major constituent of diesel exhaust operates as a carrier for different toxic chemicals, formed during fossil fuel combustion, and may be an important contributor to the health effects induced by (traffic-related) fine PM.[7] Ambient air pollution causes 3.7 million premature deaths each year, of which at least 40% are attributable to air pollution-induced cardiovascular diseases.[6] The European Union has adopted mandatory ambient air pollution levels for its member states. Nevertheless, air pollution exposure is still associated with increased cardiovascular morbidity and mortality.[8, 9] According to the Organisation for Economic Co-operation and Development (OECD), air pollution caused 509.100 and 5.663 premature deaths in respectively Europe and Belgium. The total economic cost of premature mortality due to air pollution in Europe and Belgium is estimated to be respectively 1.4 trillion and 19 billion euro.[10]

Sedentary behaviour and the associated insufficient physical activity is increasing globally and causes worldwide 5.3 million premature deaths each year of which more than 500.000 occur in Europe.[11] As a consequence, physical inactivity is the fourth leading risk factor for global mortality according to the WHO[12]. The economic costs of insufficient activity in Europe are estimated to be at least 80 billion euro as 26% of adult men and 35% of adult women are insufficiently physically active.[11] This risk factor is associated with 30% of all cardiovascular diseases, whereas regular physical activity is known to reduce cardiovascular morbidity and mortality. In order to reduce the detrimental health effects, the WHO encourages adults to be active for at least 150 minutes at moderate-intensity or for 75 minutes of vigorous activity throughout the week.[11, 12]

The cardiovascular health effects of air pollution and physical inactivity cannot be fully understood by only studying the detrimental effects on the heart and major blood vessels. These risk factors can also target the microcirculation, which makes up the bulk of the circulatory system and consists of the smallest blood vessels (small arteries, arterioles, capillaries and venules). These vessels ensure nutrient delivery to the

tissues and largely determine peripheral resistance, thus exerting a great effect on blood pressure.[13] Air pollution exposure and physical inactivity can cause oxidative stress that can cause endothelial dysfunction and vascular remodelling in the microcirculation.[14-16] These microvascular functional and structural changes are a potential underlying mechanism that drives large-vessel disease.[17 18, 19] Interestingly, these microvascular changes are often detectable before overt clinical macrovascular signs are present. Detection of these early detrimental signs is therefore important in disease treatment and prevention.

OBJECTIVES AND OUTLINES OF THE THESIS

Fundus photography and image analysis can be used to assess retinal blood vessels in vivo. Retinal blood vessels share anatomical, physiological and pathological features with the cerebral and coronary microcirculation. Vessel diameter and blood flow in these microvessels is regulated by myogenic and metabolic autoregulation, but also endothelial production and secretion of vasoactive agents such as nitric oxide (NO) plays an important role.[20-22] As a consequence, retinal microvascular dysregulation and pathologies (as seen on fundus images) hold prognostic information on the disease process and state in the coronary and cerebral circulation.[23, 24] Epidemiological research has revealed that cardiovascular risk factors are associated with retinal arteriolar narrowing and retinal venular widening. Structural changes in retinal blood vessels are a risk factor for cardio- and cerebrovascular diseases.[25-31] These associations point at an involvement of the microcirculation in cardiovascular diseases. The potential role of the microcirculation, and more specifically the retinal microcirculation, in the associations between air pollution exposure/physical (in)activity and cardiovascular health are discussed in Chapter 2.
The evidence on the impacts of air pollution and/or physical (in)activity on the retinal microvasculature is rather scarce. Before this thesis, only one study on the association between air pollution and the retinal microvasculature was published.[32] The effects of physical inactivity on retinal microcirculation have mostly been assessed in cross-sectional studies.[33, 34] Even less is known about whether the short-term effects of air pollution exposure or physical (in)activity can be tracked by measuring retinal blood vessels widths. Therefore, the general aim of this thesis is to investigate whether fundus photography can be used to assess the short-term microvascular effects of air pollution and physical (in)activity.

Our *first hypothesis* is that short-term air pollution exposure is associated with retinal arteriolar narrowing and venular widening in healthy individuals. These effects may be caused by endothelial dysfunction: the reduced NO bioavailability will cause arteriolar vasoconstriction and promote venular inflammation that is associated with a vasodilating effect. We have conducted three separate panel studies in order to investigate this hypothesis. The first panel study was conducted on 84 healthy individuals who were examined three times over a period of 5 months, including a an episode of high ambient air pollution levels (Chapter 3). Oxidative stress and inflammation are involved in the health effects of air pollution and may drive the hypothesized retinal vascular changes. Therefore, a second panel of 50 healthy individuals was monitored for five consecutive months in which the retinal microvasculature and molecular markers were assessed monthly (Chapter 4). An important issue in epidemiological research on the health effects of air pollution is exposure misclassification. This reduces statistical power and causes an underestimation of the effect size of the association.[35] In the aforementioned panel studies, nearby monitoring stations, which are representative for the study area, were used (Chapter 3 and 4). However, personal exposure measurements allow to more accurately

determine air pollution exposure. Therefore, a panel of 56 healthy nurses were equipped during one week with portable devices to measure their personal BC exposure in order to investigate the cardiovascular effects of traffic-related air pollution using retinal imaging (Chapter 5).

Our *second hypothesis* is that physical activity causes observable changes in the retinal microcirculation. We investigated the microvascular effects of (prolonged) physical inactivity in healthy persons and the effects of a cardiac rehabilitation program in cardiac patients. Bedrest is an experimental model that induces the rapid cardiovascular effects of physical inactivity (vascular deconditioning) that are associated with sedentary behaviour and the aging process. We investigated the acute and sustained retinal microvascular changes in a cross-over study in which 14 healthy persons underwent three weeks of normal (physical) activity under hypoxic circumstances, three weeks of bedrest (physical inactivity) under normoxic conditions and three weeks of bedrest under hypoxic conditions (Chapter 6). The beneficial effects of physical activity on the retinal microcirculation were investigated in 53 cardiac rehabilitation patients. These patients were enrolled in an exercise program to improve endothelial function and overall cardiac health. Endothelial dysfunction is a hallmark of cardiovascular diseases and impairs blood flow-mediated dilation. Retinal microvascular responses were assessed after a maximal endurance test at the beginning of the program and after 6 weeks of training. Endothelial dysfunction might prevent detectable retinal vascular responses after the maximal endurance test at the beginning of the rehabilitation progam. Completing the rehabilitation program might improve endothelial function and retinal microvascular responses after the maximal endurance test (Chapter 7).

A general discussion of the outcome of this thesis is presented in Chapter 8. Also, potential pitfalls, shortcomings and future perspectives are addressed.

This work contributes to a better understanding of risk factors such as air pollution and physical (in)activity on the retinal blood vessels. Retinal vessel analysis is a convenient and non-invasive technique that is proposed as a sensitive proxy for changes in the coronary and cerebral microvasculature. Retinal vessel analysis holds promise as a screening tool in epidemiological and (pre)clinical studies to detect early phenotypic changes.

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Chapter 2

General Introduction: Air pollution and physical (in)activity alter retinal microvascular function and structure

Chapter 2

THE MICROCIRCULATION IN HEALTH AND DISEASE

The overall structure of the cardiovascular system

Circulatory systems evolved as a means to transport substances such as oxygen and nutrients rapidly through the body. These systems move fluids by increasing the pressure on the fluid in one part of the body. Consequently, the fluid flows through the body following the pressure gradient. To accomplish this function, circulatory systems use three main components. First, pumps or other propulsive structures create a local area of altered pressure within the body. Second, a system of tubes, channels or other spaces through which the fluid can flow. Third, a fluid that circulates through the system.

When these principles are applied to the human cardiovascular system, the pressure gradient is generated by the contractile function of the heart, and to a lesser extent by skeletal muscle contractions. The human cardiovascular system is a closed circulation, where the blood remains within a specialized system of blood vessels. Blood vessels are hollow tubular structures consisting of a complex wall surrounding a central lumen. The vascular wall is composed of three distinct layers. The tunica intima consists of the vascular endothelium and the subendothelial layer, a basement membrane which supports the vascular endothelial cells. The tunica media is composed of smooth muscle and sheets of elastin that wrap around the tunica intima. The tunica externa surrounds these layers and is largely composed of collagen fibres that support and reinforce the blood vessel.

The macrovascular network consists of conduit arteries and venes as well as large arterioles and venules to ensure fast transportation of the blood. The microvascular network consists of small resistance arteries and arterioles, the capillary network and the venules. The microcirculation is the main determinant of peripheral vascular resistance and the main site of gas and nutrient exchange. These small vessels make up the bulk of the cardiovascular system and the surface area of the microvascular endothelium is much larger compared with large conduit vessels. This huge contact area is the primary site for risk factor mediated endothelial dysfunction and vascular inflammation[1]. Disturbances in microvascular function and structure can have dire consequences for overall cardiovascular health. Evidence of microvascular dysfunction often predates evidence of clinically recognized atherosclerosis or target organ damage in the heart or brain.[2]

Microvascular function and the endothelium

The endothelium is an important regulator of microvascular function and structure. As a physical barrier, it prevents extravasation of circulating substances and cells from blood into the vessel wall.[3] As a metabolically active system, it controls and alters vascular tone and homeostasis through the secretion of dilating [nitric oxide (NO), prostacyclin, bradykinin, endothelium-derived hyperpolarizing factor] and constricting [endothelin-1 (ET-1), superoxide anion, angiotensin II (Ang-II), thromboxane] substances (Figure 1).[4, 5] Under normal conditions, NO is constantly produced by endothelial nitric oxide synthase (eNOS) from L-arginine, in the presence of the cofactor tetrabiopterin (BH_4), and released in the lumen and vascular wall.[6] The production of NO is essential for microvascular health. NO is a potent vasodilator that opposes the effects of endothelium-derived vasoconstrictors ET-1 and Ang-II. Microvascular function relies on the uptake of NO by smooth muscle cells that causes vasodilation. Moreover, this suppresses smooth muscle cell proliferation and vascular wall remodeling in order to maintain microvascular structure.[7] NO also possess anti-inflammatory properties. Luminal NO inhibits adhesion and aggregation of circulating platelets.[8] In endothelial cells and leukocytes, NO inhibits the activation of the pro-inflammatory expression NF-κB thereby suppressing the expression of surface adhesion molecules, pro-inflammatory cytokines and chemoattractants.[9]

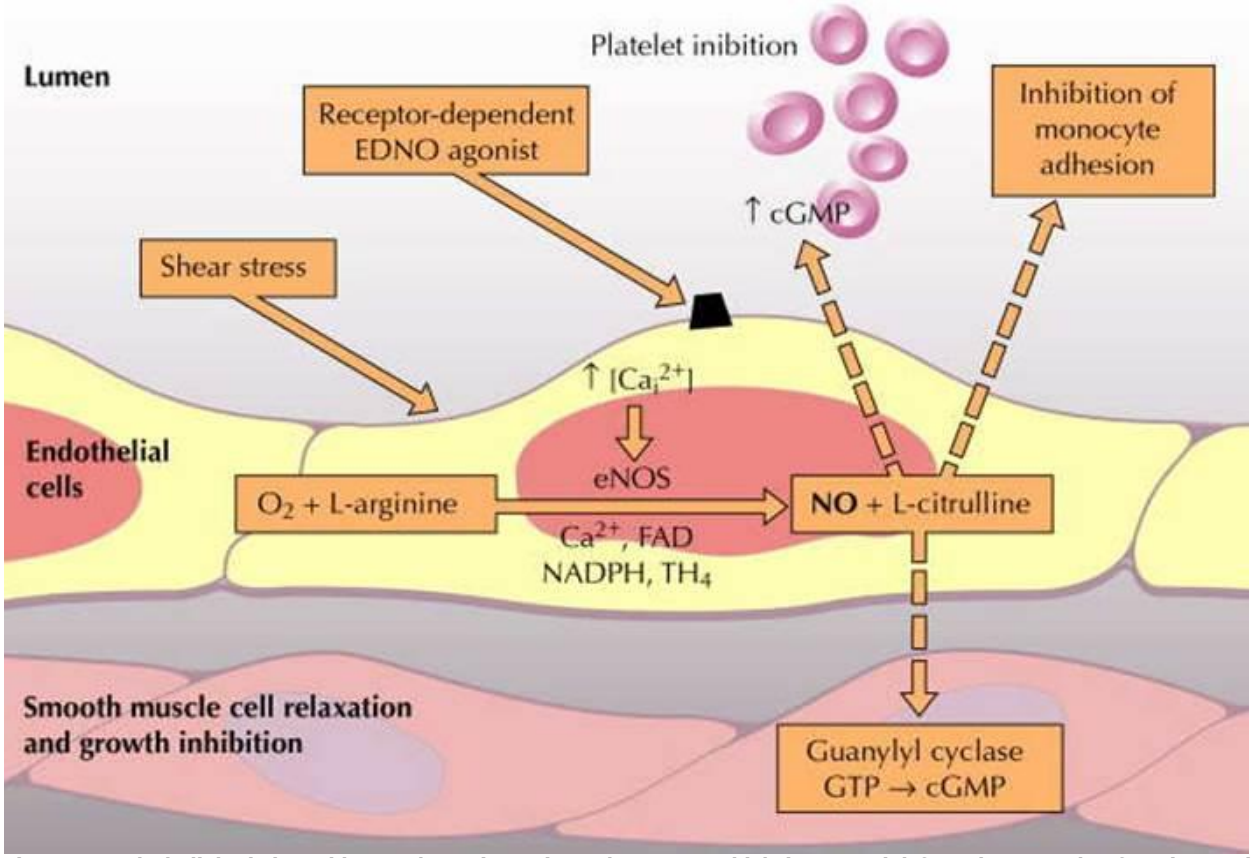

Figure 1 Endothelial Nitric Oxide Synthase (eNOS) produces NO which is essential for microvascular function and structure. eNOS activity can be regulated by shear stress and vasoactive agents. NO uptake in the smooth muscle cells causes endothelium-mediated vasodilation and inhibits smooth muscle cell proliferation. NO inhibits vascular inflammation by suppressing NF-KB expression in endothelial cells and circulating monocytes.

The nature of blood flow generates shear stress on the endothelium, which senses this force through mechanoreceptors protuding in the vessel lumen.[10] Through mechanotransduction, changes in shear stress are translated in endothelial responses. In this process, reactive oxygen species (ROS) play an important role as messengers as they activate redox-sensitive signaling pathways. In order to prevent oxidative stress, anti-oxidant defenses are also upregulated under physiological levels of shear stress.

Laminar blood flow causes laminar shear stress on the endothelium, which is essential for endothelial function as it exerts an atheroprotective effect by augmenting eNOS, and anti-inflammatory and atheroprotective gene expression in endothelial cells (Figure 2).[11] Therefore, shear stress is kept at a constant rate by dilating or constricting vessels when blood flow changes. When blood flow increases, due to increased cardiac output or downstream increased metabolic demand, laminar shear stress is also increased. This will stimulate the endothelium to produce and release NO in order to induce vasodilation and normalize shear stress levels on the endothelial cells. The opposite happens when blood flow is decreased: the endothelium will produce vasoconstrictors in order to maintain shear stress rate.[12] In contrast, oscillatory or disturbed blood flow will cause non-laminar shear stress on endothelial cells. In this situation, shear stress will fluctuate, which will disturb endothelial function and promote an endothelial proatherogenic phenotype as NO production is reduced and inflammatory and atherogenic gene expression is increased (Figure 2). In this way, endothelial-mediated vasodilation is impaired when blood flow increases in small and large vessels. Oscillatory blood flow is often observed at the bifurcation of (large) blood vessels. These regions of large vessels are prone to endothelial dysfunction which will facilitate the development of atherosclerosis.[13] As a consequence, atherosclerosis is often first observed at the bifurcations of large vessels.

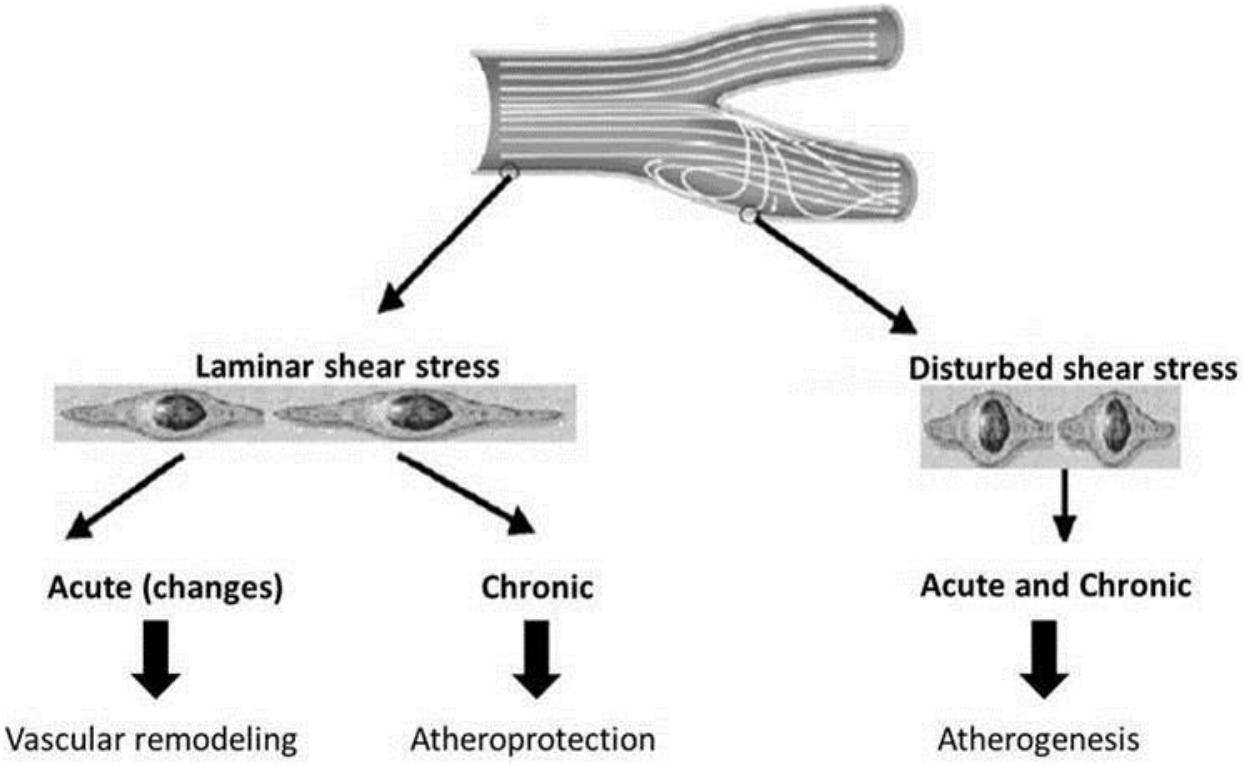

Figure 2 Shear stress patterns elicit different endothelial responses. The endothelium is constantly exposed to changes in magnitude and pattern of shear stress due to changes in blood flow. Through mechanotransduction, these mechanical forces are transduced in a biological response. Lamniar shear stress will induce an atheroprotective endothelial phenotype whereas disturbed shear stress will induce atherogenesis that facilitates the development of atherosclerosis. (Resnick et al., 2003)

The role of endothelial dysfunction in the pathogenesis of cardiovascular diseases

Several cardiovascular risk factors can disturb endothelial function and compromise microvascular function and structure, which play an important role in the genesis and progression of atherosclerosis and hypertension.[14, 15] This process often precedes clinically detectable sings of cardiovascular diseases. Endothelial dysfunction is characterized by reduced NO bioavailability and impaired

endothelium-mediated vasodilation. In this way, endothelial and microvascular dysfunction may be the first step towards development of hypertension and atherosclerosis, which are the major causes of cardiovascular diseases.

Endothelial dysfunction contributes to the development of hypertension.[16] Under normal conditions, the pulsatile nature of blood flow will induce cyclic stretch in blood vessels. Smooth muscle cells are pushed in the outside direction during cardiac systole when blood pressure and the force on the vessel wall is increased. This excessive force is absent during diastole and the smooth muscle cells will contract in order to return to their initial position. These physiological levels of cyclic stretch preserve the contractile phenotype of smooth muscle cells and prevent proliferation or atrophy of the vessel wall.[17] When endothelium-mediated vasodilation is impaired, blood flow increases are not met with proper vasodilation and the force generated on the vascular wall will increase. As a consequence, the amplitude of cyclic stretch in the vessels will increase to pathological levels. As a compensatory mechanism, which is enhanced due to the loss of NO, smooth muscle cells will proliferate and deposit elastin and collagen in the vascular wall in order to strengthen the vessel wall and cope with the increased stretch. The inward remodeling and stiffening of the vessel wall decrease vessel lumen and normalize blood flow, but this will increase vascular resistance and permanently elevate blood pressure.[17, 18] The strengthening of the vessel wall will reduce cyclic stretch properties. This will increase oxidative stress, decrease anti-oxidant defences and increase ET-1 and Ang-II expression that will further stimulate smooth muscle cell proliferation.[19, 20] In this way, microvascular functional and structural changes start a vicious circle that initiates, maintains and amplifies endothelial dysfunction and blood pressure.[21] Hypertension can reduce blood flow to target organs, cause rupture of small blood vessels and lead to target organ damage (especially in the brain), cause left-ventricular hypertrophy and heart failure and accelerate atherosclerosis in coronary vessels. The physical stress on the arterial wall also accelerate atherosclerosis in the coronary vessels and may cause plaque rupture.

Oxidative stress and endothelial dysfunction promote a shift towards a pro-atherosclerotic endothelial phenotype and are the main causes of atherosclerosis.[19] Impaired endothelium-mediated vasodilation often precedes atherosclerotic plaque formation. This is suggestive of an underlying mechanism in the microvasculature, before clinical macrovascular changes are detectable.[3] Reduced NO bioavailability has pro-inflammatory consequences as NF-κB activity is no longer inhibited. NF-κB will induce the endothelial production of vascular cytokines and adhesion molecules which attract and facilitate ingression of inflammatory cells into the vessel wall. Moreover, loss of NO inactivates fibrinolytic factors and activates platelets, which promotes thrombus formation.[3] Reactive oxygen species can easily oxidate low-density lipoprotein (LDL) cholesterol.[22] Oxidized LDL promotes monocyte adhesion and migration to the subendothelial space and intima media where these cells differentiate into macrophages. This process initiates the formation of a fatty streak in the subendothelial space when macrophages bind oxLDL via scavenger receptors to become foam cells which start releasing pro-inflammatory cytokines.[23, 24] The accumulation of leukocytes and mast cells in the subendothelial space will stimulate crosstalk between monocytes, macrophages, foam cells, and T-cells and result in cellular and humoral immune responses, further boosting the pro-inflammatory state.[25] Smooth muscle cells are attracted into the intima media and will start to produce extracellular matrix proteins that create a fibrous cap over the lesion.[24] When foam cells die inside the fibrous cap, lipids accumulate into a lipid-rich pool, known as the necrotic core.[26] The integrity of the atherosclerotic

plaque is maintained by the thickness of the fibrous cap.[27] Stable plaques have an intact fibrous cap and protrusion of these lesions in the vascular lumen causes flow-limiting stenosis, leading to tissue ischemia and usually stable angina. The fibrous cap of vulnerable plaques is very thin and are prone to erosion or rupture, caused by hypertension. Plaque rupture exposes the core of the plaque to circulating coagulation proteins, causing thrombosis, sudden partial of full occlusion of the artery lumen, often resulting in an acute coronary syndrome or stroke (Figure 3).[25, 27]

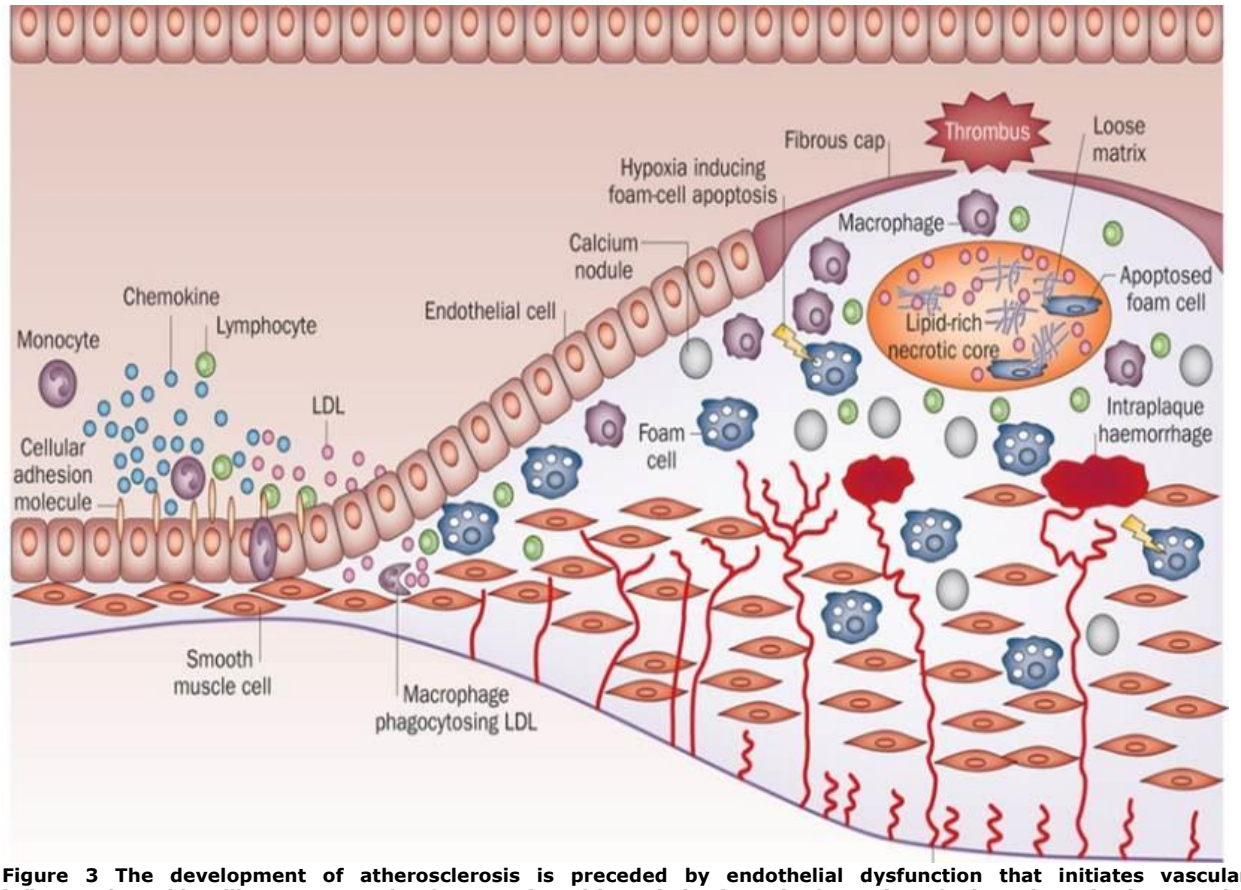

Figure 3 The development of atherosclerosis is preceded by endothelial dysfunction that initiates vascular inflammation. This will start a cascade of events that ultimately leads to the formation of atheroslecrotic plaques. The detrimental cardiovascular effects of atherosclerosis start when the size of the plaque starts occluding arteries and diminished blood flow to the downstream tissues, resulting in ischemia. Rupture of these plaques will obstruct vessel lumen and cease blood flow, resulting in tissue necrosis. (Skeoch et al., 2015)

Cardiovascular risk factors

Traditional risk factors

Aging, obesity, hypertension, diabetes, atherosclerosis are well-known traditional risk factors for cardiovascular disease. Each 5 kg/m² increase in BMI is associated with an increase in hazard ratio of 1.27 (95% CI: 1.23 to 1.31) and 1.18 (95% CI: 1.14 to 1.22) for respectively coronary heart disease and stroke[28]. Increases in blood pressure in the pre-hypertensive range were associated with increases in hazard ratios of 1.44 (95% CI: 1.35 to 1.53), 1.73 (95% CI: 1.61 to 1.85) and 1.79 (95% CI: 1.45 to 2.22) for respectively total cardiovascular diseases, stroke and myocardial infarction.[29] The presence of diabetes type 2 increases risk ratios for incident coronary heart disease with 2.82 (95% CI: 2.35 to 3.38) in women and 2.16 (95% CI: 1.82 to 2.56) in men.[330]

Air pollution exposure is an independent risk factor for cardiovascular health

Both short- and long-term exposure are associated with cardiovascular morbidity and mortality. In a recent meta-analysis, a 10 μg/m³ increase in long-term $PM_{2.5}$ exposure was associated with 6% (95% CI: 4 to 8) higher all-cause mortality and 11% (95% CI: 5 to 16) higher cardiovascular mortality.[31] In depth analyses showed that a 10 μg/m³ increase in long-term $PM_{2.5}$ exposure was associated with 3.36%, 2.12%, 2.5%, 1.05% and a 1.1% higher incidence for respectively ischemic heart disease[32], heart failure[33], myocardial infarction[34, 35], hypertension[36] and stroke[37]. The same associations have been found for PM_{10}, although the effect estimates are somewhat smaller. PM exposure is also associated with subclinical cardiovascular end points. A 10 μg/m³ increase in long-term $PM_{2.5}$ exposure was associated with a 1.39 mm Hg (95% CI: 0.87 to 1.91) increase in systolic and a 0.90 mm Hg (95% CI: 0.49 to 1.23) increase in diastolic blood pressure.[38] Similar associations were found for short-term $PM_{2.5}$ exposure where a 10 μg/m³ increase on the previous day was associated with a 3.2 mm Hg increase in systolic blood pressure.[39] In the Multi-Ethnic Study of Atherosclerosis (MESA) cohort, a 10 μg/m³ increase, measured over the 30 days preceding the exam, was associated with 0.99 mm Hg increase in systolic blood pressure.[40] Moreover, air pollution exposure is associated with atherosclerosis. In a recent meta-analysis by Provost et al., a 5 μg/m³ increase in long-term $PM_{2.5}$ exposure was associated with a 1.66% (95% CI: 0.86 to 2.46) thicker carotid intima media thickness, a marker of subclinical atherosclerosis.[41]

Unsurprisingly, air pollution has been associated with brachial flow-mediated dilation (FMD), a technique to assess endothelial function. In the MESA cohort, a 3 μg/m³ increase in long-term $PM_{2.5}$ exposure was associated with a 0.3% decrease in FMD (95% CI: −0.6 to −0.03).[42] These findings were reproduced in the Framingham Offspring Study and Third Generations Study where a 1.99 μg/m³ increase in long-term $PM_{2.5}$ exposure was associated with a 0.16% decrease in FMD (95% CI: −0.27 to −0.05).[43] Short-term increases in $PM_{2.5}$ of 30 μg/m³ were associated with a reduction of 0.5% in FMD[44]. Controlled exposure to particulate matter caused acute[45, 46] or delayed impairment of endothelium-dependent vasodilation[47-50] or acute brachial conduit artery vasoconstriction, due to increased production of ET-1, without alterations in endothelium-dependent/independent vasodilation[48, 51]. Not all studies reported these associations.[52, 53] Discrepancies between studies may be explained by differences in particle composition, study design and individual's health and susceptibility.[52, 54]

Sedentary behavior is a risk factor and physical activity promotes cardiovascular health

Self-reported sedentary time was associated with increases in hazard ratios for all-cause mortality [1.24 (95% CI: 1.09 to 1.41)], cardiovascular disease mortality [1.18 (95% CI: 1.11 to 1.26)] and cardiovascular disease incidence [1.14 (95% CI: 1.002 to 1.73)].[55] Moreover, sedentary time was associated with subclinical markers for atherosclerosis[56, 57] and endothelial dysfunction[58, 59]. In a recent meta-analysis, a dose-response relationship between physical activity and cardiovascular diseases was observed. High levels of leisure time physical activity in men and women were associated with a risk ratio of respectively 0.76 (95% CI: 0.70 to 0.82) and 0.73 (95% CI: 0.68 to 0.78) when compared to the reference group with low leisure time physical activity.[60] Walking and

cycling are also known to have population-level health benefits even after adjustment for leisure time physical activity.[61] Self-reported moderate to high levels of physical activity lowered the odds of peripheral artery disease, incident heart failure and hypertension to respectively 0.64 (95% CI: 0.63 to 0.64)[62], 0.72 (95% CI: 0.67 to 0.79)[63] and 0.81 (95% CI: 0.76 to 0.85)[64]. Physical activity had a beneficial effect on carotid intima media thickness and brachial flow-mediated dilation in adolescents[65] and adults[66].

