GENEESKUNDE

master in de biomedische wetenschappen: milieu en gezondheid

Masterproef

Mitochondrial DNA Decreases in Response to Particulate Air Pollution in Persons with Diabetes

Promotor :
Prof. dr. Tim NAWROT

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Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting milieu en gezondheid

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List of Abbreviations

Activated Partial Thromboplastin Time	aPTt
Activating Protein-1	AP-1
Airborne Particulate Matter	PM
Body Mass Index	BMI
Coarse Particles	$PM_{2.5-10}$
Copper/Zink Superoxide Dismutase	CuZnSOD
DNA Polymerase γ	POLG
Fine Particles	$PM_{2.5}$
High-Density Lipoprotein	HDL
High-sensitivity C-reactive Protein	hs-CRP
Human Ribosomal Protein LP0	RPLP0
Human β-actin	ACTB
Human β-hemoglobin	HBB
Low-Density Lipoprotein	LDL
Manganese Superoxide Dismutase	MnSOD
Mitochondrial DNA	mtDNA
Mitochondrial Single-stranded DNA-binding Protein	mtSSBP
Mitochondrial Transcription Factor A	Tfam
Mitochondrial Transcription Factor B	mTFB
Mitochondrially Encoded NADH Dehydrogenase 1	MT-ND1
Nuclear DNA	nDNA
Nuclear Factor-Kappa B	NF-κB
Nuclear Respiratory Factor	NRF
Peroxisome Proliferator-activated Receptor γ Coactivator-1α	PGC-1α
Platelet Function Analyzer	PFA
Polymerase Chain Reaction	PCR
Prothrombin Time	PT
Reactive Nitrogen Species	RNS
Reactive Oxygen Species	ROS
Thoracic Particles	PM_{10}
Ultrafine Particles	UFP
White Blood Cell	WBC

Voorwoord

In dit (meestal als eerste gelezen) hoofdstuk wil ik graag een aantal mensen bedanken die rechtstreeks of onrechtstreeks bijgedragen hebben tot het voltooien van deze scriptie.

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Abstract

BACKGROUND: Epidemiologic studies have identified oxidative stress, inflammation and thrombosis as main mechanisms by which PM (airborne particulate matter) pollutants affect cardiovascular health. Although there is growing evidence that an altered mitochondrial DNA (mtDNA) copy number is a good marker in various diseases mediated by oxidative stress, the role of this novel biomarker in the etiology of PM-related diseases has never been evaluated.

OBJECTIVES: We aimed to evaluate whether exposure to particulate air pollution was associated with relative mtDNA copy number and to investigate the possible relationship between relative mtDNA copy number and known biomarkers of inflammation and thrombosis. These objectives were investigates in a susceptible population of patients with diabetes.

METHODS: In 363 nonsmoking adults with diabetes, relative mtDNA copy number was measured by real-time quantitative PCR in peripheral leukocytes and surrogate markers for exposure to traffic-related air pollution (i.e. distance from the patient's residence to a major road and modeled regional background level PM_{10} at the participant's home address) were determined. Several markers of inflammation, thrombosis and platelet function were also investigated.

RESULTS: Each twofold increase in distance from residence to major roads was associated with a 2.18% increase (95% confidence interval (CI), 0.54 to 3.84) in relative blood mtDNA copy number. Independently from other covariates, we found that each increase of 9.4 µg/m^3 (interquartile range) in mean residential PM_{10} concentration over the preceding month was associated with a 9.09% decrease (95% CI, -13.87 to -4.03) in blood mtDNA content. A twofold increase in relative mtDNA copy number in peripheral blood was associated with a decrease of 852 leukocytes per microliter of blood (95% CI, -1280 to -424) and an increase in the PFA-100 closure time of 38.1 sec (95% CI, 18.4 to 57.8). Blood platelet count was not correlated with blood mtDNA content.

CONCLUSION: Relative blood mtDNA copy number was inversely correlated with both (sub)chronic exposure to particulate air pollution and biomolecular markers of inflammation and thrombosis. These findings suggest that mitochondrial dysfunction, reflected in lowered mtDNA copy numbers in peripheral blood, represents a potential biological pathway linking particulate air pollution to cardiovascular health effects in a susceptible subgroup of the population.

Samenvatting

INLEIDING: Studies hebben aangetoond dat blootstelling aan fijn stof (PM) een rol speelt in pro-oxidatieve, pro-trombotische en pro-inflammatoire processen, die bijdragen tot een verhoogd risico op cardiovasculaire ziekten. Men vermoedt dat een afwijkende hoeveelheid mitochondriaal DNA (mtDNA) een goede biologische merker is in ziekten gemedieerd door oxidatieve stress.

OBJECTIEVEN: Onze hypothese was dat de hoeveelheid mtDNA in witte bloedcellen enerzijds geassocieerd is met blootstelling aan fijn stof en anderzijds met trombotische en inflammatoire merkers. Deze hypothese werd getoetst bij personen die gevoeliger kunnen zijn voor de effecten van fijn stof.

METHODEN: De mtDNA-inhoud werd bepaald in leukocyten van 363 niet-rokende volwassenen met diabetes. Hiervoor werd gebruik gemaakt van kwantitatieve PCR in real time. Verder werden biologische merkers voor verkeersgerelateerde luchtverontreiniging (afstand van woonplaats tot drukke wegen en gemiddelde PM_{10}-concentratie rondom de woonplaats), trombose en inflammatie bestudeerd.

RESULTATEN: Een verdubbeling van de afstand tot drukke wegen was geassocieerd met een stijging van de hoeveelheid mtDNA met 2.18% (95% betrouwbaarheidsinterval (BI), 0.54 tot 3.84). Verder werd een daling van de mtDNA-inhoud met 9.09% (95% BI, -13.87 tot -4.03) geobserveerd bij een interkwartielafstand (9.4 µg/m^3) toename van de maandgemiddelde PM_{10}-concentratie rondom de woonplaats. Een verdubbeling van de mtDNA-inhoud in witte bloedcellen was geassocieerd met een gemiddelde daling van 852 leukocyten per microliter bloed (95% BI, -1280 tot -424) en een gemiddelde stijging van de PFA-100 closure time met 38.1 sec (95% BI, 18.4 tot 57.8). Het aantal bloedplaatjes was niet gecorreleerd met de hoeveelheid mtDNA.

CONCLUSIE: De hoeveelheid mtDNA in leukocyten was significant omgekeerd gecorreleerd met de mate van blootstelling aan verkeersgerelateerde luchtverontreiniging en met veranderingen in trombotische en inflammatoire merkers. De resultaten van deze studie suggereren dat een daling van de hoeveelheid mtDNA in witte bloedcellen een potentieel mechanisme is dat een belangrijke rol speelt in fijn stof-geïnduceerde cardiovasculaire ziekten.

1. Introduction

Air pollution has been considered dangerous to human health for centuries. Numerous studies in the past decades have observed associations between elevated air pollution levels and various health outcomes. Basic health outcomes are hospitalization and mortality due to respiratory and cardiovascular diseases, disturbed pulmonary functions, and exacerbated symptoms among people with preexisting cardiopulmonary diseases [1]. At the global level, ambient air pollution is estimated to account for 1.4% of all deaths and 0.8% of disability-adjusted life-years [2].

In recent years, most epidemiological studies have focused particularly on airborne particulate matter (PM) because the stronger correlation of adverse health effects with fine particles than with other atmospheric pollutants. Much attention has been directed both to the size and composition characteristics of these particles, and the toxicological mechanisms underlying the link between PM pollutants and adverse health effects [1, 3].

1.1. Particulate Air Pollution

Particulate air pollutants are complex mixtures of solid particles and liquid droplets that can be suspended in the air for extended periods of time. They originate from a variety of sources and may be emitted directly to the atmosphere or formed by transformation of gaseous pollutants (such as nitrogen oxides, sulfur oxides, and volatile organic compounds). Some particulates are emitted from natural sources including evaporated sea spray, windborne pollen, dust, and volcanic eruptions. However, anthropogenic sources remain the major contributors to PM emissions. Human-related sources include fossil fuel combustion in motor vehicles, industry, agriculture, construction and demolition, and fugitive dust from roads [4-6]. Particulates are classified according to their aerodynamic diameter and chemical composition, which both determine their biological effects.