Air pollution and physical inactivity disturb endothelial function

Air pollution causes oxidative stress and inflammation

Air pollution can act through several pathways to induce endothelial dysfunction. These direct and indirect mechanisms allow effects to be measured acutely or within a time course of 2 to 48 hours. Particle exposure can trigger autonomic reflexes in pulmonary receptors. The modulated autonomic neural input to the heart and vasculature will alter heart rate and blood pressure. The consequent changes in blood flow may induce acute vascular changes.[67, 68] Fine and ultrafine particles deposited on the alveolar surface may enter the systemic circulation. In this way particles are directly translocated to the target organs.[69] Uptake by inflammatory or epithelial cells may induce inflammatory reactions or generation of ROS. Moreover, the pro-oxidative potential of the particles may offset cellular redox balance in these cells, promoting the generation of ROS.[69] Particles in the lung will be processed by alveolar macrophages, bronchial and alveolar epithelial cells.[70] Macrophages will be activated by phagocytosing particles and tightly associate with the alveolar wall. These hotspots of activated cells will start producing pro-inflammatory mediators to elicit a low-grade local inflammatory response, without necessarily altering markers of overall pulmonary inflammation or damage.[71] The secretion of chemokines and cytokines from pulmonary cells in the systemic circulation will upregulate, attract and activate neutrophils and leukocytes.[70, 72] The spillover of pulmonary pro-inflammatory mediators will also activate the endothelium of the systemic circulation. Activated endothelial cells will upregulate their pro-inflammatory mediators and start interacting with circulating leukocytes. Leukocyte rolling and adherence on the endothelium will change the activity of NADPH oxidase, SOD and MPO.[73] Activated leukocytes will deposit MPO in the vascular wall. In this way, endothelial cells and leukocytes will start generating excessive amounts of ROS and pro-inflammatory mediators.

Oxidative stress alters endothelial function by lowering NO bioavailability and by enhancing the production of vasoconstrictors such as ET-1 and peroxinitrite.[48, 71, 73] In the endothelial cells, the excessive superoxide anion will react easily with NO to form peroxinitrite. Furthermore, reactions between ROS and the eNOS cofactor BH_4 will partially or fully deplete BH_4. This will cause partial or full eNOS uncoupling.[74] The "uncoupled" form of eNOS will produce superoxide anion instead of NO and significantly boost oxidative stress.[6] Luminal NO is scavenged by ROS generated by leukocytes and MPO deposits in the vascular wall. In this way, NO cannot reach the smooth muscle cells and mediate the endothelium-dependent vasodilation as observed in the aforementioned studies. When anti-oxidant defenses in the vascular wall are upregulated, endogenous NO production and endothelial function are restored.

Physical inactivity changes shear stress in blood vessels

Physical activity has a beneficial effect on endothelial function and oxidative stress through changes in the nature and magnitude of shear stress.[75] When heart rate and blood flow are elevated during physical activity, the increased laminar shear stress on the endothelium will augment endothelial NADPH oxidase activity and upregulate SOD expression to prevent oxidative stress.[76-78] In this way, the generated superoxide will be converted to H_2O_2 that will activate kinases that increase eNOS expression and activity.[79, 80] The produced NO will be used to relax smooth muscles resulting in vasodilation, but will also enhance expression of SOD and downregulate NADPH oxidase expression in endothelial cells. In this way, shear stress-induced ROS production is lowered. Regular shear stress changes and increased shear rate, associated with regular physical activity, will upregulate eNOS expression and enhance basal NO production.[81] This improves endothelium function (endothelium-dependent vasodilation and anti-inflammatory properties), increase the activity of anti-oxidant enzymes and reduces expression of pro-oxidant and inflammatory genes, restoring a natural gene expression pattern.[82] The increased shear rate will also lead to structural adaptations of the blood vessels. Vascular wall remodelling will increase vessel lumen in order to permanently normalize shear stress on the vessel wall.[83]

Endothelial dysfunction, detrimental vascular remodelling and atherosclerosis are often observed in persons with a sedentary lifestyle.[84] The changes in magnitude and patterns of shear stress, associated with exercise, are reduced with a physically inactive lifestyle.[85, 86] Physical inactivity causes an increase in the oscillatory (non-laminar) shear stress, which promotes a proatherogenic endothelial cell type and influences inward vessel remodelling.[87, 88] This will induce a dose and time dependent decrease in size of conduit, resistance and microvessels. The largest decline is observed in the beginning of the process when endothelial and smooth muscle cell functionality are altered.[89-91] Oscillatory shear stress enhances NADPH oxidase activity, but does not increase the expression or activity of eNOS and anti-oxidant enzymes.[92] The resulting oxidative stress will scavenge NO and cause endothelial dysfunction.[84] In this pro-oxidant environment, flow-mediated dilation is impaired, the production of vasoconstrictors and inflammatory markers is upregulated and inward vessel remodelling will become apparent.[93, 94] This will stimulate the process of hypertension and atherosclerosis and contribute to cardiovascular diseases.[65, 76, 84, 95, 96]

How to study the microcirculation?

Large-scale epidemiological studies have found associations between disturbed microvascular perfusion, autoregulation or structure and subsequent target organ damage. Evidence of microvascular (endothelial) dysfunction often predates the evidence of clinically recognized organ damage. However, the small size and inaccessibility of the microvessels make it difficult to investigate them. Technological advancements in the field of imaging and ultrasound have made it possible to assess the micro- and macrocirculation in vivo. The ideal technique should noninvasively give continuous, reproducible measurements, independent of tissue characteristics and provide a result in a relatively short time.

Endothelial function

Endothelial function can be measured by infusion of vasoactive drugs or post-occlusive reactive hyperaemia. Both techniques are usually applied on the fore-arm, where the vasodilation response of the brachial artery is measured with ultrasound techniques (brachial flow-mediated dilation).

Usually three kinds of vasoactive drugs are used in experimental studies. The first kind consists of substances that stimulate the endothelium to produce and release NO (acetylcholine, bradykinin, …), which will cause vasodilation of the vessel. The second kind consists of NO-donors that will directly release NO in the vessel lumen, without stimulation of the endothelium. These vasoactive drugs are often used to assess smooth muscle cell responsiveness to NO. The third kind consists of eNOS-blockers. These drugs will counter NO production and are often used to assess endothelium-independent vasodilatory properties of blood vessels.[85]

During post-occlusive reactive hyperaemia, a cuff is used to apply a pressure larger than the systolic pressure to occlude a blood vessel. Releasing the occluding pressure will restore blood flow. As a consequence, shear stress is increased and this will stimulate endothelial cells to produce and release NO which will cause vasodilation.[86]

Microvascular structure

Imaging techniques such as videomicroscopy (Orthogonal Polarization Spectral and Sidestream Darkfield imaging) and laser Doppler flowmetry have been developed to study the morphological parameters of the microcirculation. These techniques rely on dynamic measurements of microcirculatory blood flow and blood cell concentration.[97] The spatial heterogeneity of perfusion in microvascular beds makes imaging methods that cover a larger surface preferable over single-point functional measurements.[98, 99]

Orthogonal Polarization Spectral imaging (polarized light) and Sidestream Darkfield imaging (light with a specific wavelength) both use visible light to illuminate the examined tissue. The reflected (depolarized) light is captured with a CCD video camera (Figure 4). As haemoglobin absorbs specific wavelengths, the red blood cells appear dark on the image[100, 101] A direct visualization of the microcirculation allows determination of vessel diameter, vessel density and red blood cell velocity. As these devices need to be placed directly on the tissue, motion and pressure artefacts that disturb vessel blood flow can arise.[101-103] Some authors have reported that high blood flow velocities cannot be measured accurately.[104] The use on solid organs is also limited as the organ capsule needs to be very thin or has to be removed to allow penetration of the light.[102, 103] Despite these limitations, videomicroscopy has been successfully used to visualize the microcirculation of the sublingual mucosa, skin, brain, lungs, tongue, liver, …

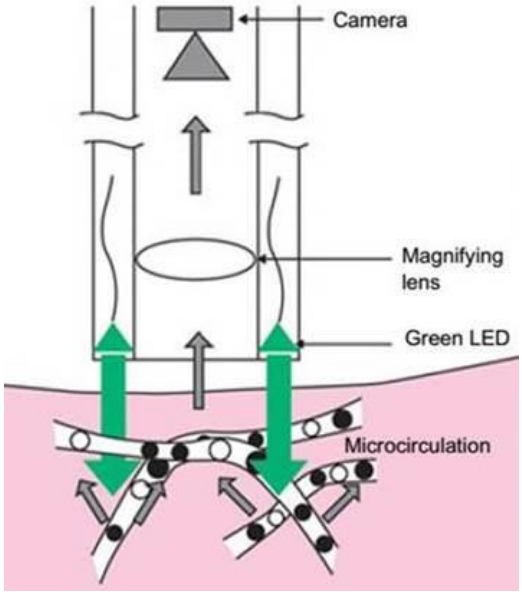

Figure 4 The principle of videomicroscopy. The imaging device is placed on the tissue to be examined. Light is sent into the tissue, of which certain wavelengths will be absorbedby haemoglobin in red blood cells. As a consequence, red blood cells will appear dark on the image and the structure of blood vesselscan be detected.

In Laser Doppler flowmetry, a coherent laser light undergoes a small shift in frequency due to the Doppler effect when striking moving particles such as red blood cells, whereas the reflected light of static tissues returns with unchanged frequency.[105] The Doppler shift is the average amount of change in frequency in the reflected light and is proportional to the product of the average speed and concentration of red blood cells. As Laser Doppler flowmetry is a non-contact method, no pressure artefacts can arise.[106] However, the average Doppler shift does not allow to evaluate absolute flow properties as this is a relative comparison of flux held against a normalized baseline.[107] Nevertheless, this technique is usable to asses blood flow in several organs, differentiate between malignant and benign skin lesions and different kinds of (healing) wounds.[108, 109]

THE RETINA: A WINDOW TO THE HEART?

The retina is a layered tissue lining the interior of the eye that enables the conversion of incoming light into a neural signal that is suitable for further processing in the visual cortex of the brain. The eye is supplied with blood from the ophthalmic artery, that branches from the internal carotid artery. It enters the eye in the optic disc, the "blind spot", void of photoreceptors, where also the optic nerve enters the retina. The inner eye has two separate vascular networks: the choroidal and the retinal blood vessels each supplying different regions of the inner eye and the retina. The activity of the photoreceptors, which are located in the inner retina, makes the retina a highly metabolically active tissue. Hence, the retinal vasculature is needed to ensure an adequate supply of blood. The choroidal vasculature supplies the outer retina, which is devoid from photoreceptors. The development of fundus photography allows to visualize the retinal microvasculature in vivo.

Development of the retinal vasculature

Interestingly, the retinal vascular network has to be developed from scratch as in the early stages of (embryological) development the inner part of the eye is supplied by the hyaloid vasculature (a branch from the ophthalmic artery). The development of the retinal blood vessels is preceded by the

migration of astrocytes into the retinal tissue.[110, 111] The astrocytes emerge from the optic nerve head and spread as a proliferating cell population across the whole inner surface of the retina, except for the fovea which will remain avascular. This mesh-like cellular network serves as a template for blood vessels in their wake.[112] As the retina is still hypoxic, the astrocytes will strongly express VEGF, a key stimulus for angiogenesis.[113, 114] In the retina (and brain), new vessels are formed when proliferating endothelial cells form new vessels sprouts and extend the vascular network from pre-existing vessels.[115] The primary plexus is formed by sprouting angiogenesis at the optic nerve head [116]. Endothelial tip cells at the leading edge of the growing vascular network will steer the vascular growth towards the retinal periphery.[117] These endothelial tip cells migrate in response to attractant and repellent guidance cues like VEGF and other (yet to be discovered) factors that ultimately result in the intricate patterning of the retinal vasculature.[118] Astrocyte-mediated VEGF expression is downregulated when the astrocytes are covered with blood vessels and oxygen is supplied. In contrast, in the hypoxic periphery of the retina that is not yet vascularized, the astrocytes will still produce VEGF. The VEGF gradient will guide sprouting vessel to the periphery. The supplied oxygen will induce apoptosis (limit astrocyte numbers) and differentiation (maturation) in the astrocytes. The mature astrocytes will stop producing VEGF.[119]

The newly-formed retinal vasculature is a dense and uniform capillary plexus extending from the optic nerve head to the periphery. The vessels will start to mature and remodel into the hierarchical vascular tree. In this process, vessels differentiate into arteries and veins and capillaries in the vicinity of the arteries will be pruned, which will create capillary-free zones.[120, 121] In this stage, astrocytes and Müller cells mediate the formation of the blood-retina barrier.[122-124] At the same when time the retinal vasculature is developing and supplying the retina with oxygen and nutrients, the hyaloid vasculature will start to regress. Ultimately, the inner portion of the retina is supplied solely by the retinal blood vessels.

The retinal microcirculation shares features with the cerebral and coronary microvasculature

During (embryological) development, the eye is formed as an extension of the diencephalon (the interbrain). This close anatomical connection will result in a similar vascularization process during development resulting in anatomical similarities in the retinal and cerebral microcirculation.[115, 125] In both organs, capillaries are denser in the metabolically more active regions[126-128] and the endothelia perform a strict barrier function to in order keep the neuronal milieu free from exogenous toxins, buffer variations in blood composition and restrict the transfer of small hydrophilic molecules, large molecules and haematogenous cells.[129-131]

The retinal, cerebral and coronary microcirculation supply three organs with a high metabolic demand. A dense capillary network ensure an adequate gas and nutrient exchange, but this network is prone to sudden increases in perfusion pressure. In these organs, blood flow is primarily controlled by myogenic or metabolic autoregulation as autonomic innervation is absent[132, 133] or its effect negligible.[134] Myogenic autoregulation is a negative feedback loop to maintain a constant blood flow. The stretch-sensitive smooth muscles in the arteriolar wall are stretched when pressure on the arteriolar wall increases. This will cause contraction of the smooth muscles that increases resistance

and decreases flow. Metabolic autoregulation will cause increases/decrease in blood flow through arteriolar vasodilation/vasoconstriction when the tissue becomes metabolically active/inactive.

NO is the key regulator of vascular tone in the retinal, cerebral and coronary microcirculation as inhibition of eNOS reduces vessel diameter and autoregulatory properties.[135, 136] NO is constantly produced and released in these vessels and keeps the vessels in a permanently widened state. This will allow a sufficient blood supply and reduce the perfusion pressure in these vessels. When blood flow needs to be increased, due to metabolic demand, NO is the primary mediator of flow mediated dilation in these vessels.[137] This vasodilator reserve can maintain flow when perfusion pressure changes[138] and ensure the delivery of nutrients in the face of a broad range of external factors such as systemic blood pressure.[139-142]

Microvascular function, structure and network is optimized in order to minimize physical properties such as shear stress and blood pressure in blood vessels.[143-145] Certain vascular conditions change the optimal geometrical topography, the structure of the vessel wall or the function of the retinal, coronary and cerebral vasculature.[146, 147]

Visualization of the retina and image analysis

Since ocular structures need to be transparent in order to capture incoming light, the retina offers a unique possibility to non-invasively visualize its microcirculation in vivo. Retinal imaging techniques have a substantial reproducibility and can be used repeatedly in the same individual for follow-up. Static retinal imaging, such as fundus imaging and optical coherence tomography (OCT), produces images used to assess retinal vascular parameters, abnormalities or retinal layer thickness. In contrast, dynamic vessel analysis allows to measure retinal endothelial function.

Fundus imaging encompasses (classic) colour fundus photography, stereo fundus photography, hyperspectral imaging, scanning laser ophthalmoscopy and adaptive optics scanning laser ophthalmoscopy, which can be differentiated by the wavelength of the light that is used. This technique sends an external light into the eye. The light traverses the pupillary plane and is reflected by the retina. The outgoing reflected light is separated by the optical apparatus, to prevent interference and corneal/lenticular reflection which will eliminate image contrast. The outgoing light is projected on an imaging plane and gives a 2D representation of the 3D retinal tissue. The static image of the retinal surface can be used for analysis of the vessel width or pattern, detection of abnormalities and manifestations of disease in the retina.[148] Therefore, it is widely used for population-based, large scale detection of diabetic retinopathy, glaucoma and age-related macular degeneration.

OCT-imaging estimates the depth at which a specific backscatter originated by measuring its time of flight. These backscatters are caused by differences in refractive index in transitions from one tissue to another. Backscatters from deeper tissues take longer to arrive at the sensor. In this way, the different layers of the retina can be visualized separately. The incoming light is absorbed by the blood vessels on the retinal surface. Hence, vessel silhouettes will appear dark on OCT images.[148] OCT can be used to determine retinal layer thickness, an additional feature of retinal health.[149] OCT images of the superficial retinal layer can also be used for analysis of blood vessel width or pattern. OCT is predominantly used in diagnosis and management of patients with diabetic retinopathy, macular degeneration and inflammatory retinal diseases.

Dynamic retinal vessel analysis is comparable to earlier described videomicroscopy techniques. Here, a small part of the retina is stimulated with flicker light, illumination alternating in brightness or colour at a frequency of approximately 1-50 Hz. This will stimulate the metabolic activity of the affected photoreceptors in the retina. Due to neurovascular coupling, blood flow to the illuminated region of the retina will be increased.[150] Metabolic vasodilation is caused by retinal endothelial release of NO. This was proven when administration of eNOS inhibitors blunted vasodilation after flicker light stimulation.[151] In this way, endothelial function (NO-mediated vasodilation) of the retinal blood vessels can be assessed non-invasively.[152]

Fundus image analysis

Localisation of retinal landmarks

The first step in fundus image analysis is the detection of the retinal landmarks (optic disc, fovea and vasculature) (Figure 5). Since the optic nerve head is the brightest component of the fundus with a confluence of blood vessel at the optic disc margin, it will be a cluster of high-intensity pixels with a high grey-scale value surrounded by pixels with a large variance in intensity.[153, 154] Principal component analysis or a geometrical parametric model allow algorithms to distinguish the optic disc from other structures.[155] The identification of the optic disc is important for localisation of other anatomical components, vessel tracking, reference length for measuring distances or registration of changes within the optic disc region due to disease.

Usually, the fovea is the darkest region on the retinal image and it is positioned at a relatively fixed distance and location from the optic disc. This avascular region results in different grey levels at its border[156]. Its position is determined using model-based methods that search for a maximum correlation between a model template and the intensity scales of the image.[155]

Retinal blood vessels are detected against their background. The cross-sectional grey-level profile of a vessel resembles a distinct Gaussian distribution against the background intensity of the surrounding retina (Figure 6A). The vessel border is identified at the half of the height of the peak of the Gaussian shaped intensity profile.[157]Three different approaches to automatically identify vessels are commonly used. First, matched filters use 2D linear structural elements which are rotated in different orientations to fit into vessels. With thresholding the vessel silhouette is extracted from the background.[158, 159] Second, vessel tracking algorithms automatically search vessel centre locations over each cross-section of a vessel along its longitudinal axis, provided a start and end point has been given (Figure 6B). Based on the Gaussian shape of the vessel's grey-level profile, the borders are detected.[160] Third, neural networks have been incorporated in image analysis. Based on mathematical weights, these algorithms decide the probability that the input date (a set of pixels) belongs to a particular output (a blood vessel).[154]

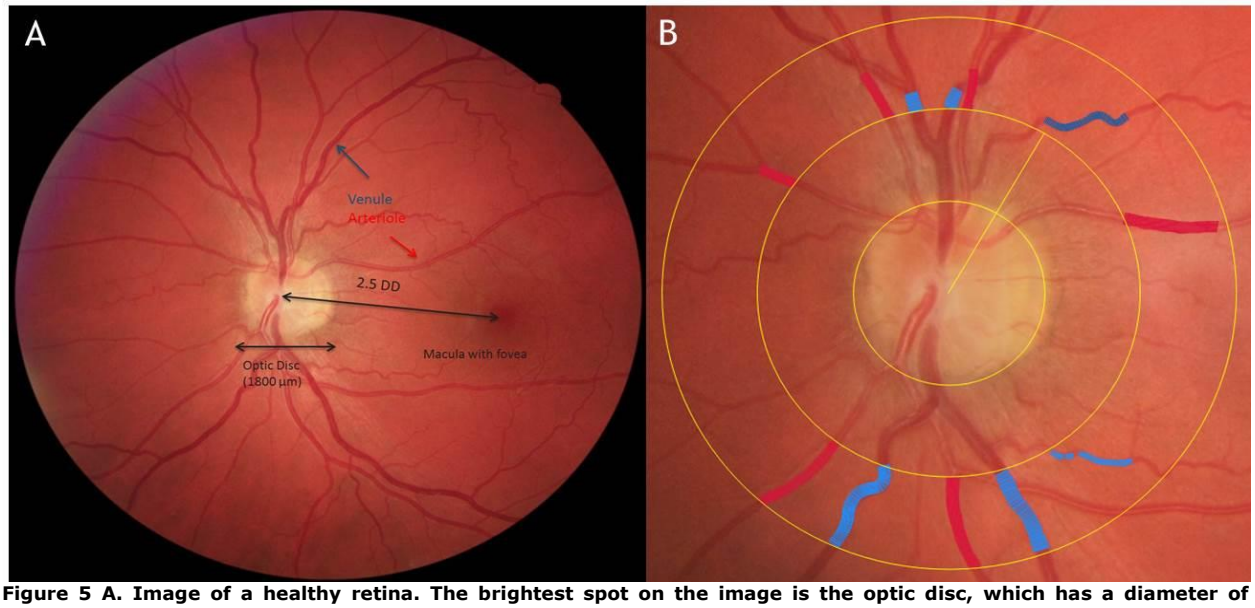

Figure 5 A. Image of a healthy retina. The brightest spot on the image is the optic disc, which has a diameter of approximately 1800 µm. On 2.5 times the disc diameter, the macula and fovea are located. The fovea is the darkest, avascular region of the image. B. Retinal vessel analysis. The revised formulas of Knudtson et al. allow to calculate retinal vessel diameters (CRAE and CRVE) based on the six biggest blood vessels of each kind (arterioles and venules).

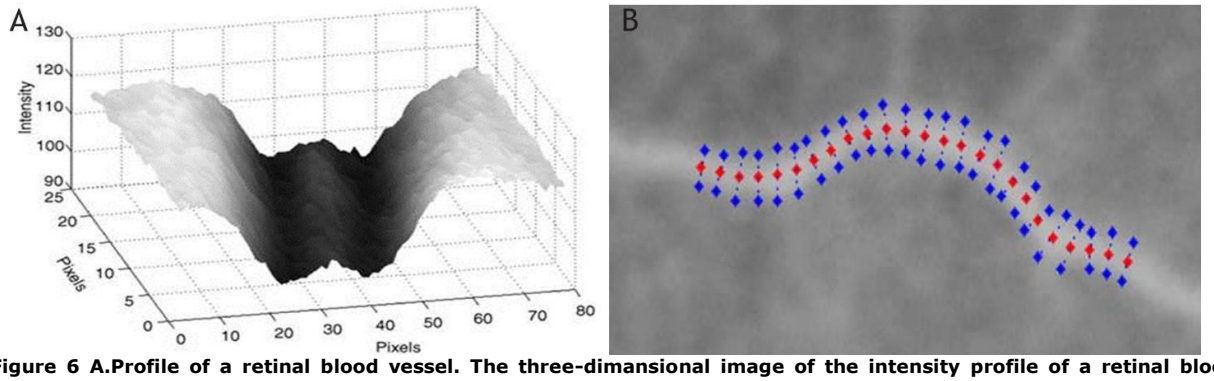

Figure 6 A.Profile of a retinal blood vessel. The three-dimansional image of the intensity profile of a retinal blood vessel of "double-Gaussian" construct. B. Grey-scale image of a retinal vessel-tracking process. The blue dots resemble the vessel borders, defined by the the half of the height of the peaks of the Gaussian shaped intensity profile. (Patton et al., 2006)

Retinal Vessel Analysis

When using computer-assisted programs, calibration is a fundamental issue in order to determine the true size of a fundus feature. Pathology studies have indicated that the true value of one standard optic disc diameter was equivalent to 1,800 - 1,900 microns and that the distance between the optic disc and macula is approximately 2.5 times the optic disc diameter (2.5 DD).[161] The standard of 1800 microns has gained relatively wide acceptance as an internal reference to compensate for the effect of camera magnification on the vessel width measurements (Figure 5).

The algorithms for automated vessel detection, briefly discussed above, are also used to measure the width of single vessels. As vessel width can vary due to heart cycle, the degree of systemic autonomic nerve stimulation or the degree of fundus pigmentation, single vessel measurements offer little information.[162-164] In this regard, the arteriovenous ratio (AVR) was developed as a general measurement of the ratio between average diameters of the arterioles with respect to the venules. Nowadays, AVR and the independent use of arteriolar and venular width, bifurcation angles, vascular tortuosity, length:diameter ratio and fractal analysis are widely used retinal vascular features that offer important information about disease status.

The AVR has been based on formulas developed by Parr and Hubbard who made estimations of respectively the arteriolar and venular trunk and branch vessels in a predefined zone. This zone, in which all measurements of vessel width are conducted, lies between concentric rings 0.5 and 1 optic disc diameter from the optic disc margin. In this zone the blood vessels are unambiguously arteriolar or venular.[163] With an iterative formula Parr et al. combined all arteriolar widths into one value: Central Retinal Arteriolar Equivalent (CRAE).[165] Hubbard et al. applied this principle to the venules: Central Retinal Venular Equivalent (CRVE).[166] The AVR was used in epidemiological research and associations between reduced AVR and hypertension or cardiovascular disease were found. It was considered a good measurement of generalized arteriolar narrowing as arterioles were thought to be more affected by narrowing than the corresponding venules in response to cardiovascular diseases. [167] However, it soon became clear that venules and arterioles had different responses to pathological conditions. Henceforth, CRAE and CRVE were used separately. Another limitation of the AVR was the significant impact of the number of vessels used in the calculation of overall AVR. This lead Knudtson et al. to revise the formulas to calculate AVR. Nowadays, only the six largest arterioles and six largest venules are used to calculate CRAE and CRVE. The "revised AVR" proved to have a greater power to detect small associations between AVR and systemic factors.[168]

Apart from vessel width, the structure of retinal vessels holds much prognostic information. First, the bifurcation angle, the angle between two daughter vessels at a vascular junction is associated with an optimal value. This optimal angle is determined by the surface, volume, drag or power and the asymmetry between the daughter vessels.[169-171] Reduced values indicate a less dense vascular network and have been associated with hypertension and increasing age.[172, 173] Second, vascular tortuosity is the ratio between the distance a vessel travels from A to B and the shortest distance between these points drawn by a straight line. Healthy blood vessels tend to be straight or show little curvature. However, pathological vessel remodelling may decrease the collagen and increase the elastin content in the vascular wall, increasing tortuosity. Increased arteriolar tortuosity around the optic disc is an early sign of disease.[157, 174] Third, the length:diameter ratio is the calculated length of a midpoint of a particular vascular bifurcation to the midpoint of the preceding bifurcation. It is expressed as a ratio to the diameter of the parent vessel at the bifurcation. Longer and/or thinner retinal arterioles are capable of greater pressure attenuation. This ratio provides information about retinal arteriolar narrowing and is increased in hypertension.[175, 176] The features discussed above are all based on single vessel width or shape. The overall pattern of the retinal vasculature also contains valuable information which can be extracted with fractal analysis. The fractal dimension indicates how thoroughly the retinal vascular network fills the 2D space of a fundus image. The normal retinal fractal dimension is approximately 1.7 and deviations from this number may indicate pathological increases or decreases in vessel density. In this way, significant biological changes can be elucidated in the early stages of disease.[177, 178]

Retinal abnormalities

Changes in vessel width or pattern are not the only features that may indicate (retinal) disease. The automated search for retinal abnormalities has been developed in diabetic retinopathy research. As diabetic retinopathy may cause blindness, the importance of a timely detection of these abnormalities cannot be overstated. Two classes of abnormalities are usually defined. The first class, (red lesions) consists of microaneurysms and small retinal haemorrhages. These were first detected in fluorescin angiograms due to their high contrast against the background.[179, 180] Current algorithms can

distinguish these abnormalities on colour fundus images using wavelet subbands that are template matched with healthy retinas. These specific algorithms do not detect normal retinal structures as the optic disc, fovea and retinal vessels, which may act as confounders. The second class consists of lesions that are brighter than the retinal background and include drusen, cotton-wool spots and lipoprotein exudates. These lesions are found in the presence of a range retinal and systemic diseases. Detection is based on comparable algorithms as used for red lesions.[181, 182] The current challenge is to correctly differentiate between different types of bright lesions as each kind of lesion is associated with a specific kind of disease (diagnostic importance) and treatment (patient management implications).

Retinal vessel caliber is associated with cardio- and cerebrovascular risk factors and outcomes

Fundus photography is being used in many population studies. With recent image analysis techniques, an objective quantitative analysis of changes in the retinal microcirculation can be detected. Since pathological changes in retinal blood vessels may reflect similar processes in the cerebral and coronary microcirculation, fundus photography has been used to investigate the associations between retinal microvascular changes/retinopathy and cardio- or cerebrovascular risk factors or endpoints in several large population cohorts, such as the Atherosclerosis Risk in Communities Study (ARIC), Beaver Dam Eye Study (BDES), Blue Mountains Eye Study (BMES), Cardiovascular Health Study (CHS), Rotterdam eye Study (RES) and Multi-Ethnic Study of Atherosclerosis (MESA), or in smaller studies that compared diseased persons with healthy controls.