1.1.1. Size of airborne particulate matter

Fine particles are classified according to their aerodynamic diameter into size fractions such as thoracic particles (PM_{10}), coarse particles ($PM_{2.5-10}$), fine particles ($PM_{2.5}$) and ultrafine particles (UFP). The aerodynamic diameter is a key determinant of the likelihood of deposition in the respiratory tract and the site of deposition after inhalation as shown in table 1. There are three major mechanisms by which particulates are deposited in the respiratory tract, namely inertial impaction, gravitational sedimentation, and diffusion. Particles greater than 2.5 µm in aerodynamic diameter are deposited in the upper airways by impaction. These particles diverge from airway streamlines and thereby come into contact with the wet airspace surface. Particles smaller than 2.5 µm penetrate deeply into the airways and have a greater likelihood of reaching the alveoli. Deposition of $PM_{2.5}$ occurs mainly by sedimentation, which is defined as the settling of particles onto airway surfaces under the force of gravity. However, as particles become smaller than 0.1 µm, deposition through impaction and sedimentation is no longer significant. These UFPs deposit in the respiratory tract via diffusion to the walls of air passages [5].

Analytical electron microscopy measurements showed that 96% of effectively retained particles had aerodynamic diameters less than 2.5 µm, which indicates that human lung parenchyma effectively retains $PM_{2.5}$. However, UFPs constituted less than 5% of the total, probably because they also deposit heavily in the nose [7].

Table 1. Classification of particles bases on size.

Particle	Diameter	Major Sources	Major sites of deposition
PM_{10}	< 10 µm		
$PM_{2.5-10}$	2.5 µm – 10 µm	Agricultural and road dust Mining operations Construction and demolition debris	Upper airways (nasal cavity, pharynx, larynx)
$PM_{2.5}$	< 2.5 µm	Fossil fuel combustion (diesel) and tailpipe emissions from mobile sources	Tracheobronchial tree (trachea, bronchi, bronchioles) and alveoli
UFP	< 0.1 µm	Wildfires Fossil fuel combustion (coal, natural gas, petroleum) in power plants	Upper airways and alveoli

Thoracic particles (PM_{10}), coarse particles ($PM_{2.5-10}$), fine particles ($PM_{2.5}$) and ultrafine particles (UFP).

1.1.2. Chemical composition of airborne particulate matter

The chemical composition of PM varies greatly and depends on many factors, such as combustion source, climate, season, and type of industrial or urban pollution. The major components of airborne particulate matter are organic carbon compounds adsorbed onto particles (e.g. polycyclic aromatic hydrocarbons and quinones), transition metals (e.g. copper, chromium, nickel and iron), ions (e.g. sulfate and nitrate), reactive gases (e.g. ozone, peroxides and aldehydes), materials of biologic origin (e.g. endotoxins, bacteria, and viruses), minerals (e.g. quartz and asbestos), and the particle core of carbonaceous material [5].

1.2. Health Effects of Particulate Air Pollution

The health effects of particulate air pollution have been extensively studied in recent years. Exposure to PM pollutants has been associated with increases in hospital admissions and mortality due to respiratory and cardiovascular diseases [1]. *In-vitro* and *in-vivo* studies have identified oxidative stress and inflammation as main mechanisms by which PM air pollutants affect respiratory and cardiovascular health [8].

1.2.1. Effects on oxidative stress

Oxidative stress is defined as a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defenses, including enzymatic and non-enzymatic systems. The perturbation of this balance may lead to the accumulation of ROS which can damage proteins, lipids and DNA [9]. Oxidative stress mediated by particulate air pollutants may arise on several ways:

- Certain organic carbon compounds (such as polycyclic aromatic hydrocarbons and quinones) and transition metals (such as copper, chromium, nickel and iron) in PM have pro-oxidant properties and may induce oxidative stress. They can generate ROS directly through their potential to redox cycle [10].
- Materials of biologic origin (such as endotoxins, bacteria and viruses) in PM pollutants can activate inflammatory cells capable of generating ROS and reactive nitrogen species (RNS), as well as oxidative DNA damage [11].
- Ambient PM exhibits direct inhibitory effects on protective enzymes (such as copper/zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD), and glutathione peroxidase and reductase) involved in oxidative stress responses, which can lead to the accumulation of ROS [12].

Additionally, particulate air pollutants can enhance inflammatory responses, which would lead to additional generation of ROS and RNS [13].

1.2.2. Inflammatory effects

In-vitro and *in-vivo* studies in human beings have revealed potent proinflammatory effects involving lung epithelial cells and alveolar macrophages [1, 8]. Inhaled particulates activate, both directly and through uptake into epithelial cells and macrophages, stress signaling pathways. This mechanism involves activation of the transcription factor nuclear factor kappa B (NF-κB) and its translocation to the nucleus. There it binds to DNA sequences in the promoters of proinflammatory genes that code for cytokines, chemokines that attract leukocytes, and adhesion molecules. These molecules increase leukocyte recruitment into the airways and alveoli, and activate them for mediator secretion. On reaching the bone marrow, cytokines and

chemokines released from the lung induce a systemic inflammatory response. This response includes stimulation of the marrow to release leukocytes and their precursors into the circulation [1]. One study has shown that this bone marrow stimulation is related to the amount of particles phagocytized by alveolar macrophages in the lung [14]. Photomicrographs of alveolar macrophages with PM_{10} particles in the cytoplasm are shown in figure 1.
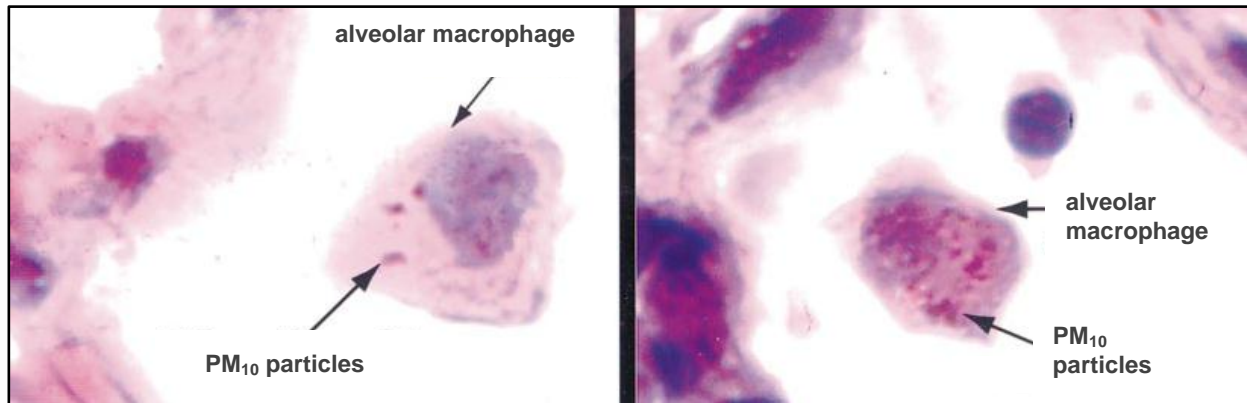
Figure 1. Two photomicrographs of an alveolar macrophage with PM_{10} particles in the cytoplasm.

1.2.3. Thrombotic effects

Exposure to particulate pollutants has been reported to result in platelet function abnormalities and hemostatic alterations that culminate in thrombotic events [15]. The mechanisms linking PM exposure to the development of thrombosis, however, have not yet been entirely elucidated. Possible prothrombotic mechanisms involved in this relation are elevation of fibrinogen, increased platelet aggregation and factors II, VII, VIII and X activity or alterations of arterial vasoconstriction [6].

1.2.4. Effects on respiratory and cardiovascular health

As already mentioned, oxidative stress and inflammation are major mechanisms by which PM air pollutants affect respiratory and cardiovascular health [8].

Adverse respiratory events may result from inflammation that occurs in the lungs in response to damage caused by the deposition of particles. Important health outcomes include decreased lung function, respiratory symptoms, aggravated asthma, chronic bronchitis, and cancer [5].

The biological mechanisms of air pollution on cardiovascular health have not yet been entirely elucidated. However, two major mechanistic hypotheses have been put forward to explain the associations between particles and effects on the cardiovascular system. One suggests that inhaled particles induce prothrombotic and systemic inflammatory effects, both playing an important role in atherosclerosis and cardiovascular disease. This association has already been observed in persons with diabetes [16]. An alternative hypothesis points to an effect on the autonomic heart-rate control, leading to an increased risk of developing an arrhythmia [17].

1.3.   Markers of Biological Ageing

1.3.1. Mitochondrial DNA content in peripheral leukocytes

The most important intracellular source and primary target of ROS is the mitochondrion. Each human cell contains several hundred to over a thousand mitochondria, each carrying 2-10 whole copies of mitochondrial DNA (mtDNA). Human mtDNA is a circular double-stranded molecule that codes for 13 subunits of the oxidative phosphorylation system, 2 ribosomal and 22 transfer RNA molecules. The different subunits of the oxidative phosphorylation system are listed in table 2. The mitochondrial genome consists predominantly of coding DNA, with the exception of the control region that has mainly regulatory functions. MtDNA has several unique features that distinguish it from nuclear DNA (nDNA). These features include exclusive maternal inheritance, lack of introns, and lack of recombination. Besides these features, it has been shown that the mitochondrial genome is more susceptible to ROS-induced damage when compared with the nuclear genome, because mtDNA lacks protective histones and has a reduced DNA repair capacity [18-19].

Table 2. The mtDNA-encoded subunits of the oxidative phosphorylation system.

Gene	Gene Product	Subunits	Function
MT-ND	mitochondrially encoded NADH dehydrogenase	1, 2, 3, 4, 5, 6, 4L	Component (Complex I) mitochondrial electron transport chain Oxidative phosphorylation
MT-CYB	mitochondrially encoded cytochrome b		Component (Complex III) mitochondrial electron transport chain Oxidative phosphorylation
MT-CO	mitochondrially encoded cytochrome c oxidase	1, 2, 3	Component (Complex IV) mitochondrial electron transport chain Oxidative phosphorylation
MT-ATP	mitochondrially encoded ATP synthase	6 , 8	ATP synthesis (Complex V) Oxidative phosphorylation

NADH dehydrogenase (MT-ND), cytochrome b (MT-CYB), cytochrome c oxidase (MT-CO) and ATP synthase (MT-ATP).

The human mtDNA content is correlated with the size and number of mitochondria, which have been shown to change under different energy demand, as well as different physiological or environmental conditions [20]. The mtDNA content is lower in muscles of diabetic subjects compared with healthy persons. This could be the result of diabetes, because this decrement is observed in both types of diabetes [21]. However, a decrease in mtDNA content in peripheral leukocytes predicted the incidence of type 2 diabetes, suggesting this could be the cause of type 2 diabetes [22]. Cells challenged with ROS, however, synthesize more copies of their mtDNA and increase their mitochondrial abundance to meet higher energy demands. The increased mitochondrial energy production is used to repair ROS-induced cell damage [23].

1.3.2. Telomere length in peripheral leukocytes

Telomeres are complex DNA-protein complexes found at the distal ends of chromosomes. They consist of long arrays of $(TTAGGG)_n$ tandem repeats that act as a cap for the ends of chromosomes. This provides protection from structural degradation, atypical recombination, and end-to-end fusion of chromosomes. At each cell division DNA polymerases fail to completely replicate telomeres due to the end-replication problem, resulting in a progressive shortening of telomeres. Consequently, the structural integrity of chromosomes becomes increasingly vulnerable with sequential cell divisions. Telomeres in somatic human cells shorten by 30-200 base pairs each cell division, and once a critical length of telomeric DNA is reached, cells are triggered into replicative senescence or become apoptotic. Telomere length thus plays a critical role in maintaining the integrity of DNA and consequently the health of cells, and can serve as a biomarker of a cell's biological age or potential for further cell division [24-25].

Cellular environment also plays an important role in regulating telomere length. Most notably, oxidative stress and inflammation can accelerate telomere shortening and biological ageing [26-28]. Peripheral leukocytes from type 2 diabetic patients are characterized by significant telomere shortening, which is likely due to oxidative DNA damage to leukocyte precursors during replication [29].

1.3.3. Concentration of circulating high-sensitivity C-reactive protein

High-sensitivity C-reactive protein (hs-CRP), a pentameric protein produced by the liver, has emerged as a marker for inflammation. It is an acute phase response protein markedly increased in both inflammatory and infectious disease. During inflammatory processes, it plays an important role in assisting complement binding to foreign or damaged cells and enhancing phagocytosis. Hypertensive patients with diabetes mellitus type 2 have higher levels of hs-CRP than normal subjects. This indicates that patients with two associated diseases have a more active inflammatory state [30].

1.4.   Objectives and Experimental Design

Numerous epidemiologic studies link adverse cardiovascular health outcomes with particulate air pollution, which to a considerable extent is caused by traffic [1]. Persons with diabetes who also have cardiovascular disease appear to be more sensitive to the effects of PM air pollution [31]. Therefore it is relevant to investigate the effects of air pollution in this more susceptible fraction of the population.

Mitochondria are semiautonomous organelles with essential importance for the energy production of the cell. They contain their own genome, which is a 16.6-kb circular structure of double-stranded DNA with several hundred to over a thousand copies per cell [18]. There is growing evidence that an altered mtDNA copy number is a good marker in various diseases that are mediated by oxidative stress (e.g. cardiovascular diseases) [32].

The primary objective of this study is to validate the use of this novel biomarker:

- as an indicator of a subject's previous exposure to traffic-related air pollution
- as an indicator of a person's inflammatory status
- as an indicator of a person's thrombotic status

To study the possible use of mtDNA content as a marker for exposure to traffic-related air pollution, a cross-sectional study of diabetic patients is performed. In this study blood mtDNA content is measured by real-time quantitative polymerase chain reaction (PCR) and surrogate markers for exposure to traffic-related air pollution (i.e. distance from residence to a major road and modeled regional background level PM_{10} at the participant's home address) are determined. To test whether the mtDNA copy number gives a good indication of a person's inflammatory and thrombotic status, known biomarkers of inflammation (i.e. blood cell counts and hs-CRP) and thrombosis (i.e. platelet reactivity and plasma factor levels) are determined.

This study gives information on the variability in blood mtDNA content within an a priori susceptible population of diabetic patients and its possible relationship with exposures to particulate air pollution. Also, the potential correlation with known biomarkers of inflammation and thrombosis is discussed. Given that inflammation and thrombosis are main mechanism by which PM air pollutants affect cardiovascular health [8], this study investigates for the first time whether mitochondrial dysfunction, reflected in content alterations of mtDNA, represents a potential biological pathway linking particulate air pollution to cardiovascular health effects. A schematic representation of the outline of this study is represented in figure 2.
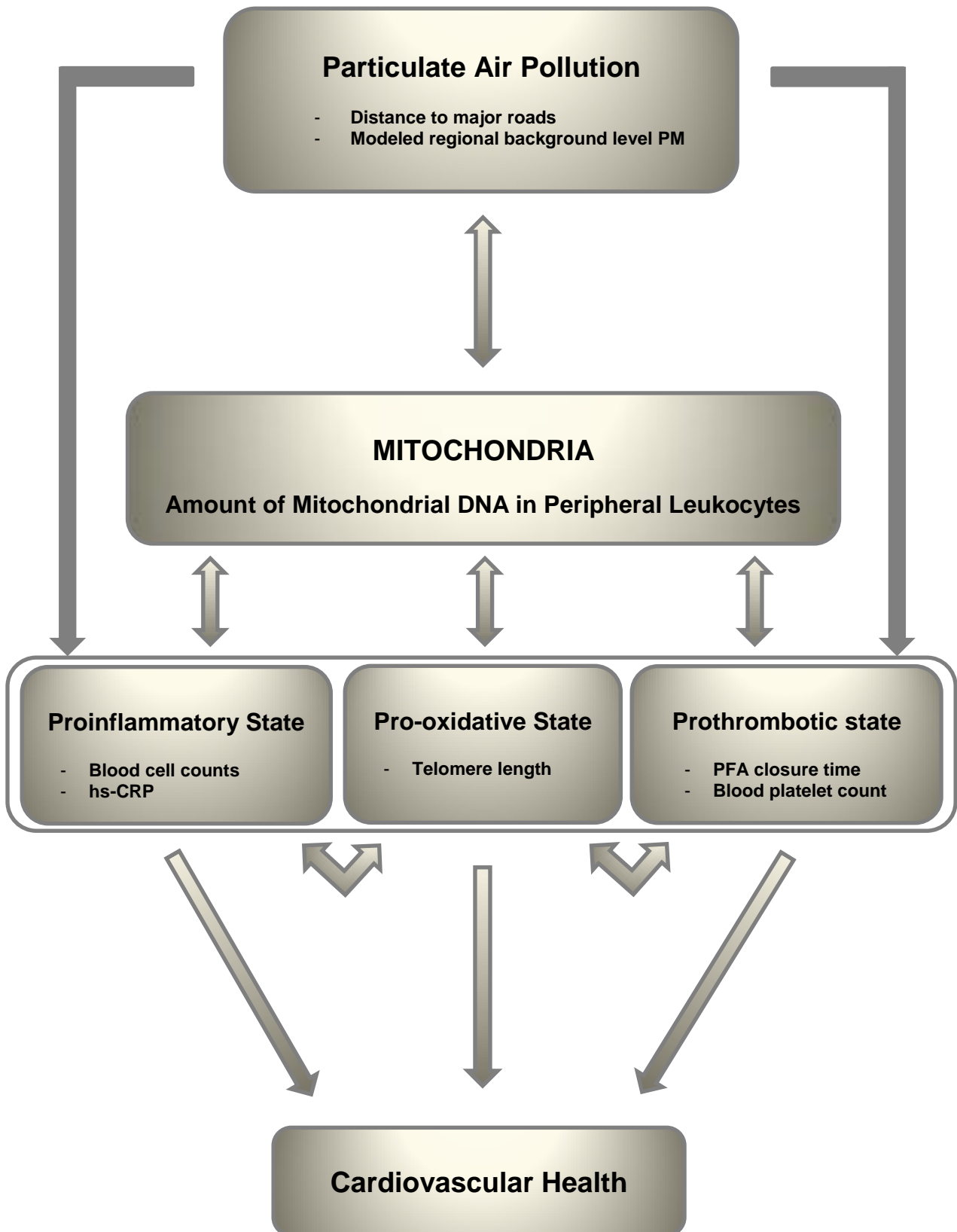
Figure 2. Schematic representation of the outline of this study. The possible use of blood mtDNA content as a marker for exposure to traffic-related air pollution is investigated. Also, the potential correlation with known biomarkers of inflammation and thrombosis is tested. Airborne particulate matter (PM), high-sensitivity C-reactive protein (hs-CRP) and platelet function analyzer (PFA).