A summary of these studies and their findings is presented in Table 1. Cardiovascular diseases, such as coronary heart disease and left ventricular remodelling, and cardiovascular mortality have been consistently associated with changes in retinal vessel caliber.[183 184 185, 186 187] Moreover, arteriolar narrowing and venular widening were predictors for future cardiovascular events.[184 188] The reported associations were mostly stronger in specific subgroups, for instance women and younger individuals, where microvascular contribution to cardiovascular disease was more prominent.[189 190, 191] Similar associations have been reported for cerebral morbidity and mortality. Changes in retinal vessels or retinopathy were associated with white matter lesions, cerebral infarcts and an increased risk of incident clinical stroke.[192-194 195]

Changes in retinal vascular caliber are also associated with traditional cardiovascular risk factors such as blood pressure/hypertension, obesity, inflammation and endothelial dysfunction, atherosclerosis and smoking. These risk factors are hallmarks of cardiovascular diseases that have a profound effect on microvascular structure and function.

Elevations in blood pressure can give rise to retinal microvascular abnormalities, arteriolar narrowing and venular widening.[196, 197 189, 198, 199 200, 201 202, 203] In a recent meta-analysis of five cross-sectional studies (19,633 adults), a 10 mm Hg increase in current mean arterial blood pressure was associated with a 3.07 µm decrease in retinal arteriolar diameter.[204] Retinal caliber not only reflects current blood pressure, but is also associated with past and future blood pressure. Lower AVR, arteriolar narrowing and venular widening were associated with past blood pressure levels, measured up to 10 years before retinal assessment.[189, 198, 205] In several longitudinal studies, retinal arteriolar narrowing preceded the onset of hypertension in normotensive individuals.[206-210] The strength of the association between retinal arteriolar narrowing and elevated blood pressure varies with age. The strongest

associations were found in the younger age categories[200] and/or in those with normal blood pressure.[211] Arteriosclerosis in older individuals progressively increases the rigidity of the blood vessels, that lose the ability to react adequately to blood pressure changes.[212]

Obesity has been associated with retinal narrowing and/or venular widening in adults[200, 213, 214 215 216]and in children.[217] Similar to blood pressure, retinal venular caliber at baseline predicted the incidence of obesity.[218] These studies indicate that the pathogenesis of weight gain also involves deleterious structural changes to the microvascular bed.[218]

Inflammation and endothelial dysfunction negatively affect the micro- and macrocirculation. Venular widening was consistently associated with systemic inflammatory diseases (obesity, metabolic syndrome, …) or with markers of inflammation (leukocyte count, interleukines) and endothelial dysfunction (impaired brachial flow-mediated dilation)[200, 213, 219-221 215 222] although not all studies report this latter association.[223]

Positive and null associations between retinal venular widening clinically established atherosclerosis have been reported.[150 220, 224 201] Retinal venular caliber has been associated with traditional risk factors for inflammation and atherosclerosis: higher total cholesterol, lower HDL-levels, higher leukocyte count, higher waist-to-hip ratio. This might hint at a similar mechanism underlying atherosclerosis and venular widening.[200] Moreover, retinal venular widening has been associated with intima-media thickness, a marker for subclinical atherosclerosis.[151 225]

The adverse macrovascular outcomes of smoking may be partially mediated by deleterious microvascular changes. Hence, smoking has been consistently linked to wider venules.[200, 201, 214, 221, 226, 227] These data are in agreement with clinical observations that show wider retinal venules in smoking. Moreover, these studies indicate that retinal venules of smokers cannot respond adequately to vasodilation stimuli.[228-230] This supports the endothelial dysfunction hypothesis as an underlying mechanism for venular widening.

Study Cohort	Year	N	Mean Age	Male	Design	Retinal measurement	Change or Quartile/quintile	… is associated with …
Cardiovascular morbidity and mortality								
Hypertensive patients[231]	1997	51	/	53%	cross-sectional	retinopathy	/	left ventricular remodeling
Hypertensive patients[232]	1998	174	51 (12)	50%	cross-sectional	retinopathy	/	left ventricular remodeling
ARIC[233]	2002	9648	59.5 (0.18)	45%	3 year follow-up	AVR	-0.08	37% increase in coronary heart disease
BDES[189]	2003	1611	61.3 (10.9)	51%	cross-sectional	arteriolar narrowing	lowest quintile of AVR	1.5 fold greater risk of cardiovascular disease related death
BMES[188]	2006	3340	65 (0.49)	44%	9 year follow-up	AVR	-0.079	1.5 fold greater risk of CHD death
						arteriolar narrowing	- 20 µm	1.5 fold greater risk of CHD death
						venular widening	+ 20 µm	1.3-2 fold greater risk of CHD death
CHS[195]	2006	1992	78.4 (4.3)	40%	5 year follow-up	arteriolar narrowing	lowest quintile of CRAE	2-fold increase in rate ratio for incident CHD
						venular widening	highest quartile of CRVE	3-fold increase in rate ratio for incident CHD
Cerebrovascular morbidity and mortality								
ARIC[234]	2002	1684	62.2 (4.5)	40%	cross-sectional	retinopathy	/	higher number of white matter lesions, clinical stroke
ARIC[235]	2006	1684	62.2 (4.5)	40%	cross-sectional	AVR	first quintile of AVR	MRI cerebral infarction
CHS[195]	2006	1992	78.4 (4.3)	40%	5 year follow-up	venular widening	highest quartile of CRVE	2.1-fold increase in incident stroke
Blood Pressure								
Current blood pressure								
ARIC[236]	1999	9648	59.5 (0.18)	45%	cross-sectional	AVR	-0.018	10 mm Hg increase in mean arterial blood pressure
BMES[205]	2004	2335	65 (0.49)	44%	cross-sectional	arteriolar narrowing	/	increase in systolic blood pressure
RES[237]	2004	5674	68 (8.2)	41%	cross-sectional	arteriolar narrowing	- 1.1 µm	10 mm Hg increase in systolic blood pressure
							- 2.1 µm	10 mm Hg increase in diastolic blood pressure
						AVR	-0.0035	10 mm Hg increase in systolic blood pressure
							-0.008	10 mm Hg increase in diastolic blood pressure
ARIC[201]	2008	8794	60 (10)	45%	cross-sectional	arteriolar narrowing	- 6.4 µm	15.3 mm Hg increase in mean arterial blood pressure
						venular widening	+ 2.6 µm	15.3 mm Hg increase in mean arterial blood pressure

BDES[238]	2012	4493	61.3 (10.9)	44%	cross-sectional	arteriolar narrowing	- 1.38 µm	5 mm Hg increase in mean arterial blood pressure
Past blood pressure								
ARIC	1999	9648	59.5 (0.18)	45%	cross-sectional	AVR	-0.01	10 mm Hg increase in past mean arterial blood pressure
BMES[205]	2004	2335	65 (0.49)	44%	5 year follow-up	arteriolar narrowing	/	increase in past systolic blood pressure
ARIC[201]	2008	8794	60 (10)	45%	cross-sectional	arteriolar narrowing	- 2.6 µm	15.3 mm Hg increase in past mean arterial blood pressure
						venular widening	+ 1.5 µm	15.3 mm Hg increase in past mean arterial blood pressure
BMES[238]	2012	4493	61.3 (10.9)	44%	5 year follow-up	arteriolar narrowing	- 0.67 µm	5 mm Hg increase in mean arterial blood pressure
Future blood pressure								
ARIC[239]	2004	5628	59.5 (0.18)	45%	3 year follow-up	AVR at baseline	lowest quintile of AVR	60% increased risk to develop hypertension
BDES[240]	2004	2451	61.3 (10.9)	44%	10 year follow-up	AVR at baseline	-0.07	30% increased risk to develop hypertension
BMES[241]	2004	1269	62.5	41%	5 year follow-up	arteriolar narrowing at baseline	/	2.6-fold increase in risk to develop hypertension
BMES[206]	2008	1952	62.5	41%	10 year follow-up	arteriolar narrowing at baseline	- 8.0 µm	10% increased risk of incident severe hypertension
Obesity								
BMES[242]	2006	3349	66.2 (9.8)	44%	cross-sectional	venular widening	highest quartile of CRVE	1.7 fold increase to be in highest quintile of BMI
					5 year follow-up	venular widening at baseline	highest quartile of CRVE	1.8 fold increase to become obese
MESA[215]	2006	5979	61.9 (10.1)	48%	cross-sectional	venular widening	+ 2.21 µm	5.4 kg/m² increase in BMI
ARIC[201]	2008	11265	59.8 (5.6)	44%	cross-sectional	arteriolar narrowing	- 0.7 µm	6.3 kg/m² increase in BMI
						venular widening	+ 0.6 µm	6.3 kg/m² increase in BMI
Inflammation and endothelial dysfunction								
ARIC[220]	2000	8772	59.5 (0.1)	45%	cross-sectional	AVR	lowest quartile of AVR	levels of fibrinogen and leukocyte count
RES[237]	2004	5674	68 (8.2)	41%	cross-sectional	venular widening	+ 2.9 µm	1.9 x 10^9 cells/L increase in white blood cell count
BDES[243]	2006	383	64 (0.5)	44%	cross-sectional	venular widening	/	CRP. IL-6. fibrinogen. serum amyloid A
MESA[215]	2006	5979	61.9 (10.1)	48%	cross-sectional	venular widening	+ 22.2 µm	inflammatory markers (hsCRP. fibrinogen. IL-6. sICAM-1. PAI-1)
MESA[244]	2010	2851	62.8 (9.8)	50%	cross-sectional	venular widening	+ 14 µm	0.25% lower brachial FMD
Atherosclerosis								

ARIC[220]	2000	5674	68 (8.2)	41%	cross-sectional	AVR	-0.004	0.15 mm increase in IMT
						AVR	-0.002	1 unit increase in carotid plaque score; 0.22 units increase in ankle-arm index
						AVR	-0.06	0.004 mm increase in intima media thickness; 0.002% increase in plaque score; 0.002 increase in ankle-arm index
						arteriolar narrowing	- 0.09 µm	0.15 mm increase in IMT
						venular widening	+ 0.04 µm	1 unit increase in carotid plaque score
						venular widening	/	higher total cholesterol. lower HDL-levels. higher leukocyte count. higher waist-to-hip ratio
Hoorn Study[223]	2006	256	71.5 (7)	52%	cross-sectional	venular widening	+ 27.1 µm	0.14 mm increase in IMT
Physical activity								
ARIC[245]	2010	12363	59.8 (5.6)	44%	cross-sectional	venular widening	highest quartile of CRVE	lower fitness
Australian Diabetes, Obesity and Lifestyle study[246]	2011	2024	55 (12)	49%	cross-sectional	venular widening	/	sedentary behavior
MESA[247]	2011	1492	6.7 (0.4)	51%	cross-sectional	arteriolar narrowing	- 1.53 µm	1h increase in sedentary behavior
Air pollution								
MESA[248]	2010	6233	64 (10)	52%	cross-sectional	arteriolar narrowing	- 0.8 µm	3 µg/m³ increase in long-term $PM_{2.5}$ concentration

Table 1 Associations between retinal vascular changes (CRAE, CRVE, AVR) and cardiovascular risk factors and outcomes.

Mechanisms of retinal vessel caliber change

The mechanism of arteriolar narrowing involves functional (endothelial dysfunction) and structural changes (vascular remodelling) and has been primarily investigated in hypertension. The autoregulatory properties of retinal arterioles will counteract an increase in blood pressure with myogenic vasoconstriction. This will decrease blood flow and perfusion pressure in the capillaries. This stage is characterized by general retinal arteriolar narrowing, without the presence of significant vascular remodelling.[249, 250] A sustained blood pressure elevation will initiate an arteriosclerotic phase: intimal thickening, tunica media hyperplasia and hyaline degradation will result in inward thickening and stiffening of the vessel wall.[250] These changes are manifested as diffuse and focal arteriolar narrowing and compression of the venules at their junctions (arteriovenous nicking). The arteriosclerotic phase is followed by an exudative phase, characterized by focal or generalized dilation. In this stage, the breakdown of the blood-retina barrier, necrosis of smooth muscle cells and endothelial cells will increase permeability of the retinal vessels and give rise to signs of severe retinopathy: exudation of blood (haemorrhages) and lipids (hard exudates).[249, 250]

The mechanism of retinal venular dilation is less understood. In several epidemiological studies, retinal venular widening was first observed in diseases and risk factors associated with active vascular inflammation and endothelial dysfunction (obesity, atherosclerosis and smoking).[213, 219, 227, 251] Activated leukocytes can activate the endothelium and/or disrupt the endothelial surface layer. This will increase intraluminal diameter and lead to observable venular widening.[200, 220]

AIR POLLUTION EXPOSURE, PHYSICAL (IN)ACTIVITY AND RETINAL MICROVASCULAR CHANGES: HYPOTHESIS AND OBJECTIVES

Air pollution

Air pollution might exert its cardiovascular health effects through the microcirculation. In epidemiological and experimental studies, air pollution exposure has been associated with endothelial dysfunction, which is an important step in the development of cardiovascular diseases. Endothelial dysfunction can lead to functional changes and structural alterations in the microcirculation. In this way, hypertension, atherosclerosis and macrovascular disease can be promoted. Detection of microvascular changes is possible with the use of fundus photography and retinal image analysis. Due to shared physiological and pathological mechanisms, retinal vessels may hold important prognostic information on cerebral and coronary vessels. Up to date, the association between air pollution exposure and the retinal microvasculature has only been investigated in the MESA cohort. Long- exposure to $PM_{2.5}$ was associated with retinal arteriolar narrowing and venular widening. An increase of 3 μg/m³ in long-term $PM_{2.5}$ concentrations was associated with 0.8 μm decrease in CRAE and a 0.9 μm increase in CRVE. These changes in retinal blood vessels were the equivalent of a 7-year increase in age.[248]

It is unknown whether short-term air pollution exposure is also associated with retinal microvascular responses. In a repeated measurements study design, the fundus of healthy study participants will be

photographed repeatedly. At the same time, air pollution data will be obtained from nearby monitoring stations that are representative for the study area or personal measurements. The static fundus images will be used to calculate the retinal vessel diameters. Subsequent fundus images can be used to study the changes in retinal vessel diameters associated with short-term air pollution exposure. Retinal vessel diameter depends on autoregulatory mechanisms and endothelial secretion of NO. Short-term air pollution exposure may promote oxidative stress and inflammation. These reactions can lower the bioavailability of NO and cause endothelial dysfunction. This might lead to retinal arteriolar narrowing and venular widening when air pollution levels increase and the opposite reactions when air pollution levels dwindle. We hypothesize that these retinal microvascular responses can be detected with repeated fundus photography.

Physical (in)activity

Physical inactivity, sedentary behaviour and low cardiorespiratory fitness, which are risk factors for cardiovascular health, are associated with changes in the microvascular structure.[216, 252, 253] Epidemiological studies have found that sedentary behaviour in children and adults is associated with retinal arteriolar narrowing[247] and venular widening. [254-256] In contrast, higher cardiovascular fitness was associated with wider retinal arterioles and higher AVR.[216]

Physical inactivity may promote microvascular dysfunction through changes in blood flow and shear stress rate. The consequent endothelial dysfunction may promote microvascular functional and structural changes. The deleterious consequences of physical inactivity need a long time to develop. However, experimental models such as bedrest induce a rapid vascular deconditioning, comparable to the vascular adaptations witnessed in physically inactive individuals. The supine position during bedrest will cause pressure loading of the retinal blood vessels and induce endothelial dysfunction. In this way, the myogenic autoregulatory properties and the role of NO of these vessels can be studied. The bedrest condition will be performed in a normoxic and a hypoxic environment. The hypoxic challenge will trigger metabolic autoregulatory vasodilation in the retinal blood vessels. In this experimental model, the combination of bedrest and the hypoxic environment will allow to assess the autoregulatory properties of retinal blood vessels. Pressure-loaded blood vessels (as seen in sedentary individuals) might not be able to adequately respond to a hypoxic stimulus. We hypothesize that these small retinal vascular changes can be visualized with repeated fundus photography.

Regular physical activity can positively influence retinal blood vessels.[216] It is known that acute isometric and dynamic exercise can induce reversible increases in retinal vessel diameters.[257, 258] It has been suggested that flow-mediated dilation causes these increases. However, it is unclear how the retinal microvasculature of cardiac patients responds to exercise and regular physical activity, as endothelial dysfunction and impaired flow-mediated dilation are often present in these patients. As cardiac rehabilitation programs can restore or improve endothelial function and flow-mediated dilation, retinal microvascular reactivity may be improved after completion of a rehabilitation program. We hypothesize that fundus photography can be used to assess retinal microvascular responses after a maximal endurance test in cardiac patients and that these responses might improve after completion of the rehabilitation program.

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Chapter 3

Retinal Microvascular Responses to Short-Term Changes in Particulate Air Pollution in Healthy Adults

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ABSTRACT

Introduction: The microcirculation plays an important role in the physiology of cardiovascular health. Air pollution is an independent risk factor for the development and progression of cardiovascular diseases, but the number of studies on the relation between air pollution and the microcirculation is limited. We examined the relationship between short-term changes in air pollution and microvascular changes.

Methods: We measured retinal microvasculature using fundus image analysis in a panel of 84 healthy adults (52% women) aged 22 to 63y between January and May 2012. Blood vessels were measured as Central Retinal Arteriolar/Venular Equivalent (CRAE/CRVE). The median number of measurements was 2 (range: 1-3). We used monitoring data on particulate air pollution (PM_{10}) and black carbon (BC). Mixed-effect models were used to estimate associations between CRAE/CRVE and exposure to PM_{10} and BC using various exposure windows.

Results: CRAE and CRVE were associated with PM_{10} and BC concentrations, averaged over 24 hours before the retinal examinations. Each 10-μg/m³ increase in PM_{10} was associated with a 0.93 μm decrease (95% CI: -1.42, -0.45; p=0.0003) in CRAE, and a 0.86-μm decrease (95% CI: -1.42, -0.30; p=0.004) in CRVE after adjustment for individual characteristics and time varying conditions such as ambient temperature. Each 1-μg/m³ increase in BC was associated with a 1.84 μm decrease (95% CI: -3.18, -0.51; p<0.001) in CRAE.

Conclusions: These findings suggest that the retinal microvasculature responds to short-term changes in air pollution levels. These results support a mechanistic pathway through which air pollution can act as a trigger of cardiovascular events at least in part through effects on the microvasculature.

INTRODUCTION

Exposure to ambient levels of air pollution increases the incidence of cardiovascular mortality and morbidity.[1, 2] Research indicates that different fractions of particulate air pollution contribute to the development of cardiovascular disease and provoke cardiovascular events.[3-5] PM_{10} (particles less than 10 µm in diameter) is a complex mixture of compounds including transition metals, sulfate and nitrate salts and black carbon.[6] Black carbon (BC) is a measure of traffic-related particles that are produced as a combustion by-product.

Although the microcirculation makes up the bulk of the circulatory system, its role in cardiovascular disease remains less clear than the influence of the macrocirculation.[7] There are two main theories about the significance of microvascular changes in the context of cardiovascular disease. First, microvascular changes could be an early marker for cardiovascular disease, secondary to the disease process.[8] Alternatively, microvascular changes could be a primary cause for the development of cardiovascular changes.[9-11]. Central Retinal Arteriolar Equivalent is a predictor of future hypertension[11]. Recent evidence suggests an association between air pollution exposures and markers of microvascular effects.[12-14]

Changes in the microcirculation can be explored non-invasively by studying retinal blood vessels that are visualized in fundus images.[15, 16] The retinal blood vessels have anatomical and physiological features that are comparable with the coronary circulation. Pathologies of the retinal blood vessels parallel changes in the coronary micro- and macrocirculation.[17-19] Retinal vessel caliber is an independent predictor for cardiovascular diseases, with arterial narrowing acting as a marker for arteriolar damage and predicting hypertension, and venular widening has been associated with inflammation, endothelial dysfunction, and markers of atherosclerosis.[8, 15, 17]

Adar and coworkers (2010) were the first to associate exposure to air pollution with arteriolar narrowing. Among 4,607 participants of the Multi-Ethnic Study of Atherosclerosis (MESA), Central Retinal Arteriolar Equivalent (CRAE) narrowed by 0.8 µm (95% CI: -1.1, -0.5) in association with an interquartile increase in long-term exposure (3 µg/m³ $PM_{2.5}$ during the 2 years preceding the clinical exam). The magnitude of this change corresponded to the change in CRAE associated with a 7-year increase in age in their study population. In a cross-sectional analysis investigating exposure on the previous day, CRAE narrowed by 0.4 µm (95% CI: -0.8, -0.04) in association with a 9-µg/m³ increase in $PM_{2.5}$.[12]

Here, we report on a study of short-term air pollution exposures and microvascular changes in healthy adults (age 22 – 63 years) using a repeated measures design.

METHODS

Study population

The study was conducted in Belgium between January 2012 and May 2012 and included employees of the Flemish Institute for Technological Research (VITO). A total of 183 persons were contacted and 84 (46%) agreed to participate in the study. Participants were 22 to 63 years old. All VITO employees undergo an annual clinical examination and all study participants were free of clinical cardiovascular diseases and diabetes before and during the study period.

Participants were not asked to fast before study visits and their post-prandial status was not recorded. On each study day, participants completed a questionnaire on their current medical history and smoking status, as well as on the use of alcohol, coffee and specific medications, and time spent in traffic during the 24 hours prior to the clinical visit. 84 persons participated in our study, of which 32 (38%) completed one visit, 7 (8%) completed two visits, and 45 (54%) participated in all three clinical visits. The visits were scheduled between 9 am and 5 pm and took place on the campus of the Flemish Institute for Technological Research. The visits were on average 16 days apart (range: 14 to 18 days). The clinical visits were scheduled on the same time of day [mean difference 1.5 hour (range: 0.2 to 2.2 hours)]. Participants gave their written informed consent. The Ethics Board of Hasselt University and University Hospital Antwerp approved the study.

Retinal photography and grading

The fundus of the right eye of each participant was photographed using a Canon 45° 6.3 megapixel digital non-mydriatic retinal camera (Hospithera, Brussels, Belgium). Participant characteristics were masked for the trained grader before review and analysis of the retinal images. IVAN retinal image analysis software was used to measure retinal vessel diameters according to previously reported protocols [20-22]. Diameters were summarized as the Central Retinal Arteriolar Equivalent (CRAE) and Central Retinal Venular Equivalent (CRVE). The equivalents represent a summary of vessel diameters within an area equal to 0.5-1 disc diameters from the optic disc margin.

Cardiovascular parameters

Systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate were measured with an automated device (Stabilograph, Stolberg, Germany), according to the guidelines of the European Society of Hypertension [23]. After the subjects had rested for 5 min, the blood pressure and heart rate were measured five times consecutively. The average of the last three measurements was calculated and used in data analyses. These cardiovascular parameters were only measured during the second and third clinical examination (n = 59).

Outdoor temperature and barometric pressure

The 24-hour mean outdoor temperature and barometric pressure measured at the nearby Retie meteorological station (N° 06464; 51°13'50.29" N, 5°3'7.64" E) were obtained from the Belgian Royal Meteorological Institute.

Air pollution levels: exposure assignment

Ambient air pollution levels were measured at a nearby official monitoring station in Dessel (N° 42N016; 51°14'2.92" N, 5° 9'45.58" E) and the data were obtained from the Flemish Environmental Agency. The distance from the monitoring station to the campus of the Flemish Institute for Technological Research is between 5.4 and 9.5 km. The station monitors ambient concentrations of a range of air pollutants, including PM_{10} and black carbon, every 30 min. PM_{10} was measured with beta-absorption, whereas black carbon was measured using reflectometry and transmission techniques.

For each participant, average air pollution concentrations were determined for the 2, 4, 6, and 24 hours before the retinal exam (lag 2h, 4h, 6h, and 24h, respectively). Air pollution levels were also assigned as a 24-hour average for the previous calendar day (lag 1d) and 48-hour average for the two calendar days preceding the retinal exam (lag 2d).

Statistical analysis

We performed pollutant-specific exposure-response analyses using mixed models that included random effects for each participant across the clinical examinations (SAS version 9.2, SAS Institute Inc, Cary, NC). This method allows each subject to serve as his or her own control over time and eliminates within-subject confounding by personal characteristics that do not change over time. Associations with exposures over different lag periods (lag 2h to lag 2d) were estimated in separate models. We did descriptive analyses to identify potential predictors of the markers of the microcirculation that could modify or confound the association between the microcirculation and air pollution exposure. All analyses were adjusted for gender, age, body mass index (BMI), smoking status, alcohol and coffee consumption during the 24 hours prior to the examination, day of the week, time of day, outdoor temperature, and barometric pressure.

In a series of sensitivity analyses, we also adjusted for blood pressure (SBP, DBP) and heart rate in a subset of 59 participants, and adjusted for fellow vessel diameter (i.e., for CRVE in models of CRAE, and vice versa). In addition, we repeated analyses with smokers (n=3) and individuals currently using medication (n=2) excluded. To explore the shape of the dose-response curves we estimated associations between average PM_{10}-concentrations over different lags and the microcirculation markers estimated using unadjusted models with exposures modeled as restricted cubic splines with 5 knots at the 5th, 25th, 50th, 75th and 95th percentiles. Finally, differences in between- and within-subject air pollution effects could be possible. Therefore, we fitted separate mixed models that included terms for within- and between- subject exposure effects in addition to the overall model. All tests were two-sided.

RESULTS

Characteristics of the study population are summarized in Table 1. 52% of included participants are women. The population had a mean age of 37 ± 9 years. All participants reported that they were free of diabetes and cardiovascular disease, though one used medication for blood pressure control (an angiotensin receptor blocker) and one used cholesterol lowering medication (a statin). Three participants were active smokers. All participants had a university or college degree. Short-term air pollution concentrations were highly variable during the study. PM_{10} concentrations (lag 24h) ranged from 9.7 to 117.7 µg/m³, with interquartile ranges (IQR) of 9.6, 39.1, and 3.7 µg/m³ for the first, second, and third visits, respectively. BC concentrations ranged from 0.37 to 6.99 µg/m³, with IQRs of 0.94, 5.64, and 0.29 µg/m³ for the first, second, and third visits. During the 5-month study period, the daily outdoor temperature ranged from -6.8 to 20.2 °C and the barometric pressure from 993 to 1031 hPa. No within-person correlation was observed for the different exposure periods. Seventy-four participants reported that they spent on average 84 min (± 20) in traffic driving a car during the previous 24 hours. Of these 74 participants, 24 participants reported driving an average of 8 min (± 22) in congested traffic. Twenty-seven participants reported riding a bicycle in traffic (mean duration 9 min ± 20).

Characteristics	Mean (+/-SD) or Number (%)
Personal characteristics	
Age (y)	37 (9)
Female (%)	44 (52%)
Race/ethnicity	
Caucasian (%)	83 (99%)
Asian (%)	1 (1%)
Smoking status	
Current	3 (4%)
General health characteristics	
Body Mass Index (kg/m²)	23 (3)
Systolic Blood Pressurea (mm Hg)	126 (11)
Diastolic Blood Pressurea (mm Hg)	75 (8)
Heart ratea (bpm)	72 (13)
Participation in traffic on day of examination	
Persons using a car	74 (88%)
Persons using a car in congested traffic	24 (30%)
Persons riding a bike or walking in traffic	27 (32%)

Table 1 Descriptive statistics of the study population

Predictors and correlates of CRAE and CRVE

Central Retinal Arteriolar Equivalent (CRAE) and Central Retinal Venular Equivalent (CRVE) averaged 136 µm (± 14 µm) and 189 µm (± 18 µm), respectively. The CRAE/CRVE ratio was 0.722 (± 0.067). CRAE did not differ significantly between men and women (p=0.95), but decreased by 0.59 µm (95% CI: -0.94, -0.23; p=0.0015) in association with a 1-year increase in age. BMI (p=0.97), alcohol use (p=0.58), coffee consumption (p=0.28), outdoor temperature (p=0.58), and barometric pressure (p=0.97) were not significant predictors of CRAE, nor was time of day (p=0.34). A 10-min increase in the amount of time spent in driving a car was associated with a 0.14 µm decrease (95% CI: -0.35, 0.07; p=0.18) in CRAE. Finally, a 1-µm increase in CRVE was associated with a 0.40 µm increase in CRAE (95% CI: 0.30, 0.51; p<0.0001). Outdoor temperature was the only statistically significant predictor of CRVE (0.98 µm decrease with a 1-°C increase in outdoor temperature, 95% CI: -1.33, -0.45; p=0.0001).

CRAE		
Exposure Time (lags)	**Model 1**	**Model 2**
PM_{10} (for each 10 µg/m³ increase)		
2 hours	-0.62 (-1.13;-0.11)[a]	-0.38 (-0.85; 0.08)
4 hours	-0.67 (-1.22; -0.13)[a]	-0.41 (-0.90; 0.09)
6 hours	-0.75 (-1.31; -0.18)[a]	-0.43 (-0.94; 0.09)
24 hours	-0.93 (-1.42; -0.45)[c]	-0.57 (-1.01; -0.12)[a]
2 days	-0.60 (-1.18; -0.02)[a]	-0.15 (-0.70; 0.40)
BC (for each 1 µg/m³ increase)		
2 hours	0.24 (-0.57; 1.05)	-0.03 (-0.75; 0.69)
4 hours	0.38 (-0.49; 1.26)	0.03 (-0.75; 0.82)
6 hours	0.52 (-0.47; 1.51)	0.10 (-0.79; 0.99)
24 hours	-1.84 (-3.18; -0.51)[b]	-1.54 (-2.69; -0.39)[a]
2 days	-0.21 (-1.13; 0.71)	-0.16 (-1.00; 0.68)

Table 2 Change in central retinal arteriolar equivalent (CRAE) in association with particulate air pollution (PM_{10}) and black carbon (BC). Estimates express the change (95% Confidence Intervals) in the retinal arteriolar blood vessels associated with a 10 µg/m³ increase in PM_{10} or a 1 µg/m³ increase in BC. In model 1, estimates were adjusted for: gender, age, BMI, smoking habits, alcohol and coffee consumption 24 hours prior to examination, time of the day and day of the week, outdoor temperature and barometric pressure. Model 2 also includes, in addition to covariates in model 1, central retinal venular equivalent. Statistical differences are expressed as: [a] <0.05, [b] <0.01, [c] <0.001. Both models include 84 persons; 25 had one measurement, 14 had 2 measurements and 45 had 3 measurements.

CRVE		
Exposure Time (lags)	**Model 1**	**Model 2**
PM_{10} (for each 10 µg/m³ increase)		
2 hours	-0.62 (-1.28; 0.04)	-0.39 (-1.00; 0.22)
4 hours	-0.77 (-1.48; -0.05)[a]	-0.49 (-1.15; 0.17)
6 hours	-0.93 (-1.67; -0.17)[a]	-0.60 (-1.28; 0.09)
24 hours	-0.86 (-1.42; -0.30)[b]	-0.60 (-1.26; 0.07)
2 days	-0.05 (-0.85; 0.75)	-0.84 (-1.61; -0.08)[a]
BC (for each 1 µg/m³ increase)		
2 hours	0.46 (-0.65; 1.57)	0.29 (-0.71; 1.31)
4 hours	0.52 (-0.68; 1.73)	0.30 (-0.80; 1.40)
6 hours	0.47 (-0.87; 1.80)	0.22 (-1.01; 1.44)
24 hours	-1.31 (-2.67; 0.07)	-0.04 (-1.77; 1.70)
2 days	0.10 (-1.36; 1.57)	-0.25 (-1.42; 0.92)

Table 3 Change in central retinal venular equivalent (CRVE) in association with particulate air pollution (PM_{10}) and black carbon (BC). Estimates express the change (95% Confidence Intervals) in the retinal venular blood vessels associated with a 10 µg/m³ increase in PM_{10} or a 1 µg/m³ increase in BC. In model 1, estimates were adjusted for: gender, age, BMI, smoking habits, alcohol and coffee consumption 24 hours prior to examination, time of the day and day of the week, outdoor temperature and barometric pressure. Model 2 also includes, in addition to covariates in model 1, central retinal arteriolar equivalent. Statistical differences are expressed as: [a] <0.05, [b] <0.01, [c] <0.001. Both models include 84 persons; 25 had one measurement, 14 had 2 measurements and 45 had 3 measurements.

Microcirculatory markers in association with changes in short-term air pollution

Unadjusted models of associations between CRAE and PM_{10} modeled using restricted cubic splines did not indicate a threshold effect (Figure 1). An increase in PM_{10} within the low concentration ranges (<30 µg/m³) was associated with a decrease in CRAE for lag 1d and lag 2d. Studying the shape of the association showed no threshold effect at higher concentrations and a linear shape (at lag 24h from 30 µg/m³ onwards) over the full exposure range (Figure 1).

After adjustment for gender, age, BMI, smoking, alcohol and coffee consumption 24 hours prior to the examination, time of the day, day of the week, 24-hour mean outdoor temperature and barometric pressure, CRAE was associated inversely with the PM_{10} and BC concentration in the hours before and the days before the clinical examination (Table 2). Each 10-µg/m³ increase in average PM_{10} during the previous 24 hours was associated with a 0.93 µm decrease (95% CI: -1.42, -0.45; p=0.0003) in CRAE (Table 2, model 1). Significant negative associations were also estimated between CRAE and average PM_{10} over shorter exposure windows, and for PM_{10} averaged over the previous 2 days. A 1-µg/m³ increase in BC during the previous 24 hours also was negatively associated with CRAE (-1.84 µm; 95% CI: -3.18, -0.51; p=0.008), but associations with shorter and longer exposure periods were not significant (Table 2, model 1). All associations with CRAE moved toward the null when adjusted for CRVE in addition to the other covariates (Table 2, model 2) but statistically significant negative associations persisted for 24h average exposures to both PM_{10} and BC.

CRVE was negatively associated with a 10-µg/m³ increase in PM_{10} during the previous 24 hours (-0.86 µm; 95% CI: -1.42, -0.30; p=0.004) and with PM_{10} exposure during other lag periods (Table 3, model 1). A 1-µg/m³ increase in BC during the previous 24h was also negatively associated with CRVE, though the association was not significant (-1.18 µm; 95% CI: -3.11, 0.75; p=0.23). Most associations moved closer to the null after adjustment for CRAE.

Sensitivity analyses

We did not find statistically significant associations between PM_{10} or BC and blood pressure components (systolic blood pressure (SBP), diastolic blood pressure (DBP), or pulse pressure (PP)) in the subset of 59 participants with blood pressure data (Supplemental Material, Table S1). When we adjusted for SBP, DBP, and heart rate, in addition to model 1 covariates and CRAE or CRVE, only the association between 24h PM_{10} and CRAE was significant (-0.50 µm; 95% CI: -0.92, -0.08; p=0.005) though a 1-µg/m³ increase in 24h BC was also negatively associated with CRAE (-1.08 µm; 95% CI: -2.21, 0.04; p=0.059) (Supplemental Material, Table S2). No significant associations between CRVE and air pollution indicators were estimated based on this model.

Associations between CRAE and 24h average PM_{10} and BC persisted when we also adjusted for time spent in traffic, and when we excluded the three smokers and two participants on anti-hypertensive and/or cholesterol medication (data not shown). The negative associations with 24h PM_{10} and BC were also confirmed when we excluded the 32 participants with only one CRAE measurement (n=52) [estimated mean decreases of 0.76 µm (95% CI: -1.32, -0.20; p=0.01) and 1.37 µm (95% CI: -2.90, 0.15; p=0.07) for a 10-µg/m³ increase in 24h PM_{10} and a 1-µg/m³ increase in BC, respectively]. Associations were of approximately the same magnitude (though no longer significant) when data from the 2nd set of study

visits, which took place during a time of relatively high PM_{10} and BC concentrations, were excluded (data not shown).

Finally, we ran models to differentiate between the within- and between-subject effects. Our overall estimates for PM_{10} were driven by the within-subject effects. Within-subject effect estimates indicated that each 10-µg/m³ increase in 24h PM_{10} was associated with a 0.66 µm decrease in mean CRAE (95% CI: -1.02, -0.30; p=0.0005) and each 1-µg/m³ increase in 24h BC was associated with a 1.08 µm decrease in CRAE (95% CI: -2.02, -0.13; p=0.03) (Supplemental Material, Table S3). Corresponding estimates for between-subject effects were -1.34 (95% CI: -2.82, 0.13; p=0.07) and -3.68 (95% CI: -6.33, -1.02; p=0.007), respectively.

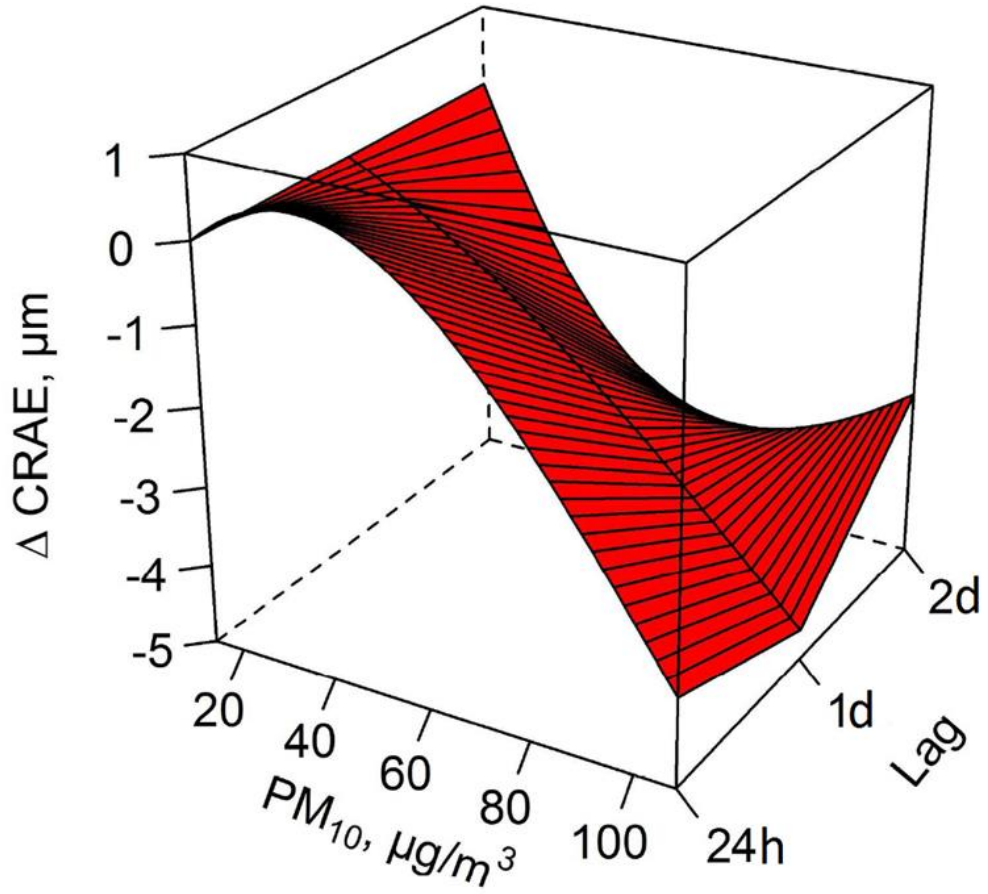

Figure 1 Microvascular responses in association with short-term changes in air pollution. Unadjusted analysis for change in Central Retinal Arteriolar Equivalent (CRAE) in association with PM_{10}. The effect was estimated using restricted cubic splines with 5 knots located at the 5th, 25th, 50th, 75th and 95th percentile for exposures on day of the examination using average exposure 24 hours before the clinical measurements (lag 24 hours), and on the 24 hour averages of the day before (lag 1) and two days before (lag 2).

DISCUSSION

We found a decrease in CRAE or Central Retinal Arteriolar Equivalent in association with exposure to PM_{10} and BC in a panel of healthy adults. These results remained significant after adjustment for gender, age, BMI, systolic and diastolic blood pressure or any of the other covariates studied. Arteriolar narrowing is an independent predictor of risk of myocardial infarction, hypertension, and cardiovascular mortality.[24-27] Other authors have reported an association between blood pressure and acute changes in air pollution.[28] Despite the decrease in retinal arteriolar vessel diameter, we did not observe statistically significant associations between PM_{10} or BC and blood pressure in the subset of participants with blood pressure data. We propose three explanations for this lack of association in our study. First, blood pressure is a highly variable phenotype, which is regulated by several control mechanisms counteracting changes in vessel diameter.[29] This study might not have sufficient power to detect such an effect. Second, the small vasoconstriction in the retinal blood vessels might not change overall peripheral resistance, thus blood pressure levels remain normal. Third, microvascular changes can be a cause or a consequence of elevated blood pressure. In our healthy population, air pollution exposure was associated with microvascular changes after adjustment for blood pressure. The microvasculature might rather be a target for primary changes that might eventually result in elevated blood pressure rather than vice versa. This is in agreement with the hypothesis that microvascular changes can be a primary cause for the development of cardiovascular changes.[9-11] In another study, inhalation of air pollution was associated with acute vasoconstriction of the forearm conduit artery without changes in systemic blood pressure[29].

Both fellow vessel diameter and blood pressure components are known to influence the microvascular changes in the retina.[25, 27] The effect estimates were attenuated by adjusting for fellow vessel diameter (i.e., including CRVE in models of associations between the exposures and CRAE, and vice versa) (Table 2 and Table 3, model 2), and much less by blood pressure (Supplemental Material, Table S2). Additional research is needed to clarify the relation between the pollutants, blood pressure and CRAE or CRVE.

It is likely that both vessel diameters are affected by an identical mechanism and respond in the same way.[30, 31] Due to their proximity, these blood vessels could interact by exchanging biologically active agents.[32] A model that accounts for fellow vessel diameter represents the independent effects of air pollution on both vessels (CRAE/CRVE), but due to their correlation over-adjustment cannot be excluded.

Exposure to air pollution has been associated with markers of pulmonary inflammation, which can cause a low-grade, systemic inflammation.[33, 34] Inflammation has been linked with endothelial dysfunction.[35] The effects of the systemic inflammation reaction may take some time to affect the retinal blood vessels. We hypothesize that inflammatory responses may alter the activity of the endothelium and initiate endothelial dysfunction, which may result in the narrowing of the retinal arterioles even up to several hours after exposure. Given the high variation in ambient air pollution levels, with intermittent peak episodes, the microvasculature is constantly adapting to a changing environment. Our findings suggest that this might occur very fast, even within 24 hours. In our first model, exposure to PM_{10} during all the hourly exposure windows was inversely associated with CRAE.

To our knowledge, only Adar et al. (2010) have previously published a study of short-term effects of air pollution on the human retinal microvasculature. The microvascular changes reported in our study complement those found by Adar and coworkers, who reported changes in the retinal microcirculation associated with long-term exposure (averaged over the previous 2 years) and short-term exposure (averaged over the previous day) in a cross-sectional analysis using the MESA cohort. Assuming that 9 $\mu g/m^3$ $PM_{2.5}$ corresponds to 12.9 $\mu g/m^3$ PM_{10}, the short-term cross-sectional association reported by Adar

et al. (-0.4 µm; 95% CI: -0.8, -0.04) per 9µg/m³ increase in average $PM_{2.5}$ on the previous day, is smaller than our estimate based on repeated measurements (-1.20 µm; 95% CI: -1.61, -0.61).[5] The effect size reported in our study may be larger than the one reported for the MESA cohort because our study population was exposed to greater variation in PM_{10} and BC concentrations. Furthermore, our study population consisted of young, healthy people with the same socio-economic status, in contrast with the much older and more diverse MESA cohort. In theory, arteriolar narrowing in response to air pollution in healthy people might be more pronounced than in susceptible people. A healthy microvasculature may respond better to changing conditions. This healthy response could result in bigger microvascular changes, whereas the response in susceptible people or people at risk might be compromised due to the already affected microvasculature.

Our results are consistent with previously reported health effects of air pollution. Toxicological studies have revealed that short-term exposure to peak levels of air pollutants is associated with microvascular responses. Animal studies conducted by Nurkiewicz et al. demonstrated that exposure to (ultrafine) particulate matter induced oxidative stress that led to eNOS-uncoupling and reduced bioavailability of the vasodilator NO.[36-38] In addition controlled exposure studies of humans have reported evidence of impaired macrovascular endothelial function in response to diesel exhaust.[13, 14]

Existing evidence suggests that air pollution is able to trigger an acute autonomic imbalance, favoring sympathetic nerve activity to the smooth muscles surrounding blood vessels.[39] Increased sympathetic activity causes smooth muscle contraction and thus vasoconstriction. Retinal blood vessels lack functional sympathetic innervations, therefore, autonomic imbalance is not likely to be the primal cause of retinal arteriolar vasoconstriction.[40] This might also explain why microvascular changes were more pronounced for the 24 hours exposure window than for the shorter lags.

Previously reported experiments on forearm conduit arteries allow assessing endothelial function, but the retinal blood vessels share more similarities in development and anatomy with the microvasculature of the heart, lungs and the brain.[41] Therefore, changes in retinal blood vessels may be related to changes in the systemic microcirculation.

Our findings may not be generalizable to the adult population as a whole. Subsequent research should therefore aim at confirming the observations in larger and more diverse populations. In addition, it would be informative to study populations that may be more susceptible to microvascular effects of air pollutants due to underlying pathologies that promote chronic inflammation. Diabetics, for example, have been shown to be a vulnerable group for the effects of air pollution.[42]

We cannot exclude some exposure misclassification. Measurements from a monitoring station close to the study site were used to estimate exposures. However, participants may have been exposed to different BC concentrations at their place of residence or while commuting.[43] The amount of time spent driving in traffic, as determined from the questionnaire, was negatively associated with arteriolar diameter, though the association was not statistically significant. Ideally, personal measurements of BC should be utilized in future studies.

The key finding of our repeated measurements study in a panel of healthy adults was that an acute narrowing of retinal arterial vessels, a marker for arteriolar damage, was associated with particulate matter air pollution. Based on our analysis, the estimated effect on CRAE, associated with a 10-µg/m³ increase in average PM_{10} during the 24 hours before the retinal examination was equivalent to the change in CRAE associated with a 1.5-year increase in age. This microvascular response to air pollution might contribute to the development or progression of cardiovascular diseases and complications, as seen in epidemiological studies. Our findings add new evidence to the cardiovascular health effects of short-term

exposure to air pollution in healthy people and suggest a mechanistic pathway through which air pollution can act as a trigger of cardiovascular events at least in part through effects on the microvasculature.

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Chapter 4

miRNA expression profiles and retinal blood vessel calibers are associated with short-term air pollution exposure

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Submitted

ABSTRACT

Introduction: Air pollution, a risk factor for cardiovascular diseases, can exert its effects through the microcirculation. Short-term PM_{10} exposure has been associated with changes in retinal blood vessels, but the underlying mechanism remains unresolved. microRNAs are key regulators of complex biological processes in cardiovascular health and disease whose expression can be affected by air pollution exposure. Studies investigating the effect of ambient air pollution exposure on miRNA expression in combination with an assessment of the microvasculature do not exist.

Methods: 50 healthy adults (50% women, 23-58 years old) were examined once a month from December 2014 until April 2015 in Flanders (Belgium). During the study visits fundus photos and venous blood samples were collected. PM_{10} data were obtained from a nearby monitoring station. Image analysis was used to calculate the width of retinal blood vessels, represented as the Central Retinal Arteriolar/Venular Equivalent (CRAE/CRVE). Total miRNA was isolated from blood and the expression of miR-21, -146a, -222 were measured using quantitative real-time PCR. Mixed models were used for statistical analysis.

Results: Each short-term increase of 10 µg/m³ PM_{10} during the 24 hours preceding the study visit was associated with a 0.58 µm decrease (95% CI: -1.16, -0.0005; p=0.056) in CRAE, a 0.99 µm increase (95% CI: 0.18, 1.80; p=0.021) in CRVE, a 6.6% decrease (95% CI: -11.07, -2.17; p=0.0038) in miR-21 expression and a 6.7% decrease (95% CI: -10.70, -2.75; p=0.0012) in miR-222 expression. Moreover, miRNA expression was associated with CRAE and CRVE. Each 10% increase in miR-21 and miR-222 expression was associated with respectively a 0.14 µm increase (95% CI: 0.0060, 0.24; p=0.046) in CRAE and a 0.28 µm decrease (95% CI: -0.50, -0.062; p=0.016) in CRVE. These associations were also found in exposure windows ranging from 2 hours to 1 week.

Conclusion: PM_{10} exposure was associated with retinal arteriolar narrowing and venular widening. PM_{10} exposure affected miRNAs involved in the gene regulation of inflammation and oxidative stress. These molecular changes may be an underlying mechanism to explain the association between PM_{10} and retinal vessel calibers, a proxy for microvascular health.

INTRODUCTION

Chronic exposure to increased levels of particulate matter air pollution (PM) is associated with adverse health effects.[1, 2] Epidemiological studies proof that acute peaks in ambient PM levels are rapidly followed by increased hospitalization rate,[3] and increased deaths,[4] both predominantly caused by cardiovascular events. PM can exert its effects via the activation of inflammatory pathways, oxidative stress and enhanced coagulation.[5-7] The large surface area of the microvasculature makes it prone to these processes that may lead to microvascular dysfunction and structural changes.[8] Therefore, microvascular changes can be an important physiological process on the trajectory of cardiovascular disease development.[9, 10]

The retinal blood vessels share anatomical and physiological features with the coronary blood vessels and non-invasive imaging of the retinal vessels can be used to assess the physiological status of the systemic microvasculature.[11] Retinal vessel caliber is an independent risk factor for cardiovascular disease. Arteriolar narrowing and venular widening are associated with an increased risk of hypertension.[12] In addition, retinal venular widening has been associated with inflammation, endothelial dysfunction and markers of atherosclerosis.[13-15] Adar et al. (2010) found an association between long-term $PM_{2.5}$ (particulate matter with a diameter equal to or smaller than 2.5 μm) exposure and retinal arteriolar narrowing and venular widening.[16] We have shown that short-term exposures to PM_{10} (particulate matter with a diameter equal to or smaller than 10 μm) and exposure to black carbon, a major component of soot, were associated with retinal arteriolar narrowing.[17] These studies suggest that the retinal microcirculation might be an intermediate pathway to analyse the association between air pollution exposure and cardiovascular disease development.

The study of the molecular events that underlie the microvascular function in health and disease is complex and the research field is only emerging. MicroRNAs (miRNAs) are small, non-coding, RNA molecules which repress target gene expression by translational inhibition or mRNA degradation. Individual miRNAs have been shown to regulate the expression of multiple genes and miRNAs have been identified as key regulators of complex biological processes linked to cardiovascular functions and pathologies.[18] The field of miRNA analysis to investigate the molecular pathways that link environmental exposures to health outcomes is growing. Changes in microRNA expression have been associated with exposure to PM, diesel particles or carbon black nanoparticles in *in vitro* or *in vivo* studies in animals and humans.[19-24] Vrijens et al. (2015) have reviewed several miRNAs that are differentially expressed as a result of smoking and air pollution exposure.[25] Interestingly, we identified at least 3 miRNAs (mirR-21, miR-146a and miR-222) that play an important role in cardiovascular processes and that are changed in expression after air pollution exposure.[18, 25] These miRNAs are involved in oxidative stress, inflammatory processes and vascular remodelling. Moreover, these miRNAs play a role in endothelial dysfunction and atherosclerosis. miR-21 plays a role in endothelial cell function and is a key switch in controlling pro- and anti- inflammatory responses.[26, 27] miR-222 regulates leukocyte adhesion and vascular remodelling.[28] miR-146a is involved in the innate immune response and is known to activate the pro-inflammatory NF-kB.[29]

In the current study, we examined the association between short-term ambient PM exposure, retinal microvasculature and miRNA expression in a panel of 50 healthy individuals using a repeated measurements study design over a 5-month period. Our objective was to study whether air pollution-induced changes in miRNA expression might be a potential pathway underlying the association between microvascular changes and air pollution exposure.

MATERIAL AND METHODS

Study Population

The study was conducted in Flanders, Belgium from December 2014 until April 2015. We recruited fifty participants between 23 and 58 years old. Participants reported to be free of cardiovascular diseases and diabetes before and during the study period. All participants had a comparable socio-economic situation. Forty (80%) persons participated in five visits and 10 (20%) persons had four visits. The visits were scheduled between 9 am and 5 pm. The visits were on average 30 days apart (range: 21 to 40 days). The clinical visits were scheduled on the same time of day [mean difference 0.5 hour (range: 0.1 to 5.5 hours)]. On each study day, persons completed a questionnaire to collect information on their weight and height, recent medical history, recent use of medication, alcohol and coffee use during the 24 hours preceding the study visit. Participants were asked which medication they had used during the 24 hours prior to the clinical visit. Retinal images, blood pressure and a 3 mL venous blood sample for miRNA analysis were collected during the clinical visit. Participants did not fast before the study visits and post-prandial status was not recorded. Participants gave their written informed consent. The Ethics Board of Hasselt University and University Hospital Antwerp approved the study.

Retinal Vessel Analysis

The fundus of the right and left eye of each participant were photographed twice using a Canon 45° 6.3 megapixel digital non-mydriatic retinal camera (Hospithera, Brussels, Belgium). Photographs were graded using retinal image analysis software developed by DCI Labs (Keerbergen, Belgium, www.dcilabs.com) and VITO. The calculation of Central Retinal Arteriolar Equivalent (CRAE) and Central Retinal Venular Equivalent (CRVE) was based on previously reported protocols.[30] The equivalents represent a summary of vessel diameters within an area equal to 0.5-1 disc diameter from the optic disc margin. Average CRAE and CRVE values were calculated based on the four images taken during each study visit. Participant characteristics were masked for the trained grader before review and analysis of the retinal images.

Determination of Blood Pressure

Systolic blood pressure, diastolic blood pressure and heart rate were measured with an automated device (Stabilograph, Stolberg, Germany), according to the guidelines of the European Society of Hypertension.[31] After the subjects had rested for 5 min, the blood pressure and heart rate were measured five times consecutively. The average of the last three measurements was calculated and used in the analyses.

Particulate Matter Exposure

Ambient air pollution levels were measured at nearby official monitoring stations in Dessel and Hasselt (minimal distance = 2.5 km, maximal distance = 10.5 km, average distance = 7.5 km). These data were obtained from the Flemish Environmental Agency. The stations measure every half hour the ambient concentrations of PM_{10} with beta-absorption. Air pollution levels were calculated as the average exposure during 2 hours (lag 2h), 4 hours (lag 4h), 6 hours (lag 6h), 24 hour (lag 24h), 48 hours (lag 48h) and 1 week (lag 1 week) before each of the clinical visits. In addition, the 24-hour mean outdoor temperature,

humidity and barometric pressure of the nearby meteorological stations of Retie and Hasselt were obtained from the Belgian Royal Meteorological Institute.

miRNA Analysis

Total RNA was extracted from venous blood collected in Tempus tubes (Fisher Scientific, Aalst, Belgium) using the Preserved Blood RNA purification kit 1 (Norgen Biotek Corp, Thorold, Canada). After purity and concentration were determined with NanoDrop, quantitative real-time RT-PCR was performed to detect and quantify miRNA expression. TaqMan MicroRNA Reverse Transcription Kit was used for the reverse transcription step. qRT-PCR was performed using TaqMan microRNA assays for miR-21-5p, miR-146a-5p and miR-222-3p (Applied Biosystems, Diegem, Belgium) using TaqMan Universal PCR Master Mix (Applied Biosystems, Diegem, Belgium). All PCR runs were performed in triplicate on a Light Cycler 480 (Roche, Vilvoorde, Belgium). The relative gene expression was calculated via the $2^{-\Delta\Delta Ct}$ method.[32] Data are presented as the relative quantity of target miRNA, normalized to endogenous control miRNAs (i.e. RNU44 and RNU48). qBase+ software was used for relative quantification analysis of miRNAs expression (Biogazelle, Gent, Belgium).

Bioinformatics Analysis

A biological interpretation of the miRNA data was done using Ingenuity Pathway Analysis (IPA) (http://www.ingenuity.com/). The IPA Knowledge Base was queried for all genes that are interacting with miR-21-5p, miR-146a-5p and miR-222-3p. A Core Analysis was run using these three gene lists. The Top Diseases and Bio Functions were filtered and manually curated for ontologies that are related to inflammatory processes, immune functions and processes related to cardiovascular system development. Finally a consensus molecular network was built in IPA's Path Designer.

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/her own control over time and it controls for potential confounding from within-subject covariates that do not change over time. The models were used to investigate the association between retinal vessel caliber (CRAE and CRVE) and PM_{10} exposure, the association between miRNA expression and PM_{10} exposure and the association between retinal vessel caliber and miRNA expression. miRNAs measurements were natural-log-transformed to improve normality. All analyses were corrected for sex, age, body mass index (BMI), alcohol and coffee consumption 24 hours prior to the examination, day of the week, hour of the study visit, location of the study visit, participants blood pressure (systolic and diastolic blood pressure), outdoor temperature and fellow vessel diameter (where applicable). The associations between microvascular markers (CRAE and CRVE) and miRNA expressions were studied with two mixed models. The first model was adjusted for the aforementioned covariates and in a second model, fellow vessel diameter was also included.

RESULTS

The characteristics of the study population are summarized in Table 1. Of the 50 participants, 50% were women. The mean age of this population was 32 ± 8 years. All participants had an university or college degree and a comparable socio-economic background. PM_{10} (lag 24 hr) ranged from 6.88 µg/m³ to 75.92 µg/m³ over the duration of the study. The average PM_{10} concentrations for the five consecutive study visits were respectively: 20.71 ± 7.18 µg/m³, 29.21 ± 7.62 µg/m³, 26.13 ± 16.42 µg/m³, 43.35 ± 19.29 µg/m³ and 24.00 ± 4.36 µg/m³.

Characteristic	Mean ± SD or n(%)
Personal Characteristics	
Age (years)	32 ± 8
Sex (%) Female	 50
Smoking status (%) Current	 4
Consumption (number of glasses per day) Alcohol Cafeine	 2.0 ± 4.0 2.0 ± 2.0
General Health Characteristics	
BMI (kg/m²)	23 ± 3
SBP (mm Hg)	122 ± 8
DBP (mm Hg)	72 ± 9
HR (bpm)	71 ± 12
CRAE (µm)	152.79 ± 13.68
CRVE (µm)	222.14 ± 21.59

Table 1. Population Characteristics

Predictors of CRAE and CRVE

The average CRAE and CRVE values were respectively 152.79 ± 13.68 µm and 222.14 ± 21.59 µm. Each 1-year increase in age of a participant was associated with a 0.72 µm decrease in CRAE (p=0.002) and a 1.17 µm decrease in CRVE (p=0.002). Systolic blood pressure was not associated with CRAE (p=0.91) or CRVE (p=0.38), but a 10 mm Hg increase in diastolic blood pressure was significantly associated with a 2.95 µm smaller CRAE (95% CI: -3.13, -2.77; p=0.003) and a 3.60 µm wider CRVE (95% CI: 1.63, 5.67; p=0.0009). Additionally, for each 1 µm increase in CRVE, CRAE widened with 0.50 µm (95% CI: 0.44, 0.56; p<0.0001). Likewise, a 1 µm wider CRAE was associated with a 0.86 µm wider CRVE (95% CI: 0.73, 0.99; p<0.0001). Sex did not significantly predict CRAE (p=0.06) or CRVE (p=0.29). BMI did not significantly predict CRAE (p=0.62) or CRVE (p=0.84). Meteorological data were not associated with CRAE and CRVE.

Microvascular markers are associated with short-term air pollution exposure

Two mixed models were constructed to investigate changes in CRAE or CRVE in association with PM_{10} exposure lags with consideration of age, sex, BMI, blood pressure, alcohol consumption, location of the study visit, day of the week, time of the day and outside temperature as covariates (model 1). The impact of additional adjustment for fellow vessel diameter was investigated in an extended model (model 2). In both models we have found significant associations between CRAE or CRVE and PM_{10} exposure for different exposure lags ranging from hours up to one week before the study visit. For example, each 10

μg/m³ increase in PM_{10} during the previous 24 hours was associated with a 0.72 μm decrease (95% CI: -1.38, -0.06; p=0.031) in CRAE (Table 2, model 1). This association was attenuated when CRVE was added to the model (Table 2, model 2), in which a 10 μg/m³ increase in PM_{10} during the previous 24 hours was associated with a 0.45 μm decrease (95% CI: -0.94, 0.053; p=0.087) in CRAE. The negative associations of CRAE with the lag 48 h and lag 1 week exposure metrics were significant in model 1 and model 2. We did not find an association between PM_{10} exposure and CRVE in model 1 (Table 2). In contrast, when CRAE was added to the model, each 10 μg/m³ increase in PM_{10} during the previous 24 hours was associated with a 0.99 μm increase (95% CI: 0.18, 1.80; p=0.021) in CRVE (Table 2, model 2).Comparable associations between CRAE/CRVE and outdoor PM_{10} concentrations were found in shorter (lag 2h, lag 4h and lag 6h) and longer (lag 48h and lag 1 week) exposure windows (Table 2, model 1 and model 2).

miRNA expression is associated with short-term air pollution exposure

We investigated the association between miRNA expression levels and the different PM_{10} exposure windows using a model adjusted for sex, age, BMI, blood pressure, alcohol consumption, location of the study visit, day of the week, time of the day and outside temperature. We found negative associations between miR-21 and miR-222 expression and PM_{10} levels in all of the exposure windows, but no significant associations for miR146a. The magnitude and polarity of the associations did not change when comparing the different exposure windows (Table 3). For example, in the 24 hours exposure window, a 10 μg/m³ increase in PM_{10} was associated with a 6.62% decrease (95% CI: -11.07, -2.17; p=0.0038) in miR-21 expression and a 6.71% decrease (95% CI: -10.70, -2.75; p=0.0012) in miR-222 expression.