2. Material and Methods

2.1. Study Population

The present study population is drawn from the ones previously described [16, 33]. Briefly, persons with either type 1 or type 2 diabetes were recruited consecutively from the diabetes outpatient clinic at the University Hospital Leuven. This dedicated clinic carries out routine follow-ups of patients with diabetes. Patients were invited to participate on days when the investigator was present. They were included if they were nonsmokers and ≥18 years of age. Of the 627 contacted persons, 363 took part in the examination (figure 3). All patients completed a questionnaire to gain information on age, occupation, socioeconomic status, exposure to tobacco smoke, alcohol use, use of medication, menopausal status, place of residence, and means of transportation to the hospital. Socioeconomic status was coded and condensed into a scale with scores ranging from 1 to 3 based on education and occupation.
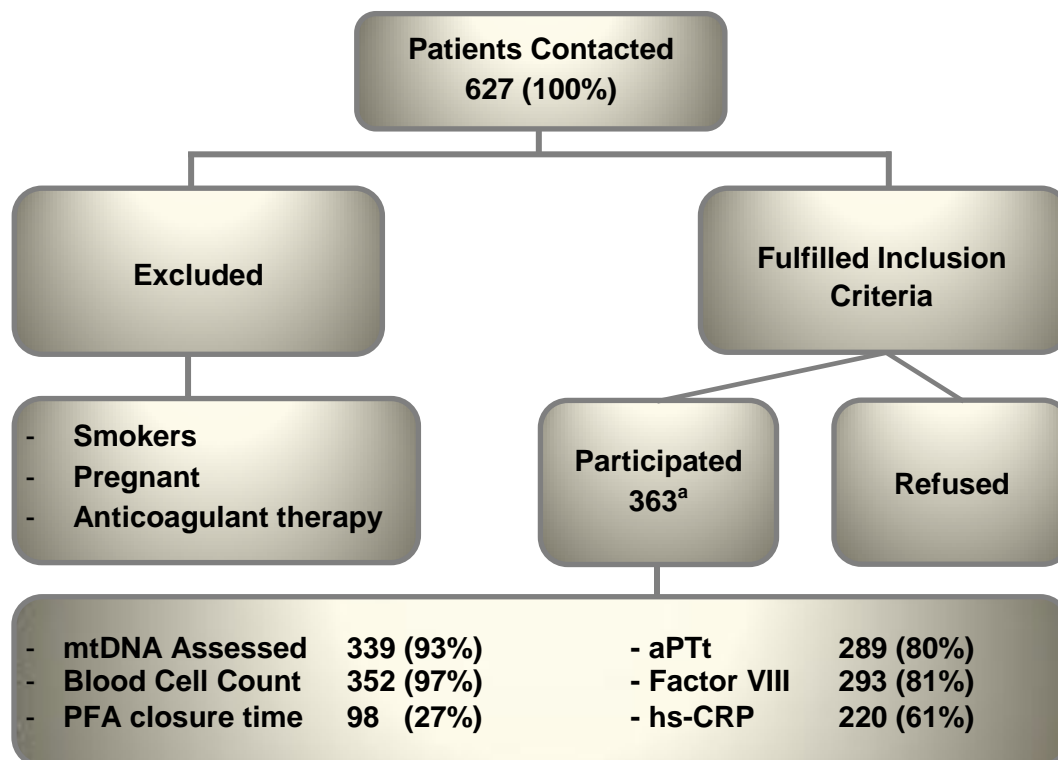
Figure 3. Flowchart of study population. Patients were consecutively recruited from the diabetes outpatient at the University Hospital Leuven. They were included if they were nonsmokers and ≥18 years of age. Platelet function analyzer (PFA), activated partial thromboplastin time (aPTt) and high-sensitivity C-reactive protein (hs-CRP). [a] The participation rate was 81%.

The Ethics Review Board of the Medical Faculty of the University of Leuven (KULeuven) approved the study. All participants gave written informed consent at recruitment.

2.2. Exposure Assessment

2.2.1. Distance to major roads

Distances from the participant's home address to major roads were calculated through geocoding (the shortest distance is 10 meters). Residing close to a major road was defined as living within 100 meters of an N-road (major traffic road) or an E-road (motorway / highway).

2.2.2. Ambient level of $PM_{2.5}$ and PM_{10}

The Aerocet 531 aerosol mass analyzer (Met One Instruments Inc.) was used to measure $PM_{2.5}$ and PM_{10} concentrations one to two hours before the patient's participation in the study. The PM concentrations were measured both outside, at the entrance of the hospital, and inside, at the waiting room. This device had been previously calibrated against a local monitoring station (Flemish Environmental Agency, Borgerhout, Belgium) [16].

2.2.3. Modeled regional background level of PM_{10}

An interpolation method was used to calculate the regional background level of PM_{10} for each participant's home address. This model provides interpolated PM_{10} values from the Belgian telemetric air quality networks in 4 x 4 km grids. The interpolation is based on a detrended kriging interpolation model that uses land cover data obtained from satellite images (Corine land cover data set, European Environment Agency, 2000) [34]. The regional background level of PM_{10} the day before recruitment was calculated, as well as the mean residential values over the preceding week, month, 3 months, 6 months and 12 months.

2.3.   Clinical Measurements

2.3.1. Peripheral Blood Collection and Analysis

Non-fasting blood samples were collected in an EDTA tube, on sodium fluoride/oxalate, and on 0.129 M (3.8%) sodium citrate. Analyses of blood cell counts and levels of glucose and glycated hemoglobin were performed on fresh full blood or plasma samples. Plasma and buffy coat samples were kept frozen at -80°C and -20°C, respectively, for future analysis.

Blood cell counts and differential leukocyte counts were determined using a Cell Dyn 3500 automated cell counter (Abbott Diagnostics) with flow differential. Routine coagulation parameters (activated partial thromboplastin time (aPTt), prothrombin time (PT), factor VII, factor VIII, factor XII, fibrinogen and D-dimers) were measured on a BCS-XP coagulation analyzer according to the manufacturer's procedures, using the manufacturer's reagents (Siemens). Blood glucose levels, glycated hemoglobin, total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides and hs-CRP were measured according to standard clinical procedures on automated analyzers. Low-density lipoprotein (LDL) cholesterol levels were calculated from the Friedewald formula [35].

2.3.2. Platelet function analyzer

The platelet function analyzer (PFA)-100 (Siemens Healthcare Diagnostics) was used to assess platelet function. This device consists of a capillary, a blood sample reservoir, and a membrane coated with collagen/epinephrine with a central aperture. Whole blood is aspirated through the capillary and the aperture, thus exposing platelets to high shear rates and collagen/epinephrine, causing platelet activation. A platelet thrombus forms at the aperture which gradually diminishes and finally arrests blood flow. The closure time, which is defined as the time from the start of aspiration until the aperture completely occludes, reflects platelet aggregation in a shear stress-dependent way [36].

2.4. Mitochondrial DNA Assessment in Peripheral Blood

2.4.1. Isolation of genomic DNA from buffy coat samples

The MagMAX™-96 DNA Multi-Sample Kit (Applied Biosystems) was used to isolate genomic DNA from buffy coat samples. This purification kit incorporates high-throughput purification of genomic DNA using magnetic bead technology. The procedures in the MagMAX™-96 DNA Multi-Sample Kit Protocol (Applied Biosystems) support 96-well reaction plate format in combination with the MagMAX™ Express-96 Well Magnetic Particle Processor (Applied Biosystems). This device significantly simplifies sample processing by eliminating the need for human participation during the washing and elution steps of the purification process.