Exposure time (lags)	CRAE		CRVE	
	Model 1	Model 2	Model 1	Model 2
2 hr	-0.71 (-1.20; -0.22)**	-0.39 (-0.78; -0.01)*	0.46 (-0.26; 1.19)	0.74 (0.11; 1.38)*
4 hr	-0.71 (-1.23; -0.20)**	-0.39 (-0.79; 0.02)	0.48 (-0.30; 1.26)	0.81 (0.13; 1.48)*
6 hr	-0.67 (-1.20; -0.14)*	-0.38 (-0.79; 0.04)	0.46 (-0.33; 1.25)	0.79 (0.10; 1.47)*
24 hr	-0.72 (-1.38; -0.06)*	-0.45 (-0.95; 0.05)	0.75 (-0.21; 1.71)	0.99 (0.18; 1.80)*
48 hr	-0.90 (-1.72; -0.07)*	-0.84 (-1.47; -0.20)*	1.22 (-0.02; 2.46)	1.63 (0.56; 2.69)*
1 week	-0.78 (-1.40; -0.16)*	-0.59 (-1.15; -0.03)*	-0.54 (-1.21; 0.12)	0.19 (-0.41; 0.78)

Table 2. Estimated change in mean CRAE (µm) or CRVE (µm) (95% CI) associated with a 10 µg/m³ increase in PM_{10}. Model 1 is adjusted for following covariates: age, gender, BMI, blood pressure, location, day of the week, alcohol and caffeine consumption and outdoor temperature. Model 2 further adjusts for fellow vessel diameter. Statistical significance is expressed as follows: *p<0.05, **p<0.01

Exposure time (lags)	miR-21	miR-222	miR-146a
2 hr	-4.04 (-7.13; -0.95)*	-4.25 (-6.83; -1.67)**	-1.26 (-4.07; 1.56)
4 hr	-4.65 (-7.88; -1.41)**	-4.79 (-7.47; -2.12)***	-1.59 (-4.52; 1.34)
6 hr	-5.01 (-8.38; -1.68)**	-5.21 (-7.93; -2.49)***	-1.87 (-4.87; 1.13)
24 hr	-6.62 (-11.07; -2.17)**	-6.71 (-10.68; -2.75)**	-1.36 (-5.79; 3.07)
48 hr	-8.27 (-14.14; -2.40)**	-10.93 (-15.87; -5.98)**	-2.58 (-8.32; 3.20)
1 week	-7.19 (-11.27; -3.11)**	-11.20 (-14.71; -7.69)***	-3.48 (-8.07; 1.11)

Table 3. Estimated change in miRNA expression (%) (95% CI) associated with a 10 µg/m³ increase in PM_{10}. All models are adjusted for following covariates: age, gender, BMI, blood pressure, location, day of the week, alcohol and caffeine consumption and outdoor temperature. Statistical significance is expressed as follows: *p<0,05, **p<0.01;***P<0.001.

Microvascular markers are associated with miRNA expression

The associations between microvascular markers (CRAE or CRVE) and miRNA expressions (miR-21, miR-146a or miR-222) were studied in a model that was adjusted for the aforementioned covariates (model 1). These associations were also investigated with additional correction for fellow vessel diameter. In this model 2, CRAE was associated with miR-21 expression: each 10% increase in miR-21 expression was associated with a 0.14 µm increase (95% CI: 0.006, 0.27; p=0.046) in CRAE (Table 4, model 2). No associations were observed for miR-146a and miR-222. In contrast, CRVE was associated with miR-222 and miR-146a expression in model 1 and model 2. Each 10% increase in miR-222 or miR-146a expression was associated with respectively 0.28 µm decrease (95% CI: -0.50, -0.06; p=0.016) or 0.30 µm decrease (95% CI: -0.49, -0.11; p=0.0034) in CRVE in model 1, and comparable associations in model 2.

	CRAE		CRVE	
	Model 1	**Model 2**	**Model 1**	**Model 2**
miR-21	0.08 (-0.09; 0.26)	0.14 (0.006; 0.27)*	-0.05 (-0.24; 0.14)	-0.06 (-0.24; 0.11)
miR-222	-0.15 (-0.36; 0.05)	0.07 (-0.08; 0.22)	-0.28 (-0.50; -0.06)*	-0.21 (-0.41; -0.01)*
miR-146a	-0.12 (-0.30; 0.07)	0.06 (-0.08; 0.21)	-0.30 (-0.49; -0.11)*	-0.19 (-0.37; -0.005)*

Table 4. Estimated change in mean CRAE (µm) or CRVE (µm) (95% CI) associated with a 10% increase in miRNA expression. Model 1 is adjusted for following covariates: age, gender, BMI, blood pressure, location, day of the week, alcohol and caffeine consumption and outdoor temperature. Model 2 further adjusts for fellow vessel diameter. Statistical significance is expressed as follows: *p<0.05

Bioinformatics analysis

The IPA Knowledge Base states that miR-21, miR-146a and miR-222 are involved in the interaction network of respectively 659 genes, 683 genes and 822 genes. These lists had 146 genes in common, with 29 genes being significantly associated (p<0.05) with cardiovascular system functioning and disease (Supplementary Table 1). We could not identify pathways involved in cardiovascular inflammation. In the miR-146a gene list, a large set of 23 genes of the was associated with the NF-κB signalling pathway, but we failed to identify genes involved in cardiovascular function. 85 genes that interact with miR-21 and miR-222 and that are related to the ontology terms *cell movement of endothelial cells* and *atherosclerosis* were used to construct a consensus interaction network (Figure 1). An overlay with the database of canonical pathways shows that most genes are involved in inflammatory pathways such as Phosphatase and tensin homolog (PTEN) and High-mobility group protein B (HMGB) signalling. The latter pathway shows cross talk with the atherosclerosis signalling pathway (Supplementary Table 2).

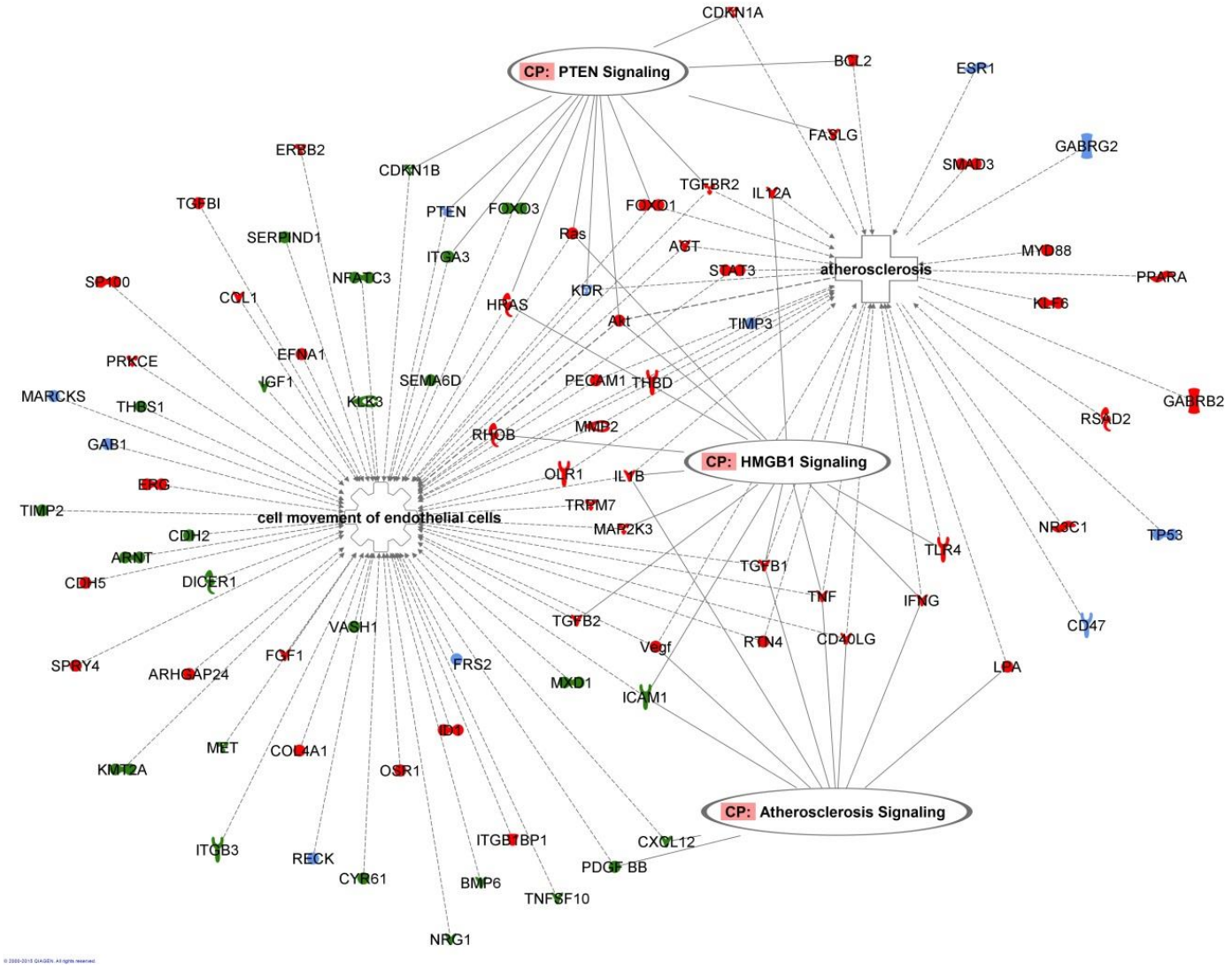

Figure 1. Consensus interaction network of miRNA's and their target genes. Symbols that are coloured in red refer to genes that interact with miR-21, genes in green interact with miR-22 and genes in blue interact both with miR-21 and miR-222. An overlay with the database of canonical pathways shows that most of the genes can be associated with inflammatory pathways such as PTEN signaling and HMGB signaling. The latter pathway shows cross talk with the atherosclerosis signaling pathway

DISCUSSION

Retinal blood vessel widths and expression of miR-21, miR-146a and miR-222, microRNAs that are involved in inflammation and oxidative stress, were associated with ambient short-term PM_{10} exposure in a repeated measurements study in healthy adults over a 5-month period. Particulate matter can induce changes in microcirculatory beds, as evidenced by retinal imaging, that may be on the trajectory of cardiovascular health effects caused by air pollution.[16, 17, 33] Furthermore, the associations between miRNA expression and PM_{10} exposure may hint at the underlying mechanism. Disturbed expression of regulatory miRNAs might transmit a molecular signal that affects downstream expression of signalling pathways that cause increased inflammatory processes and oxidative stress in the cardiovascular system.[21, 23]

The association between retinal blood vessels and air pollution has been investigated in two landmark studies. Adar et al. (2010) reported an association between arteriolar narrowing and both short-term and long-term exposure to $PM_{2.5}$ in the MESA cohort.[16] For each 9 µg/m³ increase in short-term $PM_{2.5}$ exposure on the previous day, Adar observed an arteriolar narrowing of 0.6 µm. Each 3 µg/m³ increase in long-term $PM_{2.5}$ exposure was associated with a decrease in arteriolar diameter of 0.8 µm, an effect that was calculated to be equivalent to 7 years of aging. Our research group found in a group of 84 healthy individuals that each 10 µg/m³ increase in short-term PM_{10} exposure 24 hr prior to the retinal examination was associated with an arteriolar narrowing of 0.93 µm, equivalent to 1.5 years of aging.[17] In the current study, each 10 µg/m³ increase in PM_{10} concentrations, 24 hours before the study visit, was associated with arteriolar narrowing of 0.72 µm and venular widening of 0.99 µm (after correction for fellow vessel diameter). These associations remained consistent for shorter (down to 2 hours) and longer exposure lags (up to 1 week). Our reported association is slightly larger than Adar's, assuming that 9 µg/m³ $PM_{2.5}$ corresponds to 12.9 µg/m³ PM_{10}.[34] This difference might be explained by the difference in age and health condition of the study participants. The microcirculation of young, healthy persons might react more promptly to changing conditions than the blood vessels of older, more susceptible individuals.[35, 36] In this way, air pollution might induce larger physiological effects in the retinal microvasculature of healthy individuals. The associations that are reported in this work are somewhat smaller than in our previous study and this might be explained by differences in exposure levels. In this study, peak exposure was 75 µg/m³ whereas in our previous study, these levels reached 117 µg/m³.

We have found negative associations between the expression of miR-21 and miR-222 and short-term changes PM_{10} exposure, but no association for miR-146a. Each 10 µg/m³ increase in PM_{10} levels was associated with respectively a 6.62% decrease (95% CI: -11.07, -2.17; p=0.0038) in miR-21 expression and a 6.71% decrease (95% CI: -10.70, -2.75; p=0.0012) in miR-222 expression. Other authors have published mixed results on the association between miRNA expression and air pollution exposure. In a study in foundry workers, Bollati et al. (2010) observed an upregulation of miR-21 and miR-222 after three days occupational exposure.[37] On the other hand, miR-21, miR-222 and miR-146a were negatively associated with seven-day moving averages of $PM_{2.5}$ in elderly men.[38] In vitro and in vivo studies have shown mainly downregulation of these miRNAs in the myocardium of PM-exposed rats, in cigarette-smoked exposed human airway epithelial cells and murine lungs.[19, 22, 24] Furthermore, downregulation of miR-21 and miR-146a has also been observed in smokers.[20] Our associations are much smaller compared to the study of Fossati et al. (2014). They report a 35% and 20% downregulation in miR-21 and miR-222 expression for each 3.83 µg/m³ increase in $PM_{2.5}$ levels.[38] We argue that differences in particle

composition, exposure assessment and age of our study populations may explain the differences between our studies.[39]

We are the first to report associations between expression of miRNAs and retinal blood vessels in a context of cardiovascular epidemiology. miR-21 expression was associated with retinal arteriolar width, whereas miR-222 and miR-146a expression were inversely associated with retinal venular width. It is considered that air pollution partially exerts its effects through the induction of inflammation and oxidative stress.[40] Air pollution exposure may induce a low-grade pulmonary inflammation that can spill over to the cardiovascular system. This can prime and activate leukocytes and the endothelium which can lead to endothelium dysfunction.[41] We hypothesize that air pollution-induced differential regulation of miR-21 and miR-222 could have increased oxidative stress and inflammation that in turn led to microvascular responses as evidenced by retinal vessel analysis.[38] Both miRNAs are involved in the HMGB1/RAGE pathway and PTEN signalling pathway. The first pathway leads to enhanced production of pro-inflammatory cytokines, adhesion molecules and coagulation factors. In the latter pathway, downregulation of these miRNAs may have caused upregulation of PTEN expression which may have inhibited the endothelial nitric oxide synthase (eNOS) pathway and/or increased ICAM-1 expression.[42-44] Furthermore, downregulation of miR-21 lowers endothelial nitric oxide synthase levels and the bioavailability of nitric oxide.[27] The consequent effect on the endothelium might have caused retinal arteriolar narrowing, as observed in this study. Inflammatory cells leave the circulation through the venular side as the venular vessel wall is thinner. Rolling and adhesion interactions with the endothelium will result in extravasation of the leukocytes. However, this process requires the breakdown of the endothelial surface layer which results in (retinal) venular widening.[45] miR-222 inhibits leukocyte rolling and adhesion through the downregulation of adhesion molecules. Air pollution-induced downregulation of miR-222 might have raised leukocyte-endothelial interactions that might have caused retinal venular widening.

Our results are consistent with the previously reported health effects of ambient air pollution. Epidemiological and controlled exposure studies have revealed that particulate matter exposure is associated with adverse micro- and macrovascular responses in humans and animals.[40, 46-48] Our study has strengths and limitations. First, we have worked with a panel of relatively young, healthy adults. This was a very homogenous study population in which variability in participant characteristics was limited. The use of repeated measurements, conducted on approximately the same moment each day/month, increased the chance of finding significant effects in this small population. We have been able to confirm negative associations between retinal vessels calibers and particulate matter in this independent study. Furthermore, we contribute to the field of molecular epidemiology by reporting associations between miRNA expressions and particulate matter exposure. We are the first to add this layer of molecular information that may help to explain the microvascular response to air pollution exposure. We have performed a mediation analysis to further investigate the role of miRNAs as potential intermediate phenotypic response. A proper protocol for mediation analysis for a repeated measurements study design has not been developed. Hence, we had to average our repeated measurements in order to perform the mediation analysis.[49] This approach reduced the statistical power, with a failure to further underpin the mediating effect of miRNA expression. Obviously, this small and healthy study population limits the generalizability of our findings to the whole population and more susceptible subgroups such as diabetics or cardiac patients.[50-52] Second, we also cannot exclude exposure misclassification as exposure was

calculated for the location of the study visit. Although the measurement stations are representable for the study region and study participants lived and worked close to the location of the clinical examination, time-activity patterns can influence PM_{10} exposure.[53] However, exposure misclassification would have resulted in an underestimation of the health effects.[54] Third, we did not measure expression of genes and proteins that are under the regulatory control of the studied miRNAs. Whether PM_{10} exposure-induced differential regulation of miRNAs led to downstream induction of oxidative stress and inflammatory processes remains to be further investigated.

In conclusion, we have confirmed a retinal microvascular response to recent variation in ambient particulate air pollution and we hint at a possible role for miRNAs to explain this effect at a molecular level. PM_{10} downregulated the expression of miRNAs that are controlling oxidative stress and inflammatory pathways. Differential regulation of miRNAs might have caused downstream molecular events that might have contributed to arteriolar narrowing and venular widening, which are risk factors for cardiovascular health. Our findings further contribute to the importance of the microvascular pathway through which air pollution exposure may affect or trigger cardiovascular events.

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Chapter 5

Blood Pressure Changes in Association with Black Carbon Exposure in a Panel of Healthy Adults Are Independent of Retinal Microcirculation

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ABSTRACT

Introduction: Exposure to ambient particulate matter and elevated blood pressure are risk factors for cardiovascular morbidity and mortality. Microvascular changes might be an important pathway in explaining the association between air pollution and blood pressure. The objective of the study was to evaluate the role of the retinal microcirculation in the association between black carbon (BC) exposure and blood pressure.

Methods: We estimated subchronic BC exposure based on 1-week personal measurements (µ-Aethalometer, AethLabs) in 55 healthy nurses. Blood pressure and retinal microvasculature were measured on four different days (range: 2-4) during this week.

Results: Subchronic BC exposure averaged (± SD) 1334 ± 631 ng/m³ and ranged from 338 ng/m³ to 3889 ng/m³. An increased exposure of 631 ng/m³ BC was associated with a 2.77 mm Hg (95% CI: 0.39 to 5.15, p=0.027) increase in systolic blood pressure, a 2.35 mm Hg (95% CI: 0.52 to 4.19, p=0.016) increase in diastolic blood pressure and with 5.65 µm (95% CI: 1.33 to 9.96, p=0.014) increase in central retinal venular equivalent. Mediation analysis failed to reveal an effect of retinal microvasculature in the association between blood pressure and subchronic BC exposure.

Conclusion: We found a positive association between blood pressure and subchronic black carbon exposure in healthy adults. This finding adds evidence to the association between black carbon exposure and cardiovascular health effects, with elevated blood pressure as a plausible intermediate effector. Our results suggest that the changes in a person's blood pressure as a result of subchronic black carbon exposure operates independent of the retinal microcirculation.

INTRODUCTION

Short-term and long-term exposures to particulate matter air pollution contribute to cardiovascular morbidity and mortality.[1, 2] Altered autonomic function of the heart, changes in micro- and macrovascular reactivity, induction of systemic inflammation, endothelial dysfunction and altered peripheral resistance of the blood vessels can mediate these cardiovascular effects.[3] The microcirculation determines the overall peripheral resistance and microvascular alterations may lead to blood pressure elevation and an increased risk for developing hypertension.[4, 5] Adar et al. (2010) and Louwies et al. (2013) have studied the impact of air pollution on the retinal microcirculation. These authors found that retinal arteriolar narrowing is associated with long-term and short-term exposure to air pollution.[6, 7] Additionally, retinal arteriolar narrowing has been associated with increased blood pressure and hypertension. [8] Thus, microcirculatory changes in the retina are potentially relevant in the association between air pollution exposure and blood pressure changes.

Epidemiological research and animal studies have produced positive, negative and null associations between blood pressure and ambient air pollution.[9] These outcomes can be explained by study-specific differences such as population characteristics, dose and duration of the exposure that are different between studies. Furthermore, the chemical composition of particulate matter is heterogeneous and varies between studies. For instance, $PM_{2.5}$ (particulate matter with a diameter smaller than 2.5 µm) exposure in high-traffic areas had a stronger effect on blood pressure compared with $PM_{2.5}$ in low-traffic areas.[10, 11] Furthermore, spatial and temporal variability in pollution sources may obscure associations between PM and blood pressure. Most epidemiological studies rely on central monitor data or complex models to estimate PM concentrations at the participant's residence. Exposure is however strongly related to an individual's time-activity patterns and time spent indoor and outdoor.[12] Exposure misclassification may occur with central monitor data and may lead to incorrect estimation of cardiovascular health effects associated with air pollution exposure.[13, 14]

Black carbon (BC), a by-product of fuel combustion and a constituent of particulate matter, has been associated with systemic inflammation and oxidative stress, decreased flow-mediated dilation of the brachial artery and reduced parasympathetic tone.[15-18] Mordukhovic et al. (2009) and Wilker et al. (2010) reported positive associations between short-term BC exposure, measured as the concentration averaged over the 7 days preceding each study visit, and systolic and diastolic blood pressure.[19, 20] Schwartz et al. (2012) reported an association between blood pressure and modelled long-term BC concentrations. A 0.32 µg/m³-increase in BC was significantly associated with a 2.64 mm Hg increase in systolic blood pressure and a 2.41 mm Hg increase in diastolic blood pressure.[21] Zhao et al. (2014) measured personal BC exposure using portable measuring devices in a study that investigated the effects of BC on blood pressure in 65 persons suffering from the metabolic syndrome. A short-term BC increase of 1 µg/m³, 10 hours prior to the study visit, was associated with a 0.53 mm Hg increase in systolic blood and a 0.37 mm Hg increase in diastolic blood pressure.[22]

We explore the association between blood pressure, short-term and subchronic BC exposure in this study. Subchronic BC exposure was calculated based on personal monitoring during one week with portable measuring devices and data from a reference station. During this 1-week period we repeatedly measured blood pressure and retinal vessel diameters. The retinal microcirculation was measured to

study the potential mediating effect of the microcirculation in the relationship between BC exposure and blood pressure.

METHODS

Study design

A total of 130 nurses working in the north of Belgium were invited and 99 (76%) agreed to participate. Fifty five nurses (56%) could be monitored in this study. The predominantly female participants were aged between 22 and 59 years and reported to be free of cardiovascular diseases and diabetes. Every participant was monitored during one average working week between April and May 2013. Clinical examinations were scheduled for every participant on Tuesday, Thursday, Saturday and Monday between 7 am and 9 pm [mean difference between repeated measurements was 1 hr (range, 0.1–1.9 hr)]. 75% of the participants underwent all 4 examinations, 23% completed 3 examinations, whereas 2% completed 2 examinations. Participants were not asked to fast before the visits. Blood pressure measurements and retinal images were collected during each examination. A venous blood sample was collected on the last day of the study. Gamma-glutamyl transpeptidase (γ-GT) was measured as a marker for liver function and alcohol consumption. Haemoglobin A1C was measured as a glycemic index and metabolic marker for diabetes. Participants completed a questionnaire on their smoking status, medical history and current medication use. All participants provided written informed consent. The ethics boards of Hasselt University and University Hospital Antwerp approved the study.

Blood pressure measurement

Systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) were measured with an automated device (Stabilograph, Stolberg, Germany) according to the guidelines of the European Society of Hypertension [23]. After the participants had rested in a sitting position for 5 min, SBP, DBP and HR were measured five times consecutively during each of the 4 study visits. The average of the last three measurements collected during the examinations was used in the analysis.

Retinal photography and grading

A Canon 45° 6.3 megapixel digital nonmydriatic retinal camera (Hospithera, Brussels, Belgium) was used. The fundus of the right eye and the left eye of each participant were photographed twice during each study visit. Participant characteristics were masked for the trained grader before review and analysis of the retinal images. IVAN retinal image analysis software was used to measure retinal vessel diameters according to previously reported protocols [24, 25]. Retinal vessel calibers were summarized as the Central Retinal Arteriolar Equivalent (CRAE) and Central Retinal Venular Equivalent (CRVE) in each picture. The equivalents represent a summary of vessel diameters within an area equal to 0.5–1 disc diameters from the optic disc margin. Average CRAE and CRVE values were calculated for each study visit based on the four images.

Exposure assessment

Personal black carbon exposure

Personal exposure to black carbon (BC) was measured continuously for 7 consecutive days (from Tuesday to next week's Monday) with a portable MicroAeth Model AE51 (Aethlabs, San Francisco, California, US) on a 1-min time resolution. A short tube was attached to the inlet of the aethalometer, giving the participants the opportunity to put the device in a purse or backpack while still sampling ambient air. Air was drawn over a Teflon-coated borosilicate glass fibre filter at a flow rate of 100 ml/min, resulting in BC accumulation on the filter. The attenuation of light at 880 nm was measured and converted into a BC concentration (ng/m³). The filter was replaced every two days to prevent filter saturation. The participants were instructed to carry the device with them at all times, but for indoor activities they were allowed to leave it in the room where the majority of the time was spent. Raw BC data were processed before they were used. Measurements with high attenuation (ATN >75) or an error code were excluded [12]. Next, data were smoothened with an algorithm that was developed by the Environmental Protection Agency [26].

Short-term exposure windows (24 hours and 48 hours) were calculated by taking the average of all BC measurements 24 hours or 48 hours before the clinical visit.

Calculated personal subchronic black carbon exposure

Subchronic BC exposure was calculated based on the personal BC exposure measured during the study period. This was done using the following formula: Subchronic BC exposure = Personal BC measurement x (Refsite yearly average / Refsite week average). The personal BC measurement was calculated as the average BC exposure over the whole week for each participant. This timeframe can capture the time-activity pattern during an average working week [27]. "Refsite yearly" represents the average BC concentration during the year 2013. "Refsite week average" represents the average BC concentration at the BC reference monitor during the same week as the personal BC measurements. The latter factor allows correcting for varying ambient concentrations during the study period. The monitoring station of Dessel that is operated by the Flemish Environment Agency was chosen as reference. The station is equidistant from both study locations. The personal subchronic BC exposure represented the main exposure variable in our study [27].

Outdoor temperature

The 24 hr mean outdoor temperature measured at the meteorological stations of Diepenbeek and Sint-Katelijne-Waver were obtained from the Belgian Royal Meteorological Institute (Ukkel, Belgium).

Traffic-related GIS-variables

The home address of the nurses was geocoded. The coordinates were manually adapted when they differed from the actual position of the residence. Residential distance to major roads was calculated with the Tele Atlas MultiNet dataset in ArcGIS 9.3. Attributes include name of street, route number, speed class, length and road classification (0: Motorways; 1: Roads belonging to 'Main road' major importance; 2: Other major roads; 3: Secondary roads; 4: Local connecting roads; 5: Local roads of high importance; 6: Local roads; 7: Local roads of minor importance; 8: Others). All roads of class 0, 1 and 2 were classed as major roads.

Statistical analysis

Statistical analysis was carried out using SAS software (version 9.3, SAS Institute Inc., Cary, NC, USA). Continuous data were presented as mean ± standard deviation (SD) and categorical data as percentages (%) and frequencies. We used mixed models to investigate the association between blood pressure and BC exposure, the association between retinal vessel calibers (CRAE and CRVE) and BC exposure, and the association between blood pressure and CRAE and CRVE. We used random subject effects accounting for repeated measures and we applied an unstructured covariance structure. Models were adjusted for the following fixed effects: sex, age, body mass index (BMI), smoking behaviour, use of anti-hypertensive medication, γ-GT, haemoglobin A1c, distance to major road, location where the clinical visit took place, day of the week and average weekly temperature. In a sensitivity analysis, we excluded persons that were on anti-hypertensive medication and persons with a smoking history.

To assess the role of the microcirculation in the association between blood pressure and BC exposure, we first included CRAE and CRVE as additional covariates in our previously described models. Secondly, we performed a formal mediation analysis, which decomposes the total observed effect of BC exposure on blood pressure into a direct effect (DE) and an indirect effect (IE) that acts via the mediator of interest. In this analysis we used the average blood pressure of the study week as a response variable (i.e. one value per participant) and we adjusted for the same covariates as the mixed models, except day of the week. The direct effect, indirect effect and total effect were estimated by using the SAS macro developed by Valeri and VanderWeele [28].

Characteristic	Mean ± SD or n(%)
Personal Characteristics	
Age (years)	41 ± 11
Sex	
Female	51 (93%)
Ethnicity	
Caucasian	54 (99%)
African	1 (1%)
Smoking status	
Never/Former	53 (96%)
Current	2 (4%)
Antihypertensive medication	3 (5%)
General Health Characteristics	
Body Mass Index, kg/m²	24.2 ± 4.5
Systolic blood pressure, mm Hg	116 ± 12
Diastolic blood pressure, mm Hg	73 ± 8
Heart rate, bpm,	75 ± 25
Central Retinal Arteriolar Equivalent, µm	152.15 ± 12.65
Central Retinal Venular Equivalent, µm	211.28 ± 17.35
Gamma-glutamyl transpeptidase, U/L	17.71 ± 12.21
Hemoglobine A1c, %	5.36 ± 0.25
Exposure Characteristics	
Distance to major road, m	1714 ± 1629
Personal black carbon exposure, ng/m³	866 ± 425
Subchronic black carbon exposure, ng/m³	1334 ± 631

Table 1. Population characteristics (n = 55)

RESULTS

The characteristics of the study population are summarized in Table 1. 93% of the 55 participants were women with a mean age ± SD of 41 ± 11 years. The mean BMI ± SD was 24.2 ± 4.5 kg/m². 39 study participants (71%) had never smoked and 14 persons (25)% were former smokers, whereas 2 persons

(4%) were current smokers. Three participants (5%) used β-blockers as antihypertensive medication. All participants had a similar college degree and socioeconomic background. Average values ± SD of heart rate, systolic blood pressure (SBP) and diastolic blood pressure (DBP) were 75 ± 25 bpm, 116 ± 12 mm Hg and 73 ± 8 mm Hg, respectively. Mean Central Retinal Arteriolar Equivalent (CRAE) and Central Retinal Venular Equivalent (CRVE) were 152.15 ± 12.65 µm and 211.28 ± 17.35 µm, respectively. Average values ±SD for γ-GT and haemoglobin A1C were 17.71 ± 12.21 U/l and 5.36 ± 0.25 %, respectively. None of the participants had divergent values for γ-GT or haemoglobin A1C. Participants lived on average ± SD at distance of 1714 ± 1629 m from a major road. This variable was introduced as a proxy for traffic noise exposure. The mean personal Black Carbon (BC) concentration measured during the week was 866 ± 425 ng/m³. The derived subchronic BC exposure was 1334 ± 631 ng/m³ and ranged from 338 ng/m³ to 3889 ng/m³.

Blood pressure components were significantly associated with CRAE and CRVE. All models were adjusted for sex, age, BMI, smoking behaviour, use of anti-hypertensive medication, γ-GT, haemoglobin A1c, distance to major road, location of the clinical visit, day of the week and average weekly temperature. SBP and DBP decreased with 0.33 mm Hg (95% CI: -0.49 to -0.18, p=0.0001) and 0.25 mm Hg (95% CI: -0.38 to -0.13, p=0.0002) for each 1 µm increase in CRAE. The corresponding estimates for CRVE were 0.16 mm Hg decrease (95% CI: -0.27 to -0.06, p=0.0033) and 0.14 mm Hg decrease (95% CI: -0.22 to -0.06, p=0.0014), respectively.

We did not find any association between short-term BC exposure (lag 24h and lag 48h) and SPB, DBP, CRAE or CRVE (results not shown).
In contrast to short-term exposure, subchronic BC exposure was associated with both SBP and DBP. Independent of the aforementioned covariates, a SD-increase in BC concentration was associated with a 2.77 mm Hg (95% CI: 0.39 to 5.15, p=0.027) increase in SBP and a 2.35 mm Hg (95% CI: 0.52 to 4.19, p=0.016) increase in DBP (Figure 1A, Model 1). Subsequently, the association between BC exposure and the retinal microcirculation was assessed. An association between BC exposure and CRVE was identified. A SD-increase in BC exposure was associated with a 4.76 µm (95% CI: 0.27 to 9.24, p=0.044) increase in CRVE (Figure 1B, Model 1). Addition of SBP and DBP to the model, did not change the association: a SD-increase in BC exposure was associated with a 5.65 µm (95% CI: 1.33 to 9.96, p=0.014) increase in CRVE (Figure 1B, Model 2). The association between CRVE and BC remained significant when an additional correction for fellow vessel diameter was included (Figure 1B, Model 3). No associations between BC exposure and CRAE were identified (Figure 1B).

We explored the role of the microcirculation as a mediator of the association between BC and blood pressure. In a first analysis, we tested the mediating effect of CRAE and/or CRVE by adding these factors as covariates to our previously described model. After correction for CRAE, a SD-increase in BC exposure was associated with a 2.98 mm Hg (95% CI: 0.68 to 5.28, p=0.015) increase in SBP and a 3.09 mm Hg (95% CI: 1.49 to 4.69, p=0.0005) increase in DBP (Figure 1A, Model 2). When both CRAE and CRVE were considered in the fully adjusted model, the effect estimates changed slightly. A SD-increase in BC exposure was associated with a 3.10 mm Hg (95% CI: 0.77 to 5.43, p=0.012) increase in SBP and a 3.25 mm Hg (95% CI: 1.66 to 4.85, p=0.0002) increase in DBP (Figure 1A, Model 3). Secondly, we performed a formal mediation analysis. CRVE was significantly associated with BC exposure and BP. Therefore, the mediation analysis was conducted with CRVE as a potential mediator. The results of the

mediation analysis are shown in Figure 2. The total effect of BC on SBP or DBP is decomposed into a direct and indirect effect, the latter mediated by CRVE.

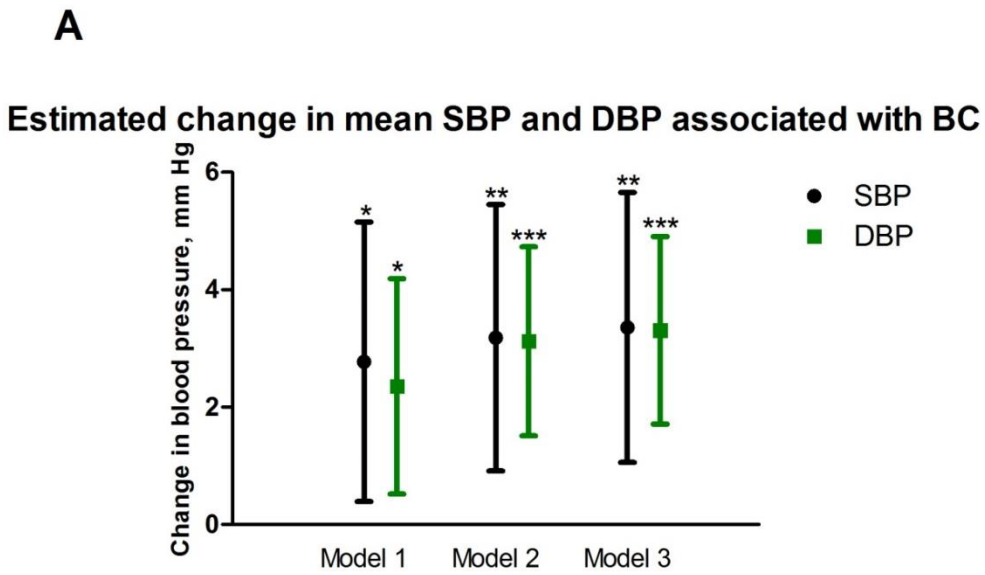

Figure 1A Effect sizes (95% confidence interval) express the change in systolic/diastolic blood pressure (SBP/DBP) (mm Hg) for an SD (631 ng/m³) increase in subchronic BC exposure. All models include 55 persons. Model 1 is corrected for sex, age, BMI, smoking behaviour, use of anti-hypertensive medication, γ-GT, haemoglobin A1c, location where the clinical visit took place, distance to major road, day of the week, average weekly temperature. Model 2, includes all aforementioned covariates and is further corrected for central retinal arteriolar equivalent. Model 3 also includes central retinal venular equivalent. Statistical significance is expressed as: *p<0.05, **p<0.01, ***p<0.001

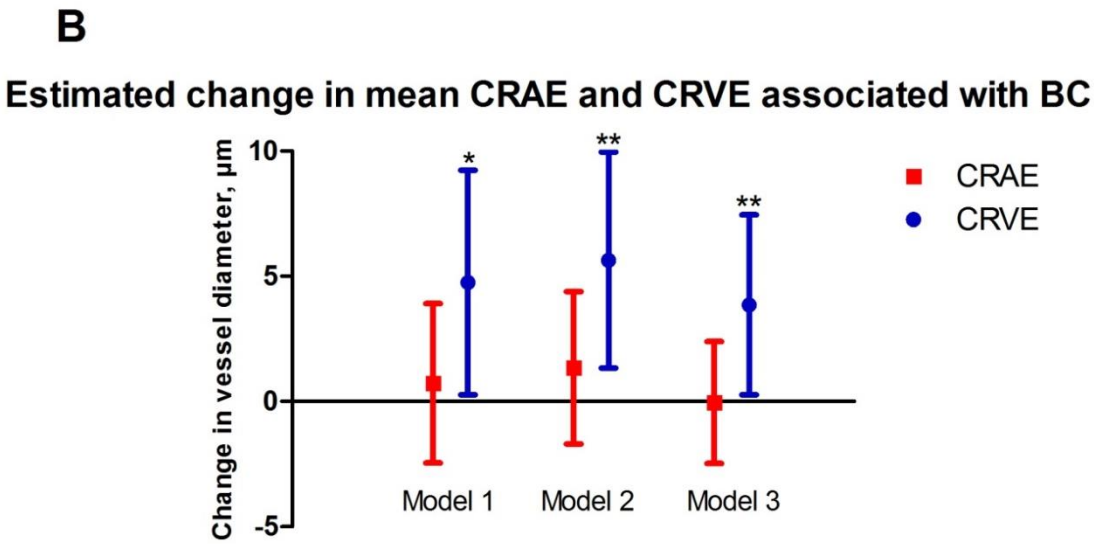

Figure 1B Effect sizes (95% confidence interval) express the change in Central Retinal Arteriolar/Venular Equivalent (CRAE/CRVE) (µm) for an SD (631 ng/m³) increase in subchronic BC exposure. All models include 55 persons. Model 1 is corrected for sex, age, BMI, smoking behaviour, use of anti-hypertensive medication, γ-GT, haemoglobin A1c, location where the clinical visit took place, distance to major road, day of the week, average weekly temperature. Model 2 also includes SBP and DBP. Model 3 additionally includes fellow vessel diameter. Statistical significance is expressed as: *p<0.05, **p<0.01

The effects of BC on blood pressure mediated by CRVE, were not significant and were respectively 0.42 mm Hg decrease in SBP (95% CI: -1.35 to 0.17) and 0.59 mm Hg decrease in DBP (95% CI: -1.44 to 0.07).

Finally, we conducted a sensitivity analysis to investigate the effect of anti-hypertensive medication use and smoking behaviour on the reported associations in our previously reported models (Model 1, Model 2 and Model 3). First, we excluded persons on antihypertensive treatment (n=3). This did not change the reported associations in any of the presented models (Supplementary Table 1). Second, we excluded current smokers (n=2) from the analysis. The associations were not affected in any of the models (Supplementary Table 2).

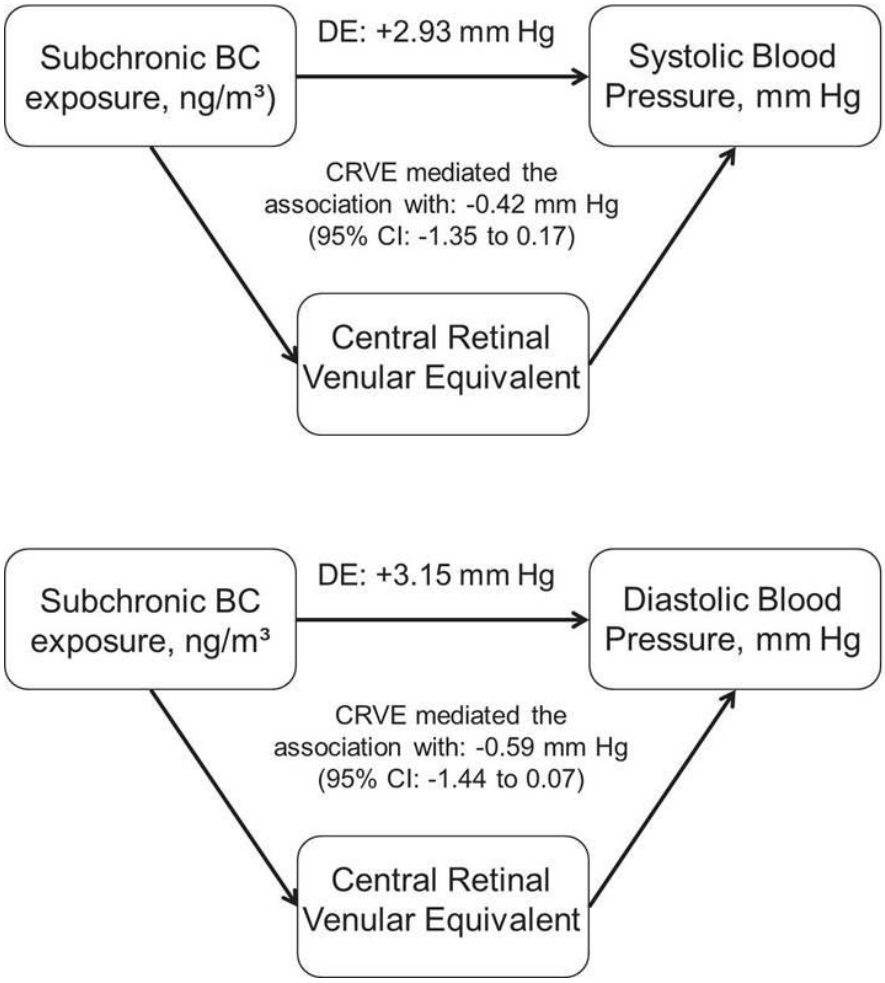

Figure 2 Mediation of the effect subchronic BC exposure (ng/m³) on blood pressure through Central Retinal Venular Equivalent. The figure shows Central Retinal Venular Equivalent as a potential mediator in the association between systolic/diastolic blood pressure and subchronic BC exposure. The estimates of the mediation through CRVE and the estimates of the direct effect (DE) of subchronic BC exposure on systolic/diastolic blood pressure are presented. The models were adjusted for sex, age, BMI, smoking behaviour, use of anti-hypertensive medication, γ-GT, haemoglobin A1c, distance to major road, location of the where the clinical visit took place and average weekly temperature.

DISCUSSION

Blood pressure in healthy nurses was positively associated with subchronic black carbon (BC) exposure. The microcirculation, assessed with retinal imaging, did not mediate the observations. The associations were identified at ambient BC exposure levels in healthy individuals. This is suggestive for the absence of a threshold value at which BC can induce health effects. Small increases in SBP or DBP in the normotensive range may eventually lead to a chronically elevated blood pressure or hypertension. The latter are associated with an increased long-term risk for cardio- and cerebrovascular events.[29, 30] A SD-increase of 631 ng/m³ in BC concentrations was associated in our model with a 2.49 mm Hg increase in SBP (95% CI: 0.08 to 4.91, p=0.049) and a 2.65 mm Hg increase in DBP (95% CI: 0.93 to 4.37, p=0.0041). Our findings have public health relevance. Assuming that BC concentrations could be lowered to background level and this leads to a population-wide reduction of 2.49 mm Hg in SBP, such an effect is then likely to result in a 9% decrease in coronary heart disease and a 13% decrease in stroke[30], and a 5 to 10% decrease of cardiovascular disease.[32, 33]

Our findings are comparable with other studies that investigated the association between long-term BC exposure and blood pressure. We recalculated the effect estimates presented in these studies in order to allow for a direct comparison with our effect estimates. For an identical increase in annual BC exposure, Wilker et al. (2010) reported increases in SBP and DBP of 2.14 mm Hg (95% CI: 0.15 to 4.14) and 1.28 mm Hg (95% CI: 0.22 to 2.33), respectively.[19] For an identical increase in annual BC exposure, Schwartz et al. (2012) found increases of 5.20 mm Hg (95% CI: 2.90 to 7.49) and 4.75 mm Hg (95% CI: 3.49 to 6.01) for SBP and DBP, respectively.[21] Zhao et al. (2014) used personal measurements of BC exposure and reported, for a comparable increase in BC, an acute increase in SBP and DBP of respectively 0.26 mm Hg (95% CI: 0.08 to 0.43) and 0.18 mm Hg (95% CI: 0.05 to 0.31).[22] We did not find an association between blood pressure changes and short-term BC exposure. Differences in study design and exposure range might explain this.
Our study was concerned with the effects of subchronic BC exposure on blood pressure. Furthermore, Zhao et al. reported an average exposure of 5.08 µg/m³ whereas our average short-term exposure was 0.87 µg/m³.

The microcirculation determines the peripheral resistance and thus exerts a great influence on blood pressure. Adverse manifestations of cardiovascular diseases are also likely to occur in microvascular beds.[5, 31] Therefore, we investigated the association between the microcirculation and BC exposure in our current study. We observed a positive association between retinal venules and BC exposure. During our previous work that focused on short-term microcirculatory effects of ambient BC exposure we observed retinal arteriolar narrowing in association with BC exposure.[7] In contrast, the current study focused on subchronic BC exposure. Our current study panel was confronted with a narrow range of BC exposure levels, whereas exposure levels were a tenfold higher in our previous study.

The influence of the microvasculature on the association between BC exposure and blood pressure was assessed by including CRAE and CRVE parameters in the statistical models. This approach did not change the associations between BC exposure and blood pressure. We also conducted a mediation analysis to formally test the interference of the microvasculature on the association between blood pressure and BC exposure. Mediation analysis requires a significant association between the exposure and the mediator, a

significant association between the mediator and the outcome, and a significant association between the exposure and the outcome.[32] In the presence of mediation, the effect of the exposure on the outcome is expected to be reduced after controlling for the mediator. Only CRVE was considered as a candidate for mediation because the significant association between CRAE and BC exposure, one of the requirements to conduct mediation analysis, was not met in our study. The mediation analysis did not reveal evidence that supported our hypothesis that the microvasculature, as measured in the retina, mediates the association between blood pressure and subchronic BC exposure. However, this statement should be interpreted with caution because of the small size of the study.

The exact pathophysiological mechanism underlying the rise in blood pressure caused by BC exposure remains to be further elucidated. BC inhalation may favour the sympathetic nerve activity via alterations in the cardiovascular autonomic nervous system.[33-35] Activation of α-adrenergic receptors leads to vasoconstriction and blood pressure increase.[36] The stress imposed on the arterial vessel walls may lead to hypertrophic remodelling and an increase of medial thickness, which can further increase peripheral resistance.[37, 38] Regular incidents that trigger a blood pressure increase may result in narrowing of retinal arterioles. In this respect, research has indicated that the narrowing of the retinal blood vessels precedes hypertension.[39, 40] At the same time it should be note that blood pressure is very tightly controlled by several feedback mechanisms. The microcirculatory response is only one effector pathway in this complex mechanism in which the renin-angiotensin pathway and the baroreceptor reflex also play an important role.[41]

The strength of our study is the combination of personal monitoring data and ambient BC concentrations from a reference station to estimate subchronic BC exposure. It has been shown before that this approach prevents exposure misclassification. The cost of personal monitoring devices typically limits the size of these personal monitoring studies over longer time periods. After correction for variation in ambient BC exposure, our subchronic exposure estimate is a reliable proxy and preferable over modelled exposure estimates. Furthermore, it has been shown that a 1-week monitoring of a representative working week can capture in a reliable way the time-activity patterns that are known to influence BC exposure.[12] Blood pressure is a highly variable phenotype and we have anticipated this by measuring blood pressure at four distinct time points with 5 measurements during each study visit. The circadian rhythm influences blood pressure. We accounted for this by measuring blood pressure of each study participant at the same time at each study visit. When the time of day was added to our models, our reported associations did not change (data not shown). Because the time difference between study visits was on average only 1 hour, blood pressure was not influenced by a circadian pattern. A third strength is that we have studied a panel of mostly female participants who reported to be free from clinically diagnosed cardiovascular diseases. A homogeneous study population reduces between-individual variability and increases the statistical power in a small panel. To our knowledge, we are introducing the first study with a retinal microvascular measurement to assess the role of the microvasculature in the association between blood pressure and subchronic BC exposure in healthy adults. We have observed a blood pressure increase associated with subchronic BC exposure. The microvasculature, assessed by retinal image analysis, did not mediate these effects in our study.

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Chapter 6

Separate and Combined Effects of Hypoxia and Physical Inactivity During Horizontal Bedrest on Retinal Vessel Diameters

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Submitted

ABSTRACT

Introduction: This study was part of a larger study investigating the separate and combined effects of physical inactivity and hypoxia on different physiological systems. Here, we report the changes in the diameters of retinal blood vessels as a measure of the microvascular response to the interventions.

Methods: Eleven healthy male subjects (age=27 ± 6 years) completed three arms of a cross-over study with wash-out periods of 5 months. There were 3 trials of 21 days: (1) normoxic horizontal bed rest (NBR; fraction of ambient O_2 (FO_2)=0.209), (2) hypoxic ambulation (HAMB; FO_2=0.141 ± 0.004), and (3) hypoxic horizontal bed rest (HBR; FO_2=0.141 ± 0.004). The fundi of subjects' eyes were repeatedly photographed: 1 day before intervention, 9 times during intervention, and on day 1 and day 2 of recovery. Retinal blood vessel diameters were summarized as Central Retinal Arteriolar and Venular Equivalents (CRAE and CRVE, respectively). Changes in CRAE and CRVE were calculated by taking day before intervention as baseline. Statistical analysis was done using linear mixed-effects models.

Results: NBR caused a significant reduction in CRAE values throughout the intervention; and a quick return to baseline values on recovery day 1. CRVE values were not significantly affected. HAMB caused an immediate and significant increase in CRAE and CRVE, with values returning to their initial values on recovery day 1. HBR caused an initial increase in CRAE; an effect that disappeared. CRVE followed the same trend during HBR as during HAMB.

Conclusions: We conclude that retinal vessel diameters are useful to study the dynamic microvascular response.

INTRODUCTION

Mader and collaborators reported that acuity changes, secondary to hyperopic shifts, are common in astronauts that participated in long-term space missions. These changes are associated with a consistent set of diagnostic findings and include globe flattening, optic disc edema, choroidal folds, and in some individuals elevated intracranial pressure resulting in permanent vision impairment[1]. Microgravity-driven cephalad fluid shift, possibly leading to elevated intracranial pressure, may be a plausible etiology for the clinical observations[2]. Additional research using quantitative and qualitative magnetic resonance techniques identified in astronauts, previously exposed to microgravity, a spectrum of intraorbital and intracranial findings similar to those seen in patients suffering from idiopathic intracranial hypertension[3, 4].

Bedrest allows investigating the physiological changes that can be expected during spaceflight and are similar to those seen during aging[5, 6]. These studies are characterized by immobilization, inactivity, confinement and elimination of gravitational stimuli, such as posture change and direction. Unloading the body's upright weight and the absence of work against gravity cause an upward fluid shift,. affect body sensors and reduce overall sensory stimulatory responses[7].For example, bedrest is associated with changes in shear stress and endothelial dysfunction[8], increased responsiveness to vasoconstrictors[9], and vascular remodeling[10].

Bedrest studies, parabolic flight experiments and studies after orbital spaceflight indicate that intraocular pressure, ocular blood flow, retinal blood vessels, ocular structures and visual function may undergo changes in low gravity environment[11]. However, current knowledge is mainly derived from scattered reports and more structured research in this field is needed, particularly since space agencies are now developing plans for long-duration human missions. In addition to the long-term exposure to reduced gravity, astronauts will be exposed to hypobaric hypoxic environments in future planetary habitats. However, not much is known about how these environmental conditions affect physiological systems and well-controlled studies to investigate the effects are unique.

The present study was part of the larger PLANHAB project investigating the separate and combined effects of physical inactivity/unloading and hypoxia on several physiological systems during a stay in a simulated planetary habitat (http://www.planhab.com/). The specific aim of the present study was to assess the separate and combined effects of 21 days of bedrest and hypoxia on ocular structures, and in particular the retinal microvasculature. Since retinal blood vessels share anatomical and physiological features with the coronary and cerebral circulation[12, 13], and their average diameters has been reported to be an independent predictor for cardiovascular and cerebrovascular events[14]. Blood flow in these microvessels is largely regulated through myogenic and metabolic autoregulation and endothelial NO secretion[15]. We hypothesize that the diameters of the retinal blood vessels during the three interventions would reflect the effect of the environmental conditions on systemic cardiovascular responses, and possibly the risk of long-term exposure to such environments on the structure and function of the eye.

MATERIAL AND METHODS

Study Population

Details about the study participants have been previously reported by Debevec and collaborators[16]. A total of 65 healthy males were initially screened for participation in the study. Besides inclusion/exclusion

criteria outlined in the standardization of bedrest condition, individuals recently (<2 months) exposed to altitudes above 2000 m were ineligible to participate. Following preliminary testing, 14 participants were selected and gave written informed consent after receiving detailed information regarding study protocol and experimental procedures. Two participants did not return for the last campaign due to personal reasons and one participant had to be withdrawn from the study during the last campaign as a result of gastrointestinal health problems. Ultimately, 11 participants completed the entire study and only their data are reported in this manuscript. All participants were healthy, near sea level residents (<500 m) with the following baseline characteristics obtained during the medical screening: age=27 ± 6 years (mean ± SD); BMI=23.7 ± 3.0 kg m^{-2}; maximal oxygen uptake=44.3 ± 6.1 mL kg^{-1} min^{-1}.

Study Protocol

The study protocol was approved by the National Committee for Medical Ethics at the Ministry of Health of the Republic of Slovenia and conformed to the guidelines of the declaration of Helsinki. The participants underwent three experimental campaigns in a counterbalanced fashion: (1) normoxic horizontal bedrest (NBR; fraction of ambient O_2 (FO_2)=0.209; partial pressure of inspired O_2, (P_iO_2)=133.1 ± 0.3); (2) hypoxic ambulatory confinement (HAMB; FO_2=0.141 ± 0.004; P_iO_2=90.0 ± 0.4; ~4,000 m simulated altitude); and (3) hypoxic horizontal bedrest (HBR; FO_2=0.141 ± 0.004; PiO_2=90.0 ± 0.4; ~4,000 m simulated altitude). The experimental campaigns were conducted at the Olympic Sport Centre Planica (Rateče, Slovenia), situated at an altitude of 940 m. The participants entered each campaign in a sequential and fixed order with two participants entering each day. Campaigns lasted 32 days and had three distinct phases. The initial testing phase comprised the first 7 days upon arrival to the facility. This phase allowed the participants to acclimate to the facility, diet and circadian cycles. All baseline measures were obtained during this period. The second phase was the 21-day confinement phase (day 1–day 21) during which the participants were exposed to their designated condition (NBR, HAMB and HBR). This was followed by a 4-day recovery phase at the Olympic Sport Centre that enabled the researchers to obtain the post-confinement measurements and this period also allowed for cautious re-ambulation of the participants. All experiments and measurements were performed on the same days and time slots during the three campaigns. A 3-month wash-out period was implemented between the campaigns. The study was highly controlled in terms of diet and physical activity levels of the study participants. More details about daily routines during the campaigns can be found in Debevec et al.[16] and Ciuha et al.[17].

Retinal Photography

A Canon 45° 6.3 megapixel digital non-mydriatic retinal camera (Hospithera, Brussels, Belgium) was used. The fundi of subjects' right and left eyes were photographed twice at distinct time points: 1 day (-1) before the intervention as baseline measurement, on day 1, 2, 3, 4, 6, 8, 10, 15, and 21 of the intervention, as well as on day 1 (R1) and day 2 (R2) of recovery. The tests were conducted at the same time of day between 16h30 and 18h30.

The procedure for obtaining a high quality image of the retina has been reported before[18]. Participant characteristics were masked for the trained grader before review and analysis of the retinal images. IVAN retinal image analysis software was used to measure retinal vessel diameters according to previously reported protocols[19]. Retinal vessel diameters were summarized as the Central Retinal Arteriolar Equivalent (CRAE) and Central Retinal Venular Equivalent (CRVE) for each image. The equivalents represent a summary of vessel diameters within an area equal to 0.5–1 disc diameters from the optic

disc margin. Average CRAE and CRVE values were calculated for each time point based on the four images.

Blood Pressure

Systolic blood pressure, diastolic blood pressure and heart rate were measured, each morning at 7.00 am, with an automated device (Stabilograph, Stolberg, Germany), according to the guidelines of the European Society of Hypertension[20]. After the subjects had rested for 5 min, the blood pressure and heart rate were measured five times consecutively. The average of the last three measurements was calculated and used in the analyses.

Statistical Analysis

Linear mixed-effects regression models were used to compare changes in CRAE and CRVE values (SAS, version 9.2; SAS Institute Inc., Cary, NC, USA). The models included the sequence in which the treatments were completed, the period when each treatment was conducted, the treatment (NBR, HAMB, and HBR), time (day of the examination), and time-by-treatment interaction as categorical fixed effects and subjects nested within sequence as random effect. The interaction term examines the effect of the treatment on changes in CRAE and CRVE and the random effect accounts for similarities across sessions for each person.

RESULTS

High-resolution retinal images were collected from both eyes of the PLANHAB participants on 12 different days during each of the three study campaigns. All images were screened by an ophthalmologist for any signs of high-altitude retinopathy. All subjects were asymptomatic during all three interventions. Responses of the retinal microvasculature to the experimental conditions were assessed by measuring changes in Central Retinal Arteriolar Equivalent (CRAE) and Central Retinal Venular Equivalent (CRVE) compared to the baseline values obtained on the day before normoxic bedrest (NBR), hypoxic ambulation (HAMB) and hypoxic bedrest (HBR) started (day of intervention-1).

The data for CRAE are depicted graphically in Figure 1. A significant vasoconstriction of 2.31 µm (95% CI: -0.20 to -5.65; p=0.035) was observed after day 1 in NBR. CRAE values reduced progressively during the intervention period and reached a maximal vasoconstriction of 7.47 µm (95% CI: -10.78 µm to -4.15 µm; p<0.0001) at day 15 of the intervention. The CRAE returned to the baseline value upon completion of the intervention, during the first day of recovery (R1). On day 1 of the HAMB intervention, CRAE increased by 7.38 µm (95% CI: 4.07 to 10.70; p<0.00001). The values remained elevated at this level throughout the 21-day intervention period and dropped to baseline values during R1. HBR (combined exposure to hypoxia and bedrest) caused a significant increase in CRAE by 3.23 µm (95% CI: 0.03 to 6.42; p=0.019) day 1. In contrast to the response observed in HAMB, the effect during HBR was transient; by day 3 of the intervention, ΔCRAE started to decrease from an average of 4.49 µm (95% CI: 1.23 to 7.75; p=0.007) towards baseline values, reaching them on day 8. CRAE values remained at baseline values thereafter. Detailed information about the changes in CRAE (ΔCRAE) on all study days is given in Supplementary Table 1.

CRVE decreased by 7.90 µm (95 CI: -13.32 to -2.48; p=0.004) on day 1 of NBR (Figure 2). CRVE values remained decreased during the intervention, but this reduction was only significant on day 15 (ΔCRVE=7.15 µm; 95 CI: -12.58 to -1.73; p=0.001). CRVE returned to baseline value during R1 of the

recovery period. HAMB caused an engorgement, which resulted in a significant increase in CRVE of 16.56 µm (95 CI: 11.14 to 21.98; p<0.001) on day 1; a significant increase that persisted during the intervention and the first recovery day. CRVE returned to the baseline on R2 (Figure 2). CRVE followed the same trend during HBR as during HAMB, increasing by 9.79 µm (95% CI: 4.57 to 15.02; p<0.001) on day 1, and remaining elevated during the entire intervention period, as well as on R1. There was a significant 7.75 µm (95% CI: -12.97 to -2.52; p=0.004) undershoot of CRVE below the pre-intervention values, on R2. Detailed information about the changes in CRVE on all study days is given in Supplementary Table 2.

Pairwise comparisons indicate that hypoxia caused a significant dilating effect during bedrest because the 95% confidence intervals of ΔCRAE and ΔCRVE do not include zero (HBR vs NBR). Under hypoxic conditions, there was a significant effect of ambulation on CRAE when comparing both hypoxic conditions, and borderline significance for CRVE during the second half of the intervention (HAMB vs HBR). The biggest effects were observed when comparing HAMB vs NBR (Figure 3).

The responses of systolic blood pressure (SBP) and diastolic blood pressure (DBP) during the three interventions are given in Supplementary Table 3 and Supplementary Table 4, respectively. SBP was increased during NBR, but this increase was only significant on day 4 [6.55 mm Hg (95 CI: 0.59 to 12.50; p=0.026)], day 6 [7.18 mm Hg (95 CI: 1.22 to 13.14; p=0.0063)], day 15 [8.45 mm Hg (95 CI: 2.50 to 14.41; p=0.011)] and day 21 [6.82 mm Hg (95 CI: 0.86 to 12.78; p=0.010)]. DBP was significantly increased on day 3 [7.36 mm Hg (95 CI: 2.35 to 12.38; p=0.0019) and remained significantly increased during the intervention. SBP and DBP were both significantly increased during HBR led to significant increases in SBP and DBP. For example, SBP and DBP were respectively increased with 8.00 mm Hg (95 CI: 1.98 to 14.02; p=0.0087) and 8.45 mm Hg (95 CI: 3.44 to 13.47; p=0.0010) on day 2. During the HAMB intervention, SBP was elevated, but reached only a significant increase on day 8 [9.31 mm Hg (95 CI: 3.19 to 15.42; p=0.0030)]. In contrast, DBP remained significantly increased from day 2 [6.80 mm Hg (95 CI: 1.66 to 11.58; p=0.0097)] for the whole study period. In an additional analysis, SBP and DBP were added as covariates to the models of CRAE and CRVE. The reported effect on the retinal microvasculature did not change.