The MagMAX™-96 DNA Multi-Sample Kit Protocol was optimized to isolate genomic DNA from buffy coat preparation containing an excess of red blood cells. The optimized protocol is attached in appendix 1. The yield and quality of purified DNA was measured immediately by the NanoDrop® ND-1000 Spectrophotometer (Isogen Life Science). The purified buffy coat DNA was stored at -20°C. DNA stocks were diluted into pure water prior to setting up real-time quantitative PCR runs. The final DNA concentration was 5 ng/µl.

2.4.2. Real-time quantitative PCR

The mtDNA content was measured in buffy coat DNA by a real-time quantitative PCR assay performed on an ABI Prism® 7900HT Sequence Detection System (Applied Biosystems). The mtDNA quantity was corrected by simultaneous measurement of the nDNA. Table 3 shows the used mitochondrial and nuclear reference genes in this study with corresponding primers for real-time quantitative PCR analysis.
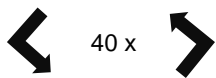
Table 3. List of the mitochondrial and nuclear reference genes with corresponding primers used to determine relative mtDNA content in peripheral leukocytes.

Gene	Gene Product	Location	Forward Primer	Reverse Primer
MT-ND1	mitochondrially encoded NADH dehydrogenase 1	Mitochondrial	5'-ATGGCCAACCTCCTACTCCT-3'	5'-CTACAACGTTGGGGCCTTT-3'
mtF3212 mtR3319		Mitochondrial	5'-CACCCAAGAACAGGGTTTGT-3'	5'-TGGCCATGGGTATGTTGTTAA-3'
RPLP0	human ribosomal protein LP0	Nuclear	5'-GGAATGTGGGCTTTGTGTTC-3'	5'-CCCAATTGTCCCCTTACCTT-3'
ACTB	human β-actin	Nuclear	5'-ACTCTTCCAGCCTCCTTCC-3'	5'-GGCAGGACTTAGCTTCCACA-3'
HBB	human β-hemoglobin	Nuclear	5'-GTGCACCTGACTCCTGAGGAGA-3'	5'-CCTTGATACCAACCTGCCCAG-3'

Mitochondrially encoded NADH dehydrogenase 1 (MT-ND1), human ribosomal protein LP0 (RPLP0), human β-actin (ACTB) and human β-hemoglobin (HBB).

The PCR was performed separately for each gene. The PCR mixture contained 5 µl Power SYBR green PCR Master Mix (Applied Biosystems), 0.3 µl forward primer (10 µM), 0.3 µl reverse primer (10 µM) and 1.9 µl RNase-free water. 2.5 µl diluted buffy coat DNA (5 ng/µl) was loaded in a 10 µl PCR reaction. All experimental samples were assayed on 96-well plates. As the number of experimental samples in this study exceeded the number of available wells in a given run, inter-run calibration was required to correct for possible technical run-to-run variation. For this purpose, several identical samples (inter-run calibrators) were measured in different runs [37]. To rule out cross-contamination of reagents and surfaces, a water control (no template) was also assayed on each 96-well plate. The thermal cycling conditions are shown in table 4. Each run was completed by melting curve analysis to confirm the amplification specificity and absence of primer dimers.

Table 4. Thermal cycling conditions for mtDNA PCR.

Temperature		Time	Process
95 °C		20 s	Activation AmpliTaq Gold® DNA-polymerase
95 °C	40 x	1 s	Denaturation DNA
60 °C		20 s	Annealing and primer extension

2.4.3. Analysis of real-time quantitative PCR data

The software program qBase^{PLUS} (Biogazelle) was used for management and automated analysis of the real-time quantitative PCR data. This software program uses an advanced relative quantification model with gene-specific amplification efficiency correction, multiple nuclear reference gene normalization and inter-run calibration [37].

2.5.   Statistical Analysis

SAS software version 9.2 (SAS Institute Inc.) was used to process the data. Non-normally distributed data were log transformed. Associations between surrogate markers for exposure to traffic-related air pollution (i.e. distance from residence to a major road and modeled average PM_{10} at the participant's home address), relative mtDNA copy number in peripheral leukocytes, and different endpoints were investigated using multiple linear regression analyses.

Three models (i.e. model A, model B and model C) were run. Results of both unadjusted analyses (in figures) and adjusted analyses (in tables) were reported.

- Model A: unadjusted
- Model B: adjusted for age, sex and type of diabetes
- Model C: adjusted for age, sex, type of diabetes, body mass index (BMI), glycated hemoglobin, use of statins, outdoor temperature and socioeconomic status

The interaction between type of diabetes and modeled PM_{10} concentration in relation to the quantity of mtDNA in blood was also explored. Finally, the possible effect modification of sex, age, type of diabetes, BMI and use of statins on the associations between surrogate markers for exposure to traffic-related air pollution and mtDNA content was studied.

3. Results

## 3.1. Mitochondrial DNA Assessment by quantitative PCR

The slope of the standard curve generated for two mitochondrial (i.e. MT-ND1 and mtF3212/R3319) and three nuclear reference genes (i.e. RPLP0, ACTB and HBB) lay between 3.27 and 3.53 (data not shown). This value represents the primer efficiency and must lie between 2.82 and 3.82 to achieve an acceptable primer efficiency of between 85 and 115%. The regression coefficient was 0.99 for all reactions. This value controls for the suitability of the linear fit. The closer this value to 1.00, the better the linear fit (table 5).

Table 5. Primer efficiencies determined by constructing standard curves for mitochondrial (i.e. MT-ND1 and mtF3212/R3319) and nuclear reference genes (i.e. RPLP0, ACTB and HBB). Standard curves were generated for mitochondrial and nuclear reference gene amplification reactions from a DNA sample serially diluted 4-fold per dilution to produce seven concentrations of DNA ranging from 9.3 to 2.3×10^{-3} ng/µl.

Gene	Primer Efficiency	Correlation Coefficient
MT-ND1	100.10%	0.99
mtF3212 mtR3319	100.99%	0.99
RPLP0	98.68%	0.99
ACTB	106.14%	0.99
HBB	103.92%	0.99

Two mitochondrial genes (i.e. MT-ND1 and mtF3212/R3319) were selected and used to determine relative mtDNA content in peripheral leukocytes. As depicted in figure 4, relative copy numbers of both mitochondrial genes were correlated positively. The Pearson correlation coefficient was 0.92 (p = <0.001).

Figure 4. Pearson correlation between relative MT-ND1 and mtF3212/R3319 copy numbers in peripheral leukocytes (based on models with log relative copy number). Both mitochondrial genes were used to calculate relative mtDNA content in peripheral blood.
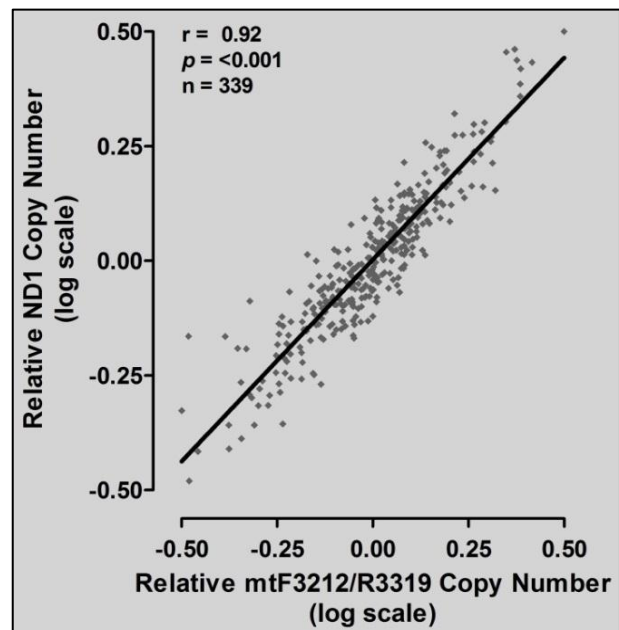
3.2. Characteristics of Study Participants

The present study population is drawn from the ones previously described [16, 33]. The characteristics of the 363 study participants (age range: 19 – 88 years) are shown in table 6. One hundred twenty-one women (66%) reported menopause, and 30 (16%) used oral contraceptives. Of the men, 63 (35%) had type 1 diabetes, compared with 82 (45%) of the women. One hundred forty-four patients with type 1 diabetes (99%) used insulin, whereas 196 persons with type 2 diabetes (90%) used insulin medication. One hundred twenty-five patients (35%) had important underlying cardiovascular disease. Outdoor arithmetic mean ± SD PM_{10} measured at the entrance of the hospital on the day of the patient's visit was 51.8 ± 27.1 μg/m^3, and the average indoor PM_{10} concentration in the waiting room was 27.8 ± 14.5. The average distance from the patient's home address to the hospital was 27.2 km (distance range: 0.5 – 138.6 km). The corresponding travel time was 21.5 min (time range: 1 – 97 min). Transportation to the hospital was by car for 79.9% of the patients.