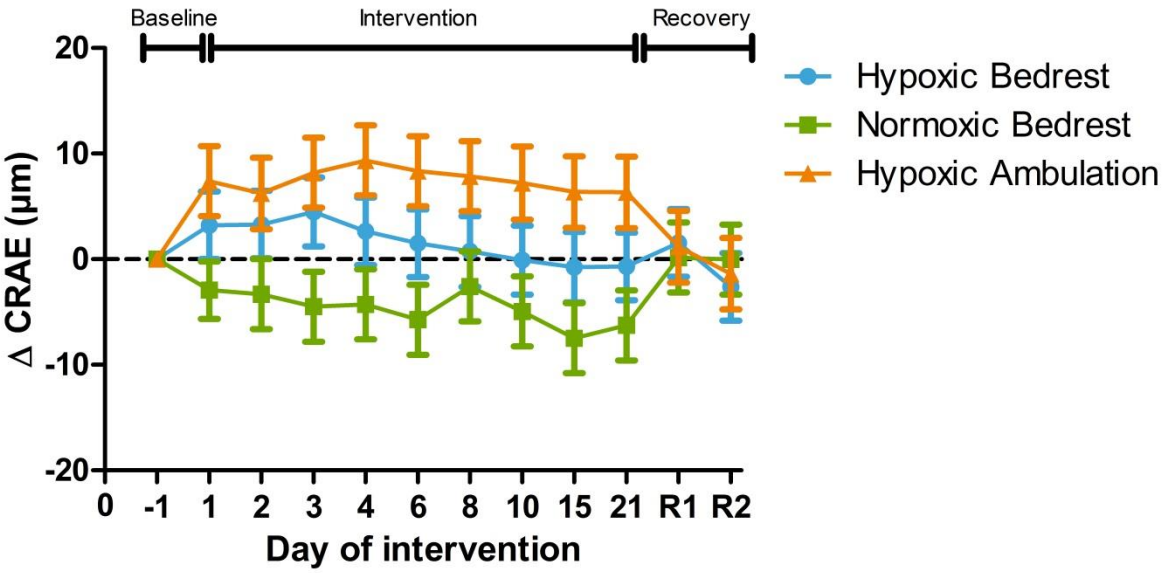

Figure 1: Retinal arteriolar response summarized as Central Retinal Arteriolar Equivalent (CRAE) during a 21-day cross-over study with hypoxic ambulation, hypoxic bed rest and normoxic bed rest as experimental conditions. Changes in CRAE (ΔCRAE and 95% confidence intervals) relative to baseline (-1) are presented for the 21-day intervention (days 1 to 21) and recovery (R1, R2). Values are statistically significant when confidence intervals do not include zero.

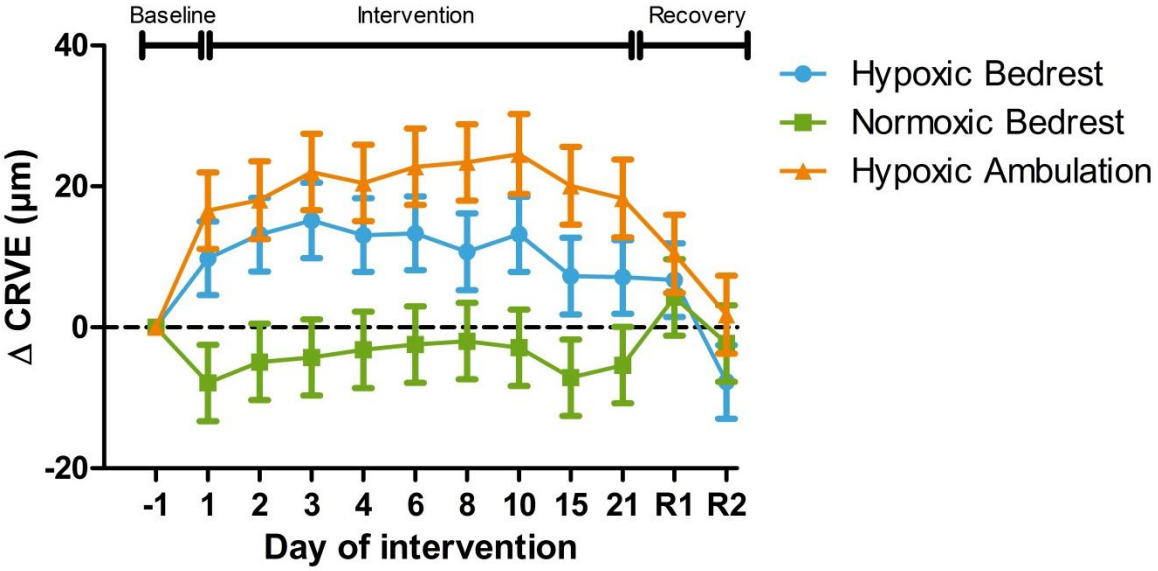

Figure 2: Retinal venular response summarized as Central Retinal Venular Equivalent (CRVE) during a 21-day cross-over study with hypoxic ambulation, hypoxic bed rest and normoxic bed rest as experimental conditions. Changes in CRVE (ΔCRVE and 95% confidence intervals) relative to baseline (-1) are presented for the 21-day intervention (days 1 to 21) and recovery (R1, R2). Values are statistically significant when confidence intervals do not include zero.

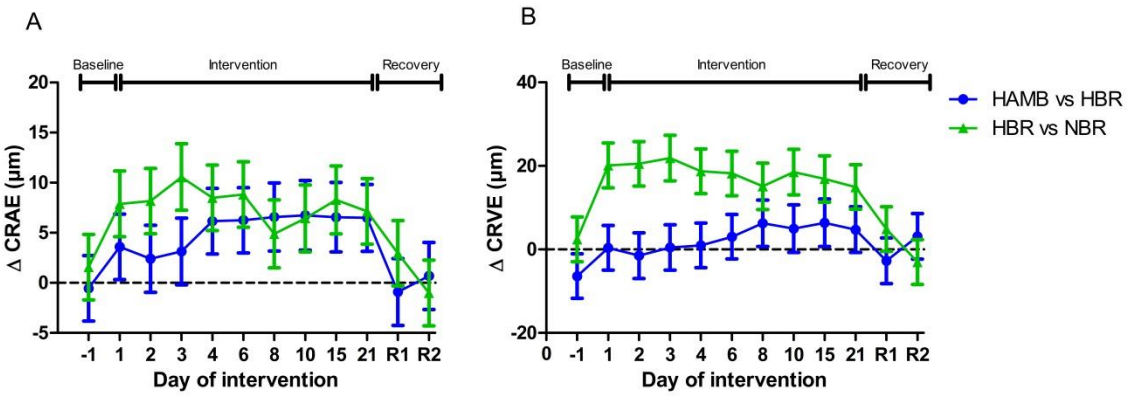

Figure 3: Differences in retinal arteriolar and venular responses between study conditions. Difference in CRAE (ΔCRAE and 95% confidence intervals) (A) and CRVE (ΔCRVE and 95% confidence intervals) (B) between study conditions are presented for every time point for each study condition. Values are statistically significant when confidence intervals do not include zero.

DISCUSSION

Recent clinical observations of functional and structural changes in the eye have renewed the interest in environmental physiology experiments that examine the impact of spaceflight factors. We have studied the effect of separate and combined exposure to horizontal bedrest and hypoxia on retinal vessel diameters. We used changes in Central Retinal Arteriolar/Venular Equivalent (CRAE/CRVE) to assess the microvascular response. The diameters of the arterioles were significantly reduced during normoxic bedrest. Normobaric hypoxia caused observable dilatation of retinal arterioles and venules. The exposure to the combined factors caused an intermediate response, with mainly a significant dilatation of the venules. All diameters returned to baseline values after a 48h-recovery period.

Normoxic bedrest (NBR) induced a non-progressive retinal arteriolar vasoconstriction in our study. This is consistent with other studies that observed decreased individual diameters of retinal arterioles and venules after 48 h in a -10 degrees position[21, 22]. We did however not observe an effect on the venular diameters. This might be due to smaller fluid shift during horizontal bedrest compared to head-down tilt. The supine position during bedrest will have increased blood pressure in the head and induced retinal myogenic autoregulation leading to arteriolar narrowing to maintain retinal blood flow[23, 24]. Prolonged physical inactivity can cause endothelial dysfunction and the production of systemic vasoconstrictors. These changes in the bioavailability of vasoactive agents may also have contributed to arteriolar narrowing[25]. Narrowing of retinal arteriolar vessels is in line with other bedrest studies that reported decreased basal arterial diameters[26, 27] and progressive peripheral vasoconstriction[28]. Very few studies have investigated how bedrest and physical inactivity may affect the retinal microvasculature. Interestingly, an epidemiological study with young children identified a significant negative association between physical inactivity and TV screen time on the one hand, and retinal arteriolar diameters on the other hand[29].

The widening of retinal arteriolar and venular diameters during hypoxic ambulation (HAMB) is consistent with the universally observed retinal changes associated with altitude hypoxia. The hypoxic condition will lead to a drop in ocular oxygenation. Subsequently, metabolic autoregulation will lead to vasodilatation of arterioles and venules to increase blood flow to the retinal tissue and ensure sufficient O_2 supply. The changes are reversible with decreasing altitude, leaving no residual effect when the hypoxic conditions are maintained for short periods of time[30]. Observations done in the retina may also hold true for other

microvascular beds[31]. For example, cerebrovascular vessels, show a transient vasodilation response in the first days of hypoxia, but return to baseline after a few days of hypoxic exposure[32, 33]. However, prolonged exposure to hypoxia may lead to vascular dysfunction and remodeling as a result of changes to vascular tone mediated through hypoxia-induced sympathoactiviation[34, 35].
Hypoxic bedrest (HBR) induced an arteriolar vasodilation, but the effect was significantly smaller than during HAMB. Two different autoregulatory responses are at work during HBR. The supine position will have elicited myogenic constriction whereas the hypoxic environment will have induced metabolic vasodilation to regulate blood flow. As a consequence, arteriolar vasodilation during HBR was smaller compared to HAMB. At day 4 of the intervention, hypoxia-induced arteriolar narrowing started attenuating. Possibly, this might have been due to increases in systemic blood pressure or endothelial dysfunction. The venular widening was comparable between HBR and HAMB. To the best of our knowledge, this is the first study that reports on the combined effects of hypoxia and bedrest, as a model of physical inactivity, on the retinal microvasculature.

The strengths of the study are the well-controlled study design that carefully controlled for the daily routines, physical (in)activity schemes and nutritional intake. Furthermore, repeated measurements have been obtained in a homogenous study population, which reduces between-individual variability and increases the statistical power in a small panel. Limitations of the study are the lack of a more integrated analysis of ocular fluid shifts. For example, it is known that head-down bedrest and stay in microgravity can increase intraocular pressure[36]. Availability of such data may be helpful in interpreting the results coming from retinal microvascular measurements. In this study, we were only able to measure intraocular pressure during the last arm of the cross-over study using a handheld tonometer. The pressures were in a normal range of 13.5 ± 1.2 mm Hg, with no obvious changes between the study groups. However, a full data set is missing in order to take intraocular pressure into account for statistical analysis. Measurements of ocular blood flows or cerebral blood flows might shed additional light on the dynamic cardiovascular responses related to changes in retinal vessel diameters. However, this requires additional burden for the study participants and is not always possible from a technical point of view, considering that bedrest patients should be measured in supine position in order to avoid immediate responses of the cardiovascular system to erect position.
In conclusion, we report for the first time the combined and separate effects of horizontal bedrest and hypoxia on retinal vessel diameters. Using a repeated measures design we have shown that both arteriolar and venular components of the retinal microvasculature acutely responded to the experimental conditions, with a return to baseline values within 48h h after the 21-day intervention. Retinal fundus photography is a convenient and easy technique to document these changes. The responses showed a normal physiological pattern in this study, but it remains to be elucidated what the long-term impact could be when these experimental conditions are sustained.

On the one hand, long-term vasoconstriction might lead to suboptimal blood flow to the retinal tissues, with the latter having been associated with the development of glaucoma and ischemic neuropathy[37, 38]. On the other hand, vasodilatation is a compensatory mechanism in hypoxia to increase blood flow to supply sufficient oxygen to the tissues. Improper vasodilatation of deconditioned blood vessels could result in inadequate blood flow patterns and could potentially intensify tissue hypoxia and aggravate hypoxia-induced cellular damage. This might cause functional and structural changes in the microvasculature that may be relevant in clinical trajectories of cardio- and cerebrovascular diseases[39]. In

this respect, it has also been suggested that changes in ocular functions (such as retinal microvasculature) are of great importance for VIIP. The syndrome could breach the clinical horizon and become symptomatic in the form visual acuity shift at the 6-week mark during spaceflight[40]. Effects of combined physical inactivity and hypoxia should be further studied because the outcome of those studies are important for people living in hypoxic environments at reduced gravity in planetary habitats, but also for patients suffering from hypoxic conditions such chronic obstructive pulmonary disease, which are highly inactive.

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Chapter 7

Retinal Microvascular Responses to Maximal Endurance Cycling in Rehabilitating Cardiac Patients

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Submitted

ABSTRACT

Introduction: Exercise-based rehabilitation is an effective measure to improve endothelial function and overall cardiac fitness of cardiac patients. Maximal endurance tests are performed to assess cardiac fitness, but may also serve to test retinal microvascular reactivity, which might be compromised in cardiac patients.

Methods: 53 cardiac rehabilitating patients (age = 62 years; 73% male), who underwent a percutaneous transluminal coronary angioplasty, percutaneous coronary intervention or atraumatic coronary artery bypass intervention, were recruited at Jessa Hospital (Belgium). These patients participated in a cardiac rehabilitation program and performed two maximal endurance tests at start and after 6 weeks of training. Fundus images were collected immediately before, 0, 5, 10, 15 and 30 minutes after these tests. Image analysis was used to calculate the width of retinal blood vessels, represented as the Central Retinal Arteriolar/Venular Equivalent (CRAE/CRVE). Mixed models were used for statistical analysis.

Results : Average CRAE and CRVE were increased and decreased with respectively 2.97 µm (95% CI: 1.98 to 3.96; $p<0.001$) and 1.88 µm (95% CI: -3.71 to -0.05; $p=0.05$) after the test. CRAE remained significantly increased whereas a trend to venular widening became apparent after the test. A similar pattern was observed after the second maximal endurance test.

Conclusions: Assessment of exercise-induced retinal microvascular changes in cardiac rehabilitation patients is possible. Microvascular vasodilation is an essential response to increased flow. Our results suggest that retinal microvascular reactivity is preserved in cardiac rehabilitation patients after a maximal endurance test. However, microvascular reactivity was not improved after the 6-week rehabilitation program.

INTRODUCTION

Physical activity is an effective measure for primary and secondary prevention of cardiovascular diseases.[1] Regular physical activity is associated with a 35% risk reduction for cardiovascular disease mortality and a 33% reduction for all-cause mortality.[2] Exercise training has proven positive effects in cardiac patients: lowered hospitalization rates and improvement of cardiovascular risk factors.[3] In patients with heart failure, exercise training improved VO2-max and peak exercise duration, reversed abnormal vasomotor tone characteristics and reduced inflammatory markers.[4, 5] Myocardial infarction survivors showed improvements in myocardial perfusion and cardiac contractile function after completion of a training program[6].

Endothelial dysfunction or impaired microvascular reactivity is a hallmark of traditional cardiovascular risk factors (diabetes, hypercholesterolemia, hypertension, atherosclerosis, smoking) and cardiovascular diseases such as heart failure, acute coronary syndrome, coronary artery disease and myocardial infarction.[7] Endothelial dysfunction is characterized by impaired endothelium-mediated vasodilation and enhanced vasoconstriction of arteries and arterioles.[8] Interestingly, several studies have proven that exercise training can restore endothelial function in cardiac patients.[5, 9-11] Exercise increases cardiac output, blood flow and shear stress on the blood vessel wall.[6] Shear stress enhances the upregulation of eNOS gene expression and protein levels, eventually reduces oxidative stress and thus limits NO scavenging by reactive oxygen species.[6 12] In this way, NO bioavailability is increased and endothelial function improved. This may be an underlying pathway of exercise-induced cardio protection.

Retinal vessel diameters are used to assess cardiovascular risk or disease state as they are affected early in the process of cardiovascular disease.[13-15] Retinal vascular caliber changes are associated with presence of cardiovascular disease, hypertension and markers of systemic endothelial dysfunction[15]. Furthermore, wider venular caliber is associated with reduced brachial artery flow-mediated dilation, a traditional marker for endothelial dysfunction.[16]

Moreover, several imaging techniques allow a non-invasive assessment of the retinal endothelial function or microvascular reactivity. Static and dynamic retinal vessel analysis before and after dynamic exercise has shown that these blood vessels respond to dynamic and isometric exercise.[17, 18] In healthy adults and seniors, retinal microvascular reactivity was preserved after dynamic exercise as retinal arteriolar and venular dilation was observed 5 minutes and persisted for at least 40 minutes after a (sub)maximal treadmill test.[18]

In this study, retinal microvascular reactivity of cardiac rehabilitation patients in response to a maximal endurance test was assessed by measuring repeatedly the response of the retinal vessel diameters using static retinal images. The patients were enrolled in an exercise training program of 6 weeks and performed two maximal endurance tests at the beginning of the program and after 6 weeks of training. First, we hypothesize that these tests will induce vasodilation of the retinal arterioles and venules. Second, we hypothesize that the exercise program will improve endothelial function and retinal microvascular reactivity, i.e. the consecutive endurance tests will show an improvement in retinal arteriolar and venular vasodilation.

MATERIAL AND METHODS

Study population

Patients with ischemic heart disease who participated in the cardiac rehabilitation program of the "Revalidatie en Gezondheid" center (Jessa Hospital, Hasselt, Belgium) were recruited between May 2015 and October 2015. In total, 60 patients were invited to participate in the study, of which 53 (88%) agreed to participate. Only patients who had a myocardial infarction or who recently underwent atraumatic coronary artery bypass surgery or percutaneous (transluminal) coronary intervention were included in the study. The patients were free of pulmonary diseases and ophthalmological complications that hampered retinal imaging. Recent data on health characteristics (diabetes, hypertension, dyslipidemia), medication use and smoking behavior were extracted from the patient's files. The study was approved by the ethics committee of Jessa Hospital and Hasselt University. Written informed consent was obtained from all study participants.

Maximal Endurance Cycling Test

Maximal endurance tests were performed on an electronically braked cycle ergometer (eBike 1.8, GE Healthcare, Diegem, Belgium) in a non-fasting condition under usual medications. After 1 minute of rest followed by 1 minute of unloading cycling, the initial load was set at 20 W for 1 minute and was increased by 10 or 20 W every 2 minutes until exhaustion. Cycle load increments were based on previous exercise testing at the hearth failure clinic, with the goal of a test duration of approximately 10 minutes. A 12-lead electrocardiogram was monitored continuously (Cardiosoft 6.6, GE Healthcare, Diegem, Belgium). Minute ventilation (V_E), oxygen uptake (V_{O_2}) and carbon dioxide output (V_{CO_2}) were acquired breath by breath and averaged over 10-second intervals. Peak V_{O_2} and peak respiratory exchange were recorded using the highest 10-seconds average obtained during the last minute of the test. The first ventilator threshold was set at the nadir of the V_E/V_{O_2} curve, and the second ventilator threshold was set at the nadir of the V_E/V_{CO_2}.

Cardiac rehabilitation program

The cardiac rehabilitation program was consistent with published recommendations[19]. Briefly, patients trained 3 times every weeks for 12 weeks. The program consisted of a 5-minute warming-up at 0 to 25W on a cycle ergometer (Gymna, Ergo-fit Ergo Cycle 157, Bilzen, Belgium) followed by progressive endurance training. Endurance training initially involved 2 cycling bouts followed by 2 bouts of walking on a treadmill (Gymna, trac 3000 S Alpin, Bilzen, Belgium). The exercise intensity in the first training session was set at the power output and heart rate achieved just before the second ventilatory threshold, as determined from the incremental cardiopulmonary exercise test, performed at the start of the program. Duration of the exercise bout was progressively increased from 6 to 8 minutes (0-6 weeks) to 8 to 10 minutes (6-12 weeks). The 4 exercise bouts were alternated with rest periods of 2 minutes.

Study design

Participants were examined during two clinical visits that were 6 weeks apart from each other. All visits were performed at approximately the same time of day (visits were on average 90 minutes apart) to avoid diurnal variation. Retinal photographs were collected immediately before as well as directly after, 5 min, 10 min, 15 min, and 30 min after the maximal endurance cycling test. Of the 53 patients, who

completed the first maximal endurance cycling test, 45 completed the 6-week rehabilitation program and returned for the second maximal endurance cycling test.

Retinal photography and vessel analysis

The fundus of the right eye, or the left eye when the fundus of the right eye could not be photographed, of each participant was photographed twice using a Canon 45° 6.3 megapixel digital non-mydriatic retinal camera (Hospithera, Brussels, Belgium) using procedures described before[20]. Photographs were graded using retinal image analysis software developed by DCI Labs (Keerbergen, Belgium, www.dcilabs.com). The calculation of Central Retinal Arteriolar Equivalent (CRAE) and Central Retinal Venular Equivalent (CRVE) was based on previously reported protocols[21]. The equivalents represent a summary of vessel diameters within an area equal to 0.5-1 disc diameter from the optic disc margin. Average CRAE and CRVE values were calculated based on the two images taken at each time point. Participant and time point characteristics were masked for the trained grader before review and analysis of the retinal images.

Statistical analysis

We used mixed models to investigate the responses of CRAE and CRVE to the maximal endurance cycle test (version 9.2, SAS Institute Inc, Cary, NC). A random effect for each participant was used across the six time points. This method allows each subject to serve as his/her own control over time and it controls for potential confounding from within-subject covariates that do not change over time. All analyses were corrected for age, sex (male/female), BMI, medication use, presence of diabetes (yes/no), hypertension (yes/no) or dyslipidemia (yes/no) and smoking status (current/former/never). In an additional model, fellow vessel diameter was added to the previously described covariates. In order to compare the differences between the endurance tests, a mixed model with an interaction effect (time point-by-endurance test) was constructed. The interaction term examines whether arteriolar and venular responses are different between the two maximal endurance cycling tests.

Characteristic	Mean ± SD or n(%)
Personal Characteristics	
Age (years)	61.7 ± 12.5
Sex Male (%)	73%
Smoking status Current	38%
General Health Characteristics	
Body Mass Index	26.6 ± 3.9
Smoking status	
Diabetes	23%
Hypertension	49%
Dyslipidemia	77%
Medication use	
Anti-hypertensive drugs	72%
Beta-blockers	51%
Statins	81%
CRAE (µm)	129.98 ± 12.3
CRVE (µm)	204.44 ± 20.2

Table 1: Population characteristics (n=53)

RESULTS

The health characteristics of the study population are summarized in Table 1. Of the 53 participants, 73% were male. The mean age of the study participants was 61.7 ± 12.5 years. Average CRAE and CRVE at baseline were respectively 129.98 ± 12.3 µm and 204.44 ± 20.2 µm. Of the 53 participants that participated in the first endurance test, only 45 participated in the second endurance test.

The results of the cardiac rehabilitation program are summarized in Table 2. We observed a significant increase in VO_2max after 6 weeks of training. Baseline CRAE and CRVE values were not changed. Baseline retinal vessel diameters did not predict VO_2max or other parameters of the endurance test, nor was any of these parameters a predictor of CRAE and CRVE responses after the test.

	Before exercise	After exercise	p-value
vO2max (mL/kg/min)	19.23 ± 5.35	20.81 ± 5.37	<0.001
Anaerobic threshold (Watt)	109.81 ± 41.81	108.48 ± 22.45	0.10
FEV1 (L)	2.92 ± 0.75	2.89 ± 0.69	0.51
FVC (L)	4.02 ± 0.92	4.02 ± 0.90	0.91
CRAE (µm)	129.60 ± 11.53	130.64 ± 11.93	0.38
CRVE (µm)	203.84 ± 20.22	204.66 ± 21.61	0.38

Table 2 Baseline parameters before and after 6-week training program

Changes in CRAE and CRVE after the endurance cycling test were investigated in a mixed model that had age, sex, BMI, medication use, presence of diabetes, hypertension or dyslipidemia and smoking status as covariates. A mixed model additionally corrected for fellow vessel diameter.

CRAE was significantly increased after the first endurance test and remained increased up to 30 minutes after the endurance cycling test (Figure 1 and Supplementary Table 1). Compared to the baseline measurement before the endurance test, CRAE was increased with 2.97 µm (95% CI: 2.09 to 3.86; p<0.0001) after exercise cessation. Thirty minutes after exercise cessation, CRAE was increased with 3.60 µm (95% CI: 2.50 to 4.70; p<0.0001) (Figure 1A and Supplementary Table 1, Model 1). Additional correction for CRVE did not change the effects (Supplementary Table 1, Model 2). Immediately after the endurance test, CRVE was decreased -1.91 µm (95% CI: -3.69 to -0.13; p=0.0413). A trend to increase in CRVE became apparent on the other time points, which resulted in a significant increase of CRVE of 2.23 µm (95% CI: 0.02 to 4.43; p=0.049) 30 minutes after exercise cessation (Figure 1B and Supplementary Table 1, Model 1). When CRAE was added to the model as a covariate, CRVE was significantly decreased after and 5 minutes after exercise cessation, but not on other time points (Supplementary Table 1, Model 2).

The second endurance test was performed after 6 weeks of training. CRAE was increased with 3.03 µm (95% CI: 2.18 to 3.88; p<0.0001) after the endurance cycling test. CRAE remained significantly increased for the whole study period and reached a maximal increase of 5.05 µm (95% CI: 3.59 to 6.51; p<0.0001) 30 minutes after exercise cessation (Figure 1A and Supplementary Table 2, model 1). Adding CRVE to the model did not change the reported effects. (Supplementary Table 2, model 2)). CRVE was significantly decreased after exercise cessation with 3.59 µm (95% CI: -6.04 to -1.15; p=0.0066). Again, a trend to increase in CRVE became significant 30 minutes of exercise cessation, where CRVE was increased with 3.54 µm (95% CI: 1.76 to 5.32; p=0.0004) (Figure 1B and Supplementary Table 2, model 1). When CRAE was added to the model, this effect became non-significant (Supplementary Table 2, model 2).

Comparison of the retinal responses between the two endurance cycling tests were done using data from the 45 participants who completed both endurance cycling tests. The responses of CRAE and CRVE after the first endurance cycling test were unaffected when these 8 participants were excluded (Supplementary Table 3). We did not observe any differences between the endurance tests in responses of CRAE or CRVE (Figure 2).

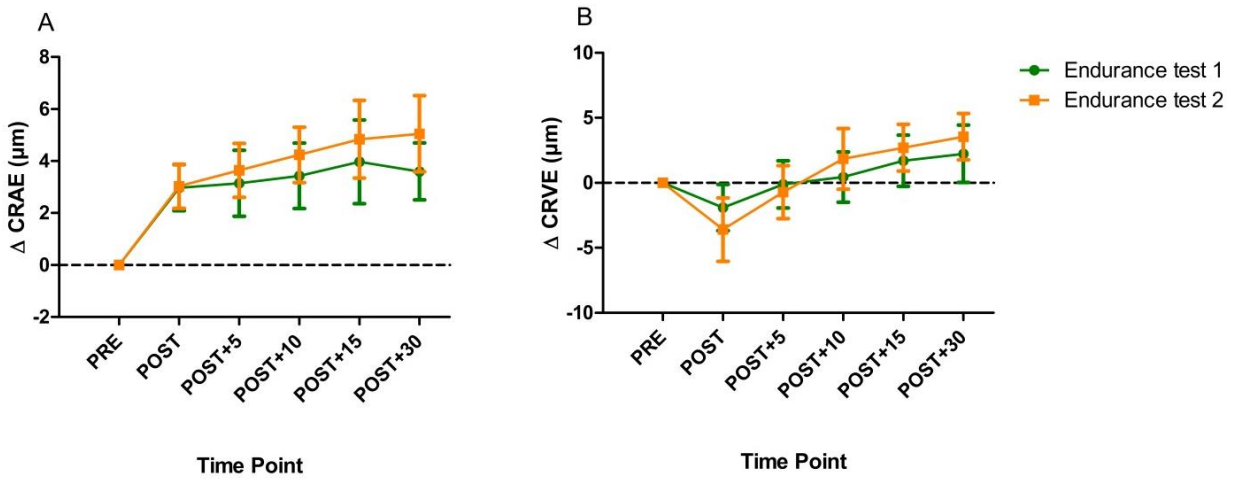

Figure 1 Retinal arteriolar and venular responses after the endurance tests. Estimated changse in mean CRAE (µm) and CRVE (µm) (95% CI) after the endurance tests compared to the baseline value, measured before the test (Time Point PRE). Fifty three patients participated in the first maximal endurance test, whereas only 45 patients completed the rehabilitation program and participated in the second maximal endurance test. The used model is adjusted for age, sex, BMI, medication use, presence of diabetes, hypertension or dyslipidemia and smoking status. Changes in CRAE and CRVE are significant when the 95% confidence intervals do not overlap with zero.

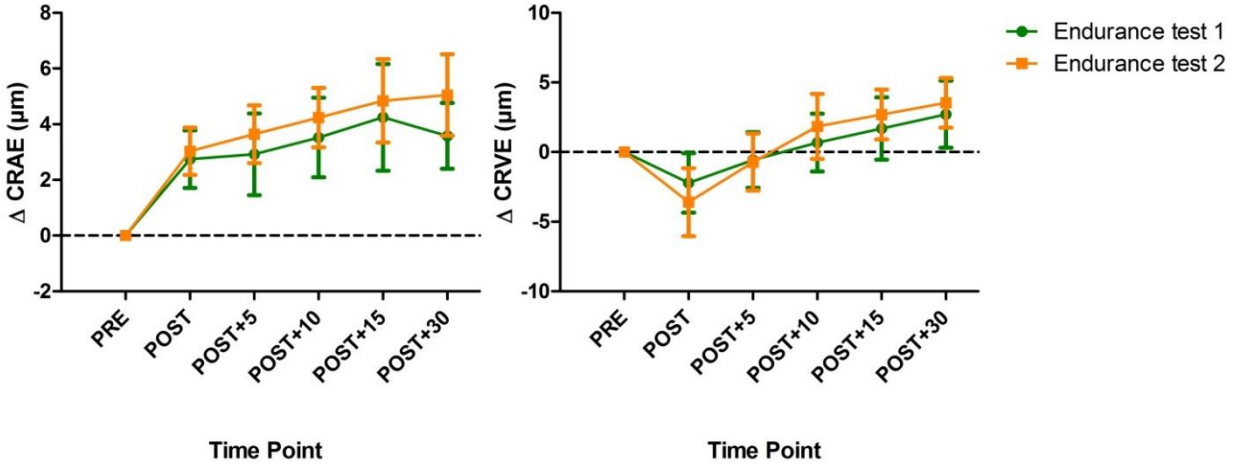

Figure 2: Retinal arteriolar and venular responses after the endurance tests. Estimated changse in mean CRAE (µm) and CRVE (µm) (95% CI) after the endurance tests compared to the baseline value, measured before the test. Only patients (n=45) who completed the rehabilitation program and both endurance tests are included in the analysis. The used model is adjusted for age, sex, BMI, medication use, presence of diabetes, hypertension or dyslipidemia and smoking status. No statistical difference in arteriolar or venular responses is observed after the endurance test.