3.3. Determinants of Mitochondrial DNA

The human mtDNA content in peripheral leukocytes was positively correlated with mean outdoor temperature on the day of participation in the study (r = 0.30; p = <0.001) and inversely correlated with age (r = -0.19; p = <0.001). The relative mtDNA copy number was also found to be significantly higher in persons with type 1 diabetes than in those with type 2 diabetes (1.07 vs. 0.97; p = 0.015) and in patients that did not use statins than in those that took statin medication (1.07 vs. 0.97; p = 0.017). Although sex, BMI, socioeconomic status and glycated hemoglobin were not significantly associated with mtDNA content, these variables, together with age, outdoor temperature, type of diabetes and statin use, were forced into the regression models.

The relative mtDNA copy number was measured on buffy coat from peripheral blood, which contains both blood leukocytes and platelets. Platelets have been shown to account for a large proportion of mtDNA content in blood DNA [38]. Therefore, blood platelet count was forced into the regression models to assess whether exposure-related changes in relative mtDNA copy number are associated with platelet activation induced by the exposures.

Table 6. Patient characteristics.

Characteristic	Total group (n=363)
Anthropometrics	
Female	183 (50)
Age (years)	57.2 ± 16.2
BMI (kg/m²)	28.7 ± 5.5
Type 1 diabetes	145 (40)
Blood glucose (mg/dl)	141.7 ± 66.8[a]
Glycated hemoglobin (%)	7.5 ± 1.1[b]
Lifestyle	
Regular alcohol use	99 (27)
Regular coffee use	268 (74)
Exposure to environmental tobacco smoke	71 (20)
Socioeconomic status	
Low	237 (65)
Middle	95 (26)
High	31 (9)
Use of medication	
Antiplatelet medication[c]	222 (61)
Statins	242 (67)
ACE inhibitor	190 (52)
Insulin	340 (94)
Oral antidiabetic medication	156 (43)
Exposure Markers	
Distance residence to major road (m)	420 (139 - 962)
Month average modeled PM_{10} (µg/m³)	26.7 ± 6.6
Year average modeled PM_{10} (µg/m³)	23.2 ± 3.1

Values are number (%) or arithmetic mean ± SD, except for distance residence to major road, which was not normally distributed, from which the median (IQR) is given.
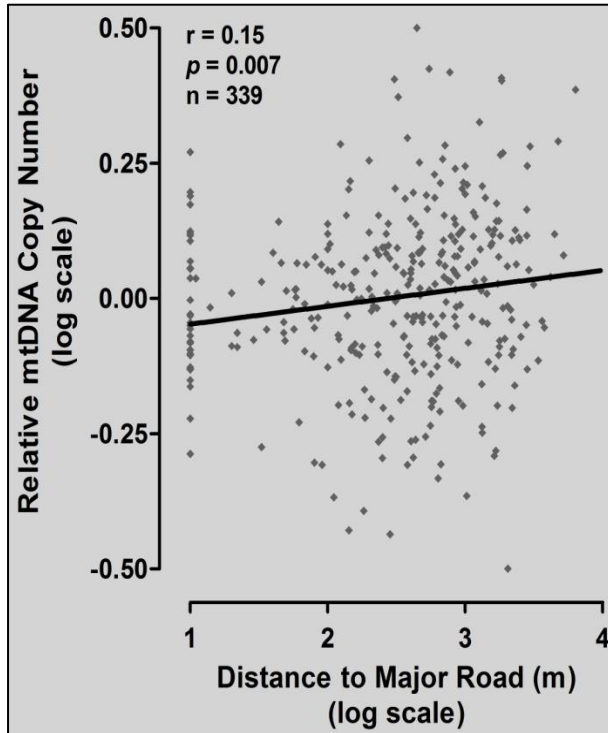[a] Data available for 354 persons
[b] Data available for 357 persons
[c] Antiplatelet medication includes acetylsalicylic acid, clopidogrel, ticlopidine or dipyridamole

3.4.   Mitochondrial DNA Content and Distance to Major Roads

The median distance from the residence to a major road was 420 meters (25th – 75th percentile: 139 to 962 meters). The relation between mtDNA content in peripheral blood, and distance between residence and major roads is represented in figure 5. Relative mtDNA content in blood was associated with distance to major roads. Each doubling in distance to major roads was associated with a significant increase – by 2.32% (95% CI, 0.63 to 4.04; p = 0.007) – in relative mtDNA content in peripheral leukocytes (table 7). Forcing blood platelet count into the regression model did not alter the reported findings significantly (data not shown). *These data validate the use of this novel biomarker as an indicator of a subject's previous exposure to traffic-related air pollution.*

Figure 5. Traffic-related exposure variable and relative mtDNA copy number in peripheral leukocytes. Pearson correlation between distance from the residence to a major road (the shortest distance to a major road is 10 meters) and relative mtDNA content in peripheral blood.

3.5.   Mitochondrial DNA Content and Exposure to PM_{10}

The regional background level of PM_{10} was calculated for each participant's home address. The mean residential values over the preceding month, 3 months, 6 months and 12 months were calculated. The mean month and year averages PM_{10} were 26.7 ± 6.6 µg/m^3 and 23.2 ± 3.1 µg/m^3, respectively. As depicted in figure 6, the relative mtDNA content in peripheral blood was inversely correlated with the mean residential PM_{10} concentration over the preceding month (r = -0.24; p = <0.001), but not with the year average PM_{10} (r = 0.02; p = 0.76). A significant decrease – by 11.74% (95% CI, -16.41 to -6.81) – in relative mtDNA content in peripheral leukocytes was associated with each interquartile range increase in modeled month average PM_{10} at the participant's residence (table 7).
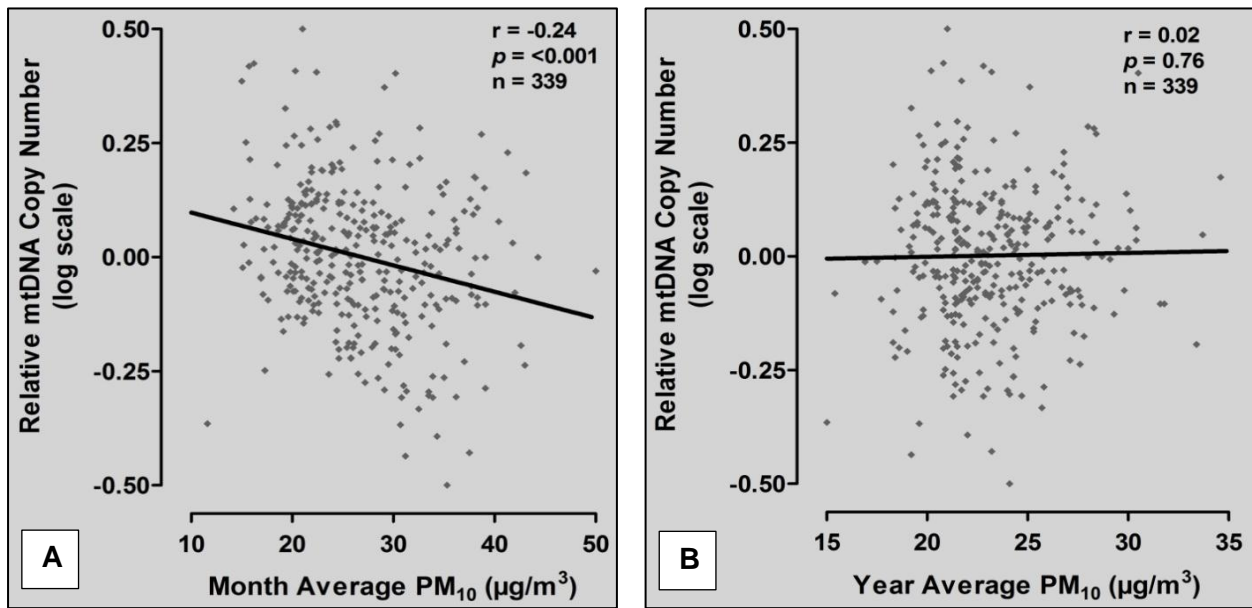
Figure 6. Traffic-related exposure variables and relative mtDNA copy number in peripheral leukocytes. An interpolation method was used to calculate the regional background level of PM_{10} for each participant's home address. The mean residential values over the preceding month and 12 months were calculated. Pearson correlation between month (A) or year average PM_{10} (B), and mtDNA content.

Forcing sex, age, type of diabetes, BMI, glycated hemoglobin, use of statins, outdoor temperature and socioeconomic status into the models did not alter the reported findings significantly. *This further confirms that this novel biomarker gives a good indication of person's previous exposure to traffic-related air pollution.*
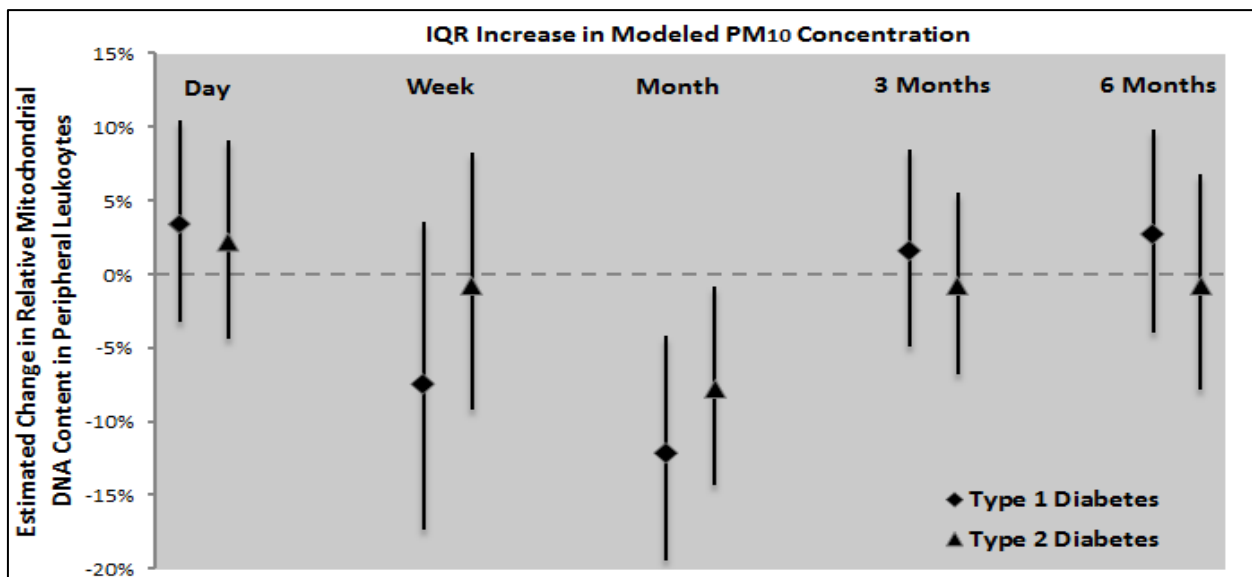
Figure 7. Estimated change in relative mitochondrial DNA content for an interquartile range (IQR) increase in modeled PM_{10} concentration by type of diabetes. The regional background level of PM_{10} the day before recruitment was used (IQR = 15 $\mu g/m^3$), as well as the mean residential values over the preceding week (IQR = 17.1 $\mu g/m^3$), month (IQR = 9.4 $\mu g/m^3$), 3 months (IQR = 4.2 $\mu g/m^3$) and 6 months (IQR = 3.6 $\mu g/m^3$). Estimated changes were adjusted for age, sex, BMI, glycated hemoglobin, socioeconomic status, use of statins, outdoor temperature and blood platelet count. The interaction terms between type of diabetes and PM_{10} concentration in relation to mtDNA content in peripheral leukocytes did not reach statistical significance (p > 0.34).

After adjustment for age, sex, BMI, glycated hemoglobin, socioeconomic status, use of statins, outdoor temperature and blood platelet count, relative mitochondrial copy number was negatively associated with the mean residential PM_{10} concentration over the preceding month both in persons with type 1 ($p = 0.004$) and type 2 diabetes ($p = 0.030$). As represented in figure 7, each interquartile range increase in modeled month average PM_{10} was associated with a 12.1% (95% CI, -4.2 to -19.4) decrease in mtDNA content in persons with type 1 and a 7.8% (95% CI, -0.8 to -14.2) decrease in persons with type 2 diabetes. However, no significant changes were observed in association with the mean day, week, 3 month or 6 month averages PM_{10}.

Table 7. Estimated change in relative mitochondrial copy number in association with distance from residence to major road or modeled regional background level of PM_{10} for each participant's home address.

Exposure Marker	Model	Change	95% CI	*p*-value
Distance from Residence to Major Road				
Relative mtDNA copy number (%)	A	2.32	0.63 to 4.04	0.007
	B	2.17	0.50 to 3.88	0.011
	C	2.18	0.54 to 3.84	0.010
Month Average Modeled PM_{10}	**+9.4 µg/m^3 (interquartile range)**			
Relative mtDNA copy number (%)	A	-11.74	-16.41 to -6.81	<0.001
	B	-10.92	-15.61 to -5.97	<0.001
	C	-9.09	-13.87 to -4.03	<0.001
Year Average Modeled PM_{10}	**+3.6 µg/m^3 (interquartile range)**			
Relative mtDNA copy number (%)	A	0.92	-3.60 to 5.66	0.70
	B	0.77	-3.69 to 5.44	0.74
	C	0.23	-4.17 to 4.84	0.92

Estimates calculated for a twofold increase in distance from residence to major road (based on a model with log distance) or for an interquartile range increase in PM_{10} concentration. CI = confidence interval.
Model A: Unadjusted
Model B: Adjusted for age, sex and type of diabetes
Model C: Adjusted for age, sex, type of diabetes, BMI, glycated hemoglobin, use of statins, outdoor temperature and socioeconomic status

3.6.   Mitochondrial DNA Content and Inflammatory Status

Total blood cell and differential blood leukocyte (i.e. neutrophil, monocyte, eosinophil and lymphocyte) counts were determined. The number of leukocytes per microliter of blood was significantly higher in persons with type 2 diabetes than in those with type 1 diabetes (7270 vs. 6327; p = <0.001). However, no association was found between the total amount of blood cells and blood glucose or glycated hemoglobin. Both before adjustment (figure 8) and after adjustment for sex, age, type of diabetes, BMI, glycated hemoglobin, use of statins, outdoor temperature and socioeconomic status (table 8), the total number of leukocytes was correlated negatively with mtDNA content. Each doubling in mtDNA copy number was associated with a decrease of 1000 leukocytes per microliter of blood (95% CI, -1408 to -592; p = <0.001).