DISCUSSION

The primary focus of this study was to investigate whether retinal imaging could be used to assess acute retinal microvascular responses in rehabilitating cardiac patients that performed maximal endurance cycle tests. Retinal blood vessels of cardiac patients are able to dilate immediately in response to a maximal endurance test and remain dilated for at least 30 minutes after completion of the test. These findings are in line with others who also observed retinal vasodilation immediately after dynamic exercise in healthy adults.[18] The vasodilatory effects were observed at the beginning of the exercise rehabilitation program and after 6 weeks of training, with no difference in response between the two time points. Static vessel analysis before and after dynamic exercise is feasible and may be useful to assess retinal microvascular reactivity. Retinal microvascular reactivity is envisioned to be clinically relevant. The retinal and cerebral microvessels share several anatomical and functional features.[22-24] Hence, studying retinal responses after exercise might offer information on the cerebral microvascular reactivity. Metabolic and hemodynamic changes cause a decrease in peripheral and an increase in cerebrovascular resistance during and after exercise. The increase in cerebrovascular resistance is achieved through myogenic vasoconstriction.[25] This will prevent a rise in cerebrovascular blood flow during and after exercise, protecting the brain from an overshooting of flow and potentially hemorrhage. Increased microvascular reactivity, due to exercise training, may contribute to this protective effect.

Cardiac output and systolic blood pressure increase during exercise. At the same time, peripheral vasodilation decreases vascular resistance in order to further increase blood flow. This mechanism does not fully apply to the autoregulated retinal microcirculation that lacks autonomic innervation. Retinal vasomotor tone and vessel diameter regulation depend on vasodilatory effects, such as shear stress-mediated NO release from the endothelial cells, and vasoconstrictive effects such as smooth muscle cell mediated myogenic autoregulation, changes in blood gases and local vasoactive peptides.[24] The increased perfusion pressure during high-intensity exercise will ensure domination of retinal myogenic vasoconstriction over the vasodilatory effects. Also, hyperventilation-induced hypocapnia may have further stimulate retinal vasoconstriction. Both mechanisms will ensure a constant blood flow during exercise.[24] However, after exercise cessation, when perfusion pressure, hemodynamic and metabolic stimuli normalize, the suppressed vasodilatory mechanisms will predominate. The increase in shear stress during exercise can provoke acute endothelium-mediated release of NO and cause a delayed upregulation of NO production. As a potent vasodilator, NO will induce vasodilation after exercise cessation to provide a steady blood flow.[26] The retinal venular responses after exercise cessation first show a significant decrease before a trend to venular widening starts. Venular constriction after the exercise might be a delayed response. Increased venous return will supply the heart with sufficient blood, that during exercise was forced into the arterial circulation. As similar hemodynamic and metabolic mechanisms affect arterioles and venules, the trend for retinal venular vasodilation corresponds with the arteriolar pattern. In the venules, shear stress will also have stimulated the endothelium to release NO which overrules the myogenic response. Also, arteriolar shear stress mediated release of NO might have a paracrine effect in the venules, contributing to the observed delayed vasodilation.

The retinal vascular responses after dynamic exercise have only been documented in healthy adults and seniors. Nussbaumer et al. reported that CRAE increased with 3.7 µm (95% CI: 1.9 to 5.5). We report similar changes in CRAE of cardiac rehabilitation patients. These distinct retinal vasodilatory responses

are somewhat surprising. Endothelial dysfunction is often observed in cardiac patients. As impaired flow-mediated dilation is a hallmark of endothelial dysfunction, smaller vasodilatory responses after dynamic exercise were expected when compared to healthy individuals.[27, 28] However, high-intensity exercise causes high shear stress on the endothelium that will be stimulated to release NO.[29] Moreover, smooth muscle cells responsiveness to NO is not altered when endothelial function deteriorates. Taken together, the increase in vessel diameter may still be caused by shear-mediated release of NO from the retinal endothelium. Aerobic exercise training is a proven measure to restore endothelial function in cardiac patients. Several investigators reported improvements in endothelial function, measured as brachial flow-mediated dilation, in different populations of cardiac patients after completion of a cardiac rehabilitation program of six, eight or twelve weeks.[30-32] Exercise causes an increase in blood flow and the associated increases in shear stress will augment NO bioavailability by increasing eNOS mRNA expression.[6,33] Regular exercise during cardiac rehabilitation can cause a persistent upregulation of eNOS, but this may take up to 10 days or several months.[34] Increased eNOS expression is associated with a constant NO synthesis and maintenance of basal vascular tone, but is also related to the vasodilatory responses of retinal arterioles to increased flow.

We used repeated measures of retinal microvasculature to assess the dynamic retinal response as a proxy for endothelial function. Despite an improvement in VO_2max, our data do not support that the 6-week exercise training program improved microvascular reactivity or caused vascular remodeling. During the rehabilitation program, exercise training will repeatedly and periodically increase and decrease shear stress and perfusion pressure in the retinal blood vessels. This may have improved retinal microvascular reactivity by increasing basal NO production and stimulating myogenic autoregulatory responses.[18, 35] Retinal blood flow is tightly regulated. Therefore, improved smooth muscle cell contractility might have countered enhanced NO-mediated vasodilation after the second endurance test. As a consequence, both endurance tests show similar retinal microvascular responses.

Our study had limitations. The retinal blood vessels could not be monitored continuously. Instead, retinal images were taken just before the endurance test and immediately after, 5, 10, 15 and 30 minutes after exercise cessation. The use of standardized time points makes the retinal vascular responses inter-and intra-individually comparable and reduces the need for continuous monitoring. As we did not further monitor the retinal blood vessels, we do not know how long the effect lasted. Exploring the molecular mechanism of increased NO bioavailability was beyond the scope of this study. Therefore, we solely relied on the measurements of retinal blood vessel diameters to analyze vascular reactivity. Furthermore, we did not include other measurements of endothelial function, such as brachial artery flow-mediated dilation. As a consequence, our assumptions about improved endothelial function are speculative. Our study did not have a control group who did not participate in the rehabilitation program. Hence, we cannot conclude whether microvascular reactivity improved or not due to the rehabilitation program or whether a 6-week rehabilitation program is too short to induce significant improvements in retinal endothelial function.

In conclusion, static retinal vessel diameter analysis can be used to assess retinal vascular reactivity after an endurance test in cardiac patients. Our results indicate that retinal vascular reactivity is preserved in these patients, but need not to be improved after a 6-week rehabilitating program.

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Chapter 8

General Discussion

CONTRIBUTIONS TO EXISTING RESEARCH

We investigated the influence of air pollution exposure and physical (in)activity on the retinal microvasculature using fundus photography. Air pollution exposure and physical inactivity are two independent cardiovascular risk factors, that can affect microvascular function and structure.[1, 2] These microvascular alterations may have important physiological consequences and predispose the circulatory system towards cardiovascular disease development. Retinal microvascular changes may provide insights into these vascular mechanisms as retinal arteriolar narrowing and venular widening have been associated with several cardiovascular risk factors.[3, 4] Furthermore, retinal microvascular changes often precede clinically detectable cardiovascular disease and consequently may hold prognostic information about disease development and be used as a phenotypical marker.

Within the European context, air pollution levels are relatively high in Flanders. Vehicle exhaust emissions generated by high traffic volumes on a dense road network contribute significantly to the high levels of PM and BC.[5] Despite serious improvements in air quality, the European air quality standards (annual limit of 40 µg/m³ for PM_{10} and daily limit of 50 µg/m³ on maximum 35 days per year for PM_{10}) are still exceeded at certain locations.[6] Ambient air pollution has been consistently associated with excess mortality, even in a population living in Flanders.[7] The difference between the highest season-specific PM_{10} quartile versus the lowest quartile was associated with 7.8% (95% CI: 6.1 to 9.6) and 1.4% (95% CI: 0.06 to 2.9) increase in mortality in respectively summer and winter. Since long-term and short-term air pollution exposure has been consistently associated with cardiovascular mortality and mortality,[8] it was unsurprising that cardiovascular disease-related mortality was the most important contributor to the excess mortality.

Microvascular dysfunction may help to explain the association between air pollution and cardiovascular diseases. Since endothelial dysfunction and vascular remodeling in the microvessels may precede macrovascular endothelial dysfunction and structural alterations, the microcirculation may hold important prognostic information. However, the evidence on microvascular changes is rather scarce. The size and inaccessibility of the microvessels has long limited their functional and structural investigation. As a consequence, endothelial function is frequently being assessed in larger vessels such as the brachial artery. The most evidence of "microvascular dysfunction" comes from experimental studies that assessed brachial artery flow-mediated dilation or responses to vasoactive agents after controlled air pollution exposure. Advancements in imaging techniques have made several microvascular beds accessible of which the retina allows a non-invasive assessment of the microcirculation in vivo. As retinal microvessels are associated with cardiovascular risk factors and diseases, they may help to elucidate the microvascular link between air pollution and cardiovascular diseases. At the start of this PhD scholarship, the association between long-term $PM_{2.5}$ exposure and the retinal microvasculature had only been investigated in the MESA cohort. Adar et al. reported that a 3 µg/m³ increase in $PM_{2.5}$ was associated with a decrease in CRAE of 0.8 µm (95% CI: -1.1 to -0.5).[9] This association was quantitatively similar to a 7-year increase in age or a 3 mm Hg increase in diastolic blood pressure.

We report and confirm that also short-term air pollution exposure is associated with retinal microvascular changes. In the first panel study (n=84, mean age= 37 ± 9, 60% females), the retinal microvasculature was assessed up to three times over a period of 5 months (January-May 2012) in which an air pollution episode occurred. The average levels of PM_{10} and BC were respectively 46.92 ± 37.17 µg/m³ and 3.07 ± 2.19 µg/m³. Each 10 µg/m³ increase in PM_{10} or each 1 µg/m³ increase in BC was associated with retinal

arteriolar narrowing of respectively 0.93 µm (95% CI: -1.42 to -0.45) and 1.84 µm (95% CI: -3.18 to -0.15). These changes in retinal arteriolar diameter were equivalent to a 1.5 year increase in age (Chapter 3).

In the second panel study (n=50, mean age= 32 ± 8, 50% females), the retinal microvasculature was assessed monthly between December 2014 and April 2015. The average level of PM_{10} was lower than our first panel study: 29.45 ± 15.68 µg/m³. In order to investigate a potential underlying mechanism, blood samples were collected during each study visit to test the changes in microRNA expression profiles. The expression of miRNAs has been associated with air pollution exposure.[10, 11-13] Changes in miRNA profiles will influence their gene regulatory capacity which can promote the expression of inflammatory or redox genes.[14, 15] In this way, increased inflammation and oxidative stress can be a potential pathway that negatively affects (retinal) microvascular responses.[16] Short-term PM_{10} exposure was associated with retinal arteriolar narrowing, venular widening and changes in the expression of miR-21 and miR-222. Each 10 µg/m³ increase in PM_{10} was associated with a decrease in CRAE of 0.72 µm (95% CI: -1.38 to -0.06), an increase in CRVE of 0.99 µm (95% CI: 0.18 to 1.80) and a downregulation of 6.62% (95% CI: -11.07 to -2.17) and 6.71% (95% CI: -10.68 to -2.75) in respectively miR-21 and miR-222 expression. Interestingly, miRNA-expression was also associated with retinal microvascular changes. Each 10% increase in miR-21 and miR-21 was associated with respectively an increase in CRAE of 0.14 µm (95% CI: 0.006 to 0.27) and a decrease in CRVE of 0.21 µm (95% CI: -0.41 to -0.01). These miRNA are involved in inflammatory pathways and endothelial dysfunction,[17, 18] suggesting that these underlying pathways may contribute to retinal microvascular changes. The changes in retinal arteriolar diameter were equivalent to a 1.4 year increase in age (Chapter 4). The effect sizes are somewhat larger in the first panel study compared with the second. These differences might be explained by the composition of the study population and the difference in exposure levels. First, it has been suggested that women are more vulnerable for air pollution-related cardiovascular morbidity and mortality[19] and that microvascular changes contribute more to cardiovascular events in women than in men.[20] The higher number of women may have increased the effect size. Second, the exposure contrast was larger in the first panel study than the second. It is likely that higher exposure levels elicit larger microvascular effects.

The exact pathophysiological mechanism of air pollution is still unclear, but the role of endothelial dysfunction will likely be very important. Endothelial dysfunction is caused by a diminished NO bioavailability. Experimental studies have indicated that air pollution may decrease NO levels in animals and humans by upregulating oxidative stress and inflammation.[16, 21, 22] The retinal blood vessels rely on an adequate endothelial NO production to ensure proper blood flow[23]. When air pollution upregulates inflammatory mediators and reactive oxygen species, they will scavenge NO in the microcirculation and induce endothelial dysfunction. The loss of this vasodilator might have caused the observed retinal arteriolar narrowing. In contrast, air pollution-related inflammatory reactions may cause vasodilation in the retinal venules as breakdown of the endothelial barrier and extravasation of leukocytes might have led to the observed venular widening. In our healthy population, the microvascular effects of air pollution exposure disappeared when pollution levels dwindled. This might have been due to the restoration of basal NO production and endothelial function.

The observed molecular and microvascular changes may influence the development of cardiovascular disease in the long term.[24, 25] If the same adverse microvascular responses are present in susceptible individuals, it might explain some of the cardiovascular complications associated with air pollution in these individuals.[26-28]

The previously discussed panel studies relied on air pollution measurements from nearby monitoring stations. Thus a degree of exposure misclassification was inevitably introduced in these studies. In our first panel study, we observed larger effect estimates for BC compared with PM_{10}. These traffic-related particles are part of the $PM_{2.5}$ fraction, which has a larger surface area per volume unit compared with PM_{10}. Fine particles have an increased potential to capture chemicals, toxins and redox-sensitive elements and can be deposited deeper in the lungs where they may provoke more severe oxidative stress and inflammation.[29] Personal BC exposure depends on an individual's time-activity patterns as there is a large spatial and temporal variation in BC levels.[30] Therefore, we set up a panel study with 56 healthy nurses in which personal BC exposure was measured during a normal working week. The average weekly exposure was used to calculate the subchronic exposure to BC, as proxy for long-term BC exposure. Each 425 ng/m³ increase in subchronic BC exposure was associated with increases in SBP, DBP and CRVE of respectively 2.77 mm Hg (95% CI: 0.39 to 5.15), 2.35 mm Hg (95% CI: 0.52 to 4.19) and 4.76 µm (95% CI: 0.27 to 9.24) (Chapter 5). These associations are similar to those reported by others.[31, 32] Retinal venular widening might have been caused by a BC-induced low-grade systemic inflammation. Interestingly, retinal venular widening did not mediate the association between BC and blood pressure. Possibly, our small panel study lacked the statistical power to formally conduct a mediation analysis. Our repeated measurements needed to be averaged in order to conduct the mediation analysis, further reducing statistical power. The lack of an association between BC exposure and retinal arteriolar narrowing raises several questions. Other effector pathways, independent of microvascular changes, might have increased blood pressure as blood pressure is a highly regulated phenotype.[33] The BC-induced changes in blood pressure might have been too small to induce a statistically detectable effect in the retinal arterioles. A recent meta-analysis indicated that long-term blood pressure increases of 10 mm Hg are associated with a 3.07 µm decrease in retinal arteriolar diameter.[34]

In contrast to our first panel study, we did not find an association between short-term BC and retinal microvascular changes. There was little variation in exposure levels due to meteorological circumstances. As a consequence, our personally measured average BC exposure was 866 ± 424 ng/m³. Dons et al. used a similar study design and measured an average personal BC exposure of 1592 ± 468 ng/m³ in the same study area.[35] Also, exposure levels in our first study were up to 10-fold higher. Conducting personal exposure measurements is costly, time-expensive, logistically more challenging and requires more effort from the study participants to measure BC exposure in a reliable manner. There are only a limited number of devices available that address all these concerns. For instance, the used µ-aethalometer allows to assess personal BC exposure with relative ease. However, the unforeseen low exposure levels and the lack of exposure variation may have required a much larger study population to obtain sufficient statistical power. The choice for personal exposure measurements to avoid exposure misclassification limited our study size as conducting more personal measurements was not feasible at that time. This is an important difference with cohort studies that rely on central monitoring data or exposure models. In these studies, a high number of individuals is included to limit the effects of exposure misclassification this may drive associations towards zero-value.

Sedentary behavior or physical inactivity is a risk factor for cardiovascular diseases whereas regular exercise or physical activity can promote cardiovascular health.[1, 36] The effects of physical inactivity on the retinal microvasculature were tested during a bedrest study, which is an experimental model that rapidly induces vascular physiological changes that are associated with physical inactivity. In a cross-over study, 14 healthy individuals participated in two periods of bedrest (one period in normoxic and one

period in hypoxic conditions) and in one period of ambulation in hypoxic conditions (Chapter 6). The combination of these hypoxic and/or bedrest conditions allowed to test the myogenic and metabolic autoregulatory capacities of the retinal blood vessels. In a supine position, the perfusion pressure in the retina will increase, because the posture change eliminates gravitational pull, and retinal vessels will become pressure loaded and experience an increased blood flow. As a consequence, myogenic autoregulation will cause vasoconstriction to maintain blood flow.[37, 38] This was observed as an immediate and persistent retinal arteriolar narrowing during the normoxic bedrest protocol. Sustained physical inactivity in bedrest study participants and sedentary individuals has been associated with changes in blood flow. Non-laminar blood flow lowers shear rate and induces endothelial dysfunction and vasoconstrictor production. This may lead to peripheral vasoconstriction and increase systemic blood pressure,[39-41] further elevating retinal perfusion pressure and the myogenic vasoconstrictor response, or directly provoke vasoconstriction of retinal arterioles as the potent vasodilator NO is lost.

The retinal microvascular responses during hypoxic ambulation were attributable to metabolic autoregulation. Retinal tissue has a high metabolic activity and O_2 demand. Decreases in O_2 tension will elicit a vasodilatory response in the retinal blood vessels to increase blood flow in order to ensure an adequate O_2 supply.[42] These changes have been observed after short-term hypoxia exposure. We demonstrated that retinal blood vessels remained dilated as long as the hypoxic stimulus was present.

Both myogenic and metabolic autoregulation were active during hypoxic bedrest. The supine position will have induced myogenic vasoconstriction, whereas the hypoxic environment will have caused metabolic vasodilation. In this condition, arteriolar vasodilation was significantly smaller than during hypoxic ambulation, possibly due to the opposite effect of myogenic vasoconstriction on hypoxia-induced metabolic vasodilation. This might have reduced blood flow to the retinal tissues. Sustained bedrest even attenuated arteriolar vasodilation, returning arteriolar diameters to their baseline levels. This might have been caused by an increased systemic blood pressure or a mechanism that favored endothelial dysfunction due to the decreased retinal blood flow.

These findings may help to explain the susceptibility of sedentary individuals for cardiovascular disease events as myogenic and metabolic autoregulation are important regulators of blood flow in in coronary and cerebral microvessels. Physical inactivity has been associated with endothelial dysfunction, narrower blood vessels and increased blood pressure. When these individuals encounter a hypoxic challenge (for instance during or after ischemia or partial occlusion of conduit vessels in atherosclerosis), their microvasculature might not be able to respond adequately. Improper vasodilation will limit the necessary increase in blood flow and O_2 supply. This might induce to tissue hypoxia, prolong and possibly aggravate hypoxic tissue damage.[43-46]

In contrast to physical inactivity, regular physical exercise has many beneficial effects and is a useful measure to improve (cardiovascular) health. Cardiac rehabilitation programs are used to improve endothelial function, muscle strength and overall cardiac fitness in order to decrease the risk of future cardiovascular complications in cardiac patients.[47-49] We are the first to investigate whether these programs also improve the retinal microvascular reactivity. We assessed retinal vascular responses before and repeatedly after a maximal endurance test, that was performed at the beginning and after 6 weeks in the rehabilitation program (Chapter 7). Our data suggest that retinal vascular responses are preserved in cardiac patients and are similar in magnitude and progression to those of healthy persons.[50] However, after 6 weeks in the rehabilitation program, baseline retinal vascular parameters and vascular reactivity after the endurance exercise did not show an improvement. Retinal microvascular responses

after a maximal endurance test might not be the appropriate way to evaluate endothelial function. Studies that reported improvements in endothelial function usually assessed brachial artery flow-mediated dilation. Retinal endothelial function can be assessed with dynamic vessel analysis after flicker light stimulation. These procedures can be performed independently from a maximal endurance test. The only other study that assessed retinal microvascular reactivity in a similar way, showed that retinal responses to a maximal endurance test were similar in young adults and seniors.[50] However, when a submaximal endurance test was performed, the microvascular reactivity of young adults was much better compared to seniors. It is likely that the increase in blood flow during a maximal endurance test is too large to detect small differences in endothelial function. A submaximal endurance test or other tests that challenge the retinal microvasculature (the administration of vasoactive agents that activate eNOS) might be more appropriate to assess improvements in retinal microvascular reactivity after a cardiac rehabilitation program.

STRENGTHS, LIMITATIONS AND FUTURE PERSPECTIVES

The major strength of our studies is the use of the repeated measurements study design. This design increased the statistical power because many potential covariates remain constant during the study period and did not need to be accounted for in the statistical models. As a consequence, we were able to detect retinal microvascular changes in small panels. The limited panel sizes are at the same time a weakness of our studies as the small sample size obviously limits overall generalizability. We observed several associations between short-term changes in air pollution or physical (in)activity and retinal microvascular changes, but we did not collect data to extrapolate our findings to long-term micro- and macrovascular physiological effects.

Using repeated fundus measurements (a sequence of static retinal images), we were able to observe reversible effects of air pollution exposure and physical (in)activity in the retinal blood vessels. These changes were observed in a relatively short time period and thus provide indirect evidence of functional alterations. Short-term changes in vessel diameters are more likely to be attributable to functional changes (endothelial dysfunction or autoregulation) instead of structural alterations that require a longer time to develop. Whether these retinal microvascular functional changes are truly caused by endothelial dysfunction can be addressed in two ways. First, in epidemiological studies, endothelial dysfunction could be assessed in the retina using dynamic vessel analysis[51] or in other vascular beds using infusion of vasoactive drugs or reactive hyperaemia to assess flow-mediated dilation.[52] However, an important trade-off is the feasibility of these techniques in large-scale research. As these techniques are more costly and time-consuming they may limit study compliance. Second, experimental studies can be used to investigate causality and underlying mechanisms, which cannot be done in epidemiological research. Experimental studies such as controlled air pollution exposure or physical (in)activity during bedrest or maximal endurance tests are useful to investigate the underlying molecular pathways. In these well-controlled experimental studies, a targeted approach can be used to measure the changes in the expression profiles of genes, gene regulators and proteins that are involved in inflammation, oxidative stress and endothelial dysfunction. This additional layer of information can help to unravel the underlying molecular mechanisms.

Future long-term studies in larges population should therefore focus on two domains. First, short-term retinal microvascular changes should be frequently assessed to address the question whether repeated

short-term microvascular effects add up to long-term microvascular structural changes and thus contribute to macrovascular disease. It would be interesting to look at microvascular role in the development of hypertension and atherosclerosis or cardiovascular endpoints such as coronary heart disease or stroke. Second, despite the shared anatomical and physiological features, retinal microvascular changes are only a proxy for cerebral and coronary microvascular function and structure.[53, 54] Technologies exist to measure cerebral and coronary blood flow and the structure of larger cerebral and coronary blood vessels. A combined assessment of retinal microvascular parameters and cerebral/coronary blood flow/vessel structure may provide a better understanding of the link between these different microvascular beds and how changes in one type of blood vessel may be related to changes in other blood vessels. Moreover, this will allow to draw conclusions whether retinal microvascular changes are a good proxy for other less-accessible microvascular beds as the relative difficulty of coronary and cerebral blood flow assessment limits their use in large population studies.
Fundus photography allows a non-invasive assessment of the retinal microvasculature in vivo. However, a single static image can only be used to assess structural vascular parameters. Of note is that retinal diameters can be affected by the heart cycle and blood pressure. Although the influence of the heart cycle is limited (the maximal difference between early systole and early diastole were 3.5% and 4.8% for respectively arterioles and venules), retinal images should be ideally taken at exactly the same moment in the heart cycle, with the use of an electrocardiogram.[55] Our retinal images were taken at random points during the heart cycle. This might have introduced a random over- and underestimation of retinal vessel diameters which would have compensated each other. In addition, we addressed this issue of heat cycle by taking two retinal images shortly after each other and averaging the values. Blood pressure is another important determinant of retinal vessel diameter and should always be measured when assessing the retinal microvasculature. Short-term increases in blood pressure may trigger retinal myogenic autoregulation whereas long-term increases cause retinal arteriolar narrowing.[38, 56] Measuring blood pressure and adding this as covariate in the statistical analysis will present an association independent from this important covariate.

We used a nearby monitoring station to estimate participant's exposure and can therefore cannot excluded exposure misclassification(Chapter 3 and 4). Since air pollutants, such as BC, have a high spatial and temporal variation, exposure is determined by time-activity patterns.[30, 57] However, we assessed the short-term effects of air pollution when air pollution levels were spatially increased or decreased. In this way we will have missed the individual's temporal component of exposure and sudden peak exposures, but this might only have further increased the individual's air pollution exposure.[30, 58, 59]
The use of personal exposure measurements is the only way to prevent exposure misclassification (Chapter 5). As discussed above, feasibility of these measurements is still limited in large-scale epidemiological studies as the use of portable air quality monitors restricts the number of study participants that can be investigated and requires an intense cooperation of study participants.

CONCLUSION

The results obtained in this PhD project suggest that the effect of cardiovascular risk factors such as ambient air pollution and physical inactivity can be assessed in the retinal microvasculature using repeated fundus photography. The effects of air pollution contributes to the notion that there is no safe threshold for air pollution exposure. Recent work indicates that the cardiovascular effects of air pollution are occurring at concentrations below the limit values set by the European Union.[60] Our experimental work on physical (in)activity suggests that microvascular changes can be provoked by physical (in)activity.

We propose fundus photography as a convenient tool in epidemiological and experimental studies to assess acute microvascular changes. There is a rapid evolution in fundus image capturing techniques and fundus image analysis. Nowadays, small handheld cameras and smartphone applications are commercially available and they can be used to detect retinal changes. In combination with automated analysis software and cloud-based solutions, retinal vessel changes can be assessed faster and more accurately using a larger panel of vascular features. These developments will broaden the applicability of fundus photography in large-scale screening programs and the technique may offer added-value as bedside testing to support doctor diagnosis and patient stratfication. In combination with other clinical analyses, the non-invasive assessment of the retinal microcirculation may hold important prognostic information on the changes in cerebral and coronary microvascular beds.[61-63]

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Curriculum vitae

CURRICULUM VITAE

Tijs Louwies was born on the 27th of April 1988 in Lommel (Belgium). In 2009, he obtained the degree of Bachelor in Science – Biology at Hasselt University. In 2011, he finished his Master degree in Biomedical Sciences – Clinical and Molecular Sciences at Hasselt University. He obtained a PhD scholarship at the Flemish Institute for Technological Research. Since January 2012, he is working as a PhD student at the Centre of Environmental Research at Hasselt University and the Unit Environmental Risk and Health of the Flemish Institute for Technological Research.

LIST OF PUBLICATIONS

Articles in peer-reviewed journals

Carotid intima-media thickness is positively associated with subchronic personal exposure to black carbon: A study in a panel of healthy adults. Eline B Provost, Tijs Louwies, Jos op 't Roodt, Evi Dons, Luc Int Panis, and Patrick De Boever , and Tim S Nawrot.
Artery Research: **doi:10.1016/j.artres.2014.09.122**

Blood pressure changes in association with black carbon exposure in a panel of healthy adults are independent of retinal microcirculation. Tijs Louwies, Tim Nawrot, Bianca Cox, Evi Dons, Joris Penders, Eline Provost, Luc Int Panis, and Patrick De Boever
Environment International; **doi:10.1016/j.envint.2014.11.006**

Fundus Photography as a Convenient Tool to study Microvascular Responses to Cardiovascular Disease Risk Factors in Epidemiological Studies. Patrick De Boever, Tijs Louwies, Eline Provost, Luc Int Panis, and Tim S. Nawrot
J.Vis. Exp. (92), e51904, **doi:10.3791/51904**

Retinal Microvascular Responses to Short-Term Changes in Particulate Air Pollution in Healthy Adults. Tijs Louwies, Luc Int Panis, Michal Kicinski, Patrick De Boever, and Tim S. Nawrot
Environ Health Perspect; **doi:10.1289/ehp.1205721**

Conference Material

Retinal Microvascular Responses to Short-Term Changes in Particulate Air Pollution in Healthy Adults. Tijs Louwies, Luc Int Panis, Michal Kicinski, Patrick De Boever, and Tim S. Nawrot. **Poster Presentation** at Microcirculation – Oxford, United Kingdom, 4 -6 July 2013. **Poster Presentation** at Environment Health – Boston, United States of America, 3 -6 March 2013. **Oral presentation** at Environment and Health (Conference of ISEE, ISES and ISIAQ)– Basel, Switzerland, 19 - 23 August 2013.

Blood pressure changes in association with personal black carbon exposure are not mediated through microcirculatory responses. Tijs Louwies, Tim Nawrot, Bianca Cox, Evi Dons, Joris Penders, Eline Provost, Luc Int Panis, Patrick De Boever. **Poster Presentation** at ISEE Europe conference – Barcelona, Spain, 20 and 21 October 2014.

PlanHab: In vivo retinal images for a non-invasive analysis of the microcirculation during hypoxia and unloading/inactivity. Tijs Louwies, Patrick De Boever, Bianca Cox, Stelios Kounalakis, Polona Jaki Mekjavic, Ola Eiken, Igor Mekjavic. **Oral presentation** at International Society for Gravitational Physiology – Waterloo, Canada, 15 - 20 June 2014.

Changes in miRNA expression and retinal blood vessels are associated with short-term air pollution exposure. Tijs Louwies, Luc Int Panis, Bianca Cox, Karen Vrijens, Caroline Vuegen, Tim S. Nawrot, Patrick De Boever. **Poster presentation** at Methods in Epidemiology symposium – Leuven, Belgium – 17 September 2015.

Microvascular responses in association with recent and chronic exposure to particulate air pollution in school children. Eline B Provost, Nelly D Saenen, Michal Kicinski, Tijs Louwies, Karen Vrijens, Luc Int Panis, Patrick De Boever, Tim S Nawrot. **Oral presentation** at Healthy Living conference – Maastricht, The Netherlands – 25 to 27 June 2015. **Poster presentation** at Methods in Epidemiology symposium – Leuven, Belgium – 17 September 2015. **Oral presentation** at ISEE Europe conference – Utrecht, The Netherlands, 2 and 3 November 2015.

Short-term fluctuations in personal black carbon exposure are associated with rapid changes in carotid arterial stiffening. Eline B Provost, Tijs Louwies, Jos op 't Roodt, Evi Dons, Luc Int Panis, Patrick De Boever , Tim S Nawrot. **Poster presentation** at ISEE Europe conference – Utrecht, The Netherlands, 2 and 3 November 2015.


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