The same negative correlation was found between mtDNA copy number in peripheral blood and the amount of blood monocytes or neutrophils. The Pearson correlation coefficients were -0.21 (p = <0.001) and -0.27 (p = <0.001), respectively. However, no significant changes were observed in blood eosinophils and lymphocytes (data not shown). *These results validate the use of mtDNA content in circulating peripheral leukocytes as an indicator of a person's inflammatory state.*
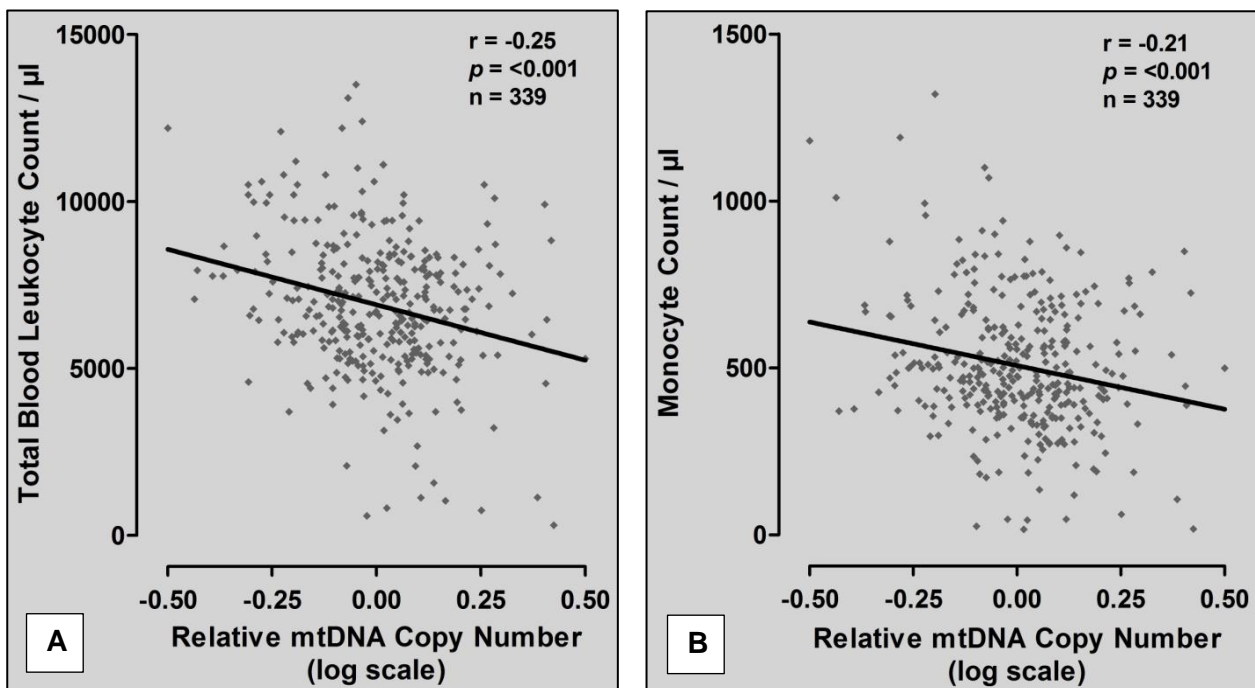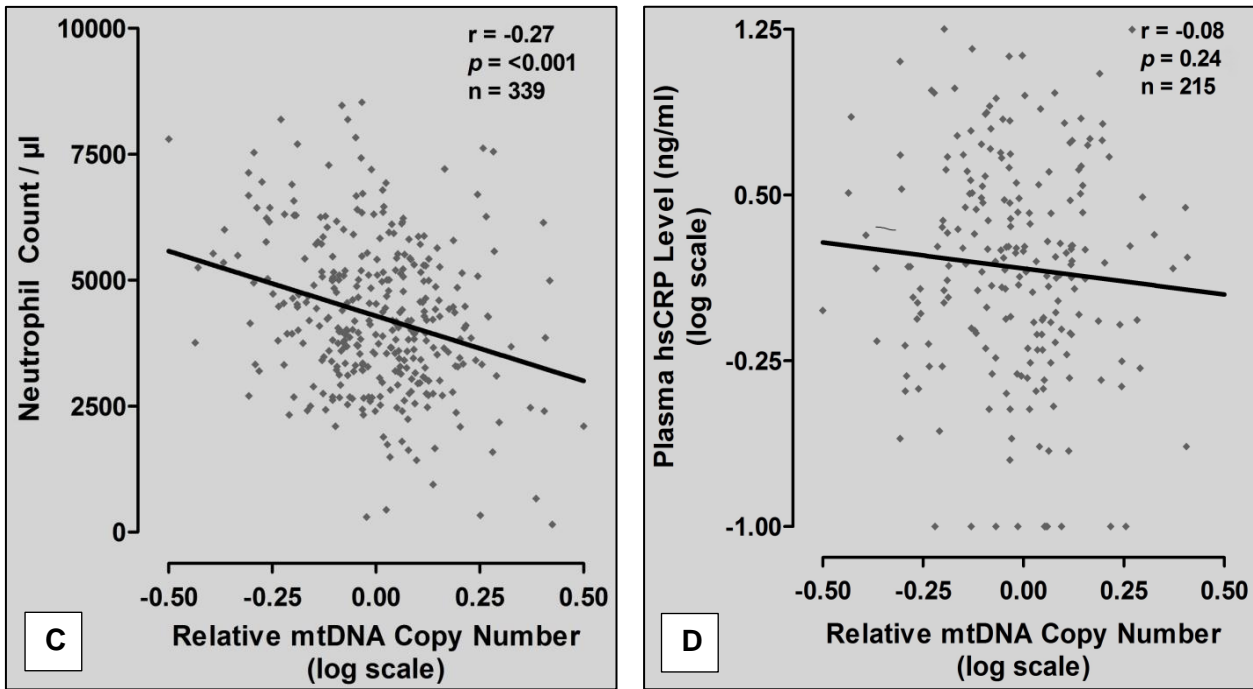
Figure 8. Inflammatory status and mtDNA content in blood. Pearson correlations between relative mtDNA content in peripheral leukocytes and total (A) or differential blood leukocyte (i.e. neutrophil and monocyte) counts (B, C). Plasma hs-CRP level, taken as a marker of inflammation, was measured according to standard clinical procedures on an automated analyzer. Pearson correlation between relative mtDNA copy number in blood and plasma hs-CRP level (D).

Plasma hs-CRP, taken as a marker of inflammation, was measured. However, no correlation was found between mtDNA content in peripheral leukocytes and plasma hs-CRP levels (based on a model with log plasma hs-CRP level).

3.7. Mitochondrial DNA Content and Thrombotic Status
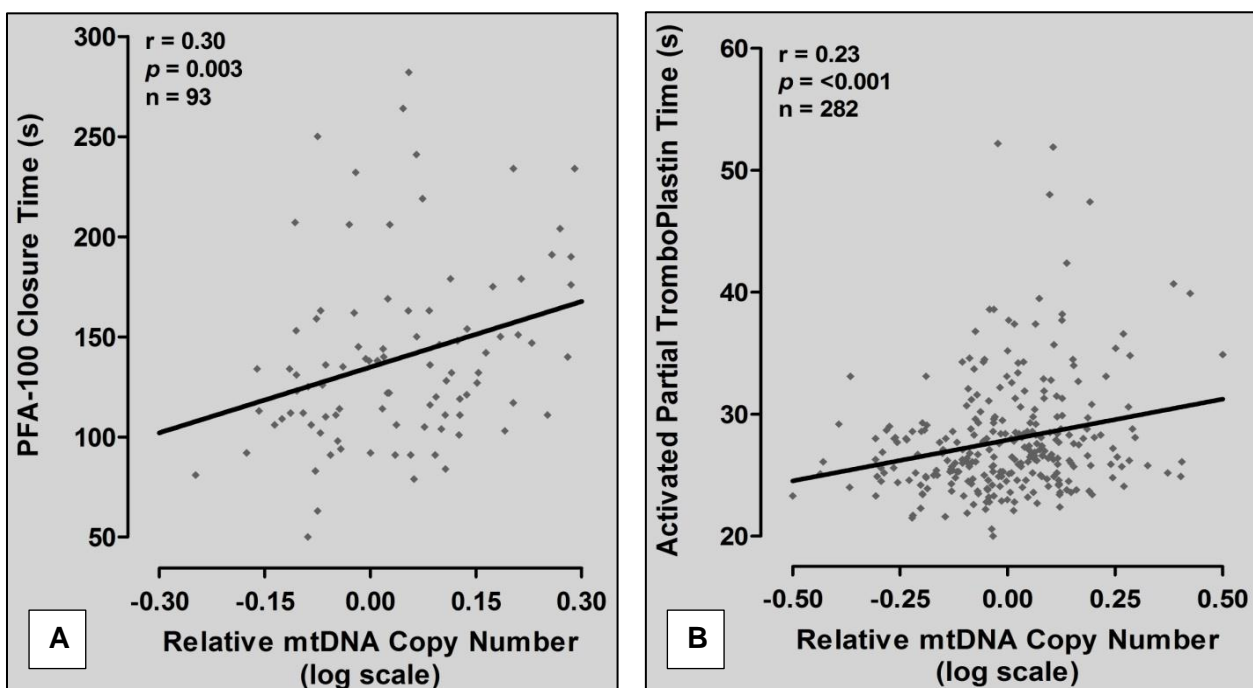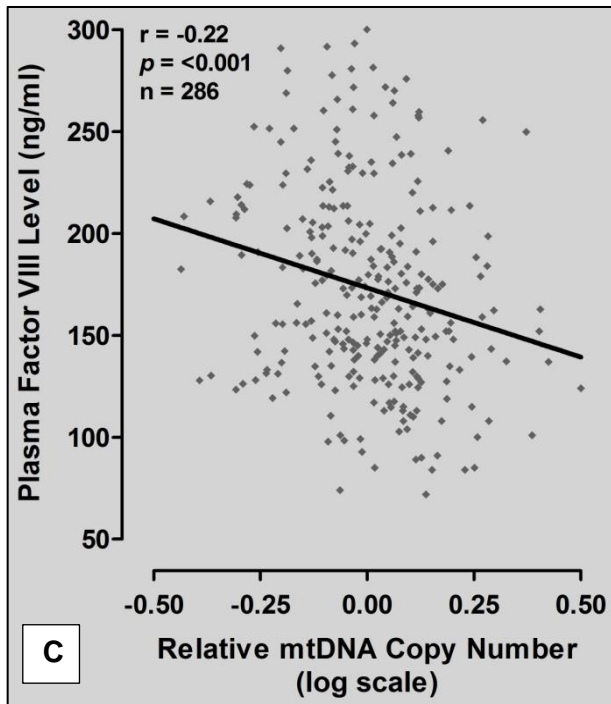
Figure 9. Thrombotic status and mtDNA content. Pearson correlations between mtDNA copy number in peripheral blood and platelet function (A) or coagulation parameters (i.e. activated partial thromboplastin time (B) and plasma factor VIII level (C)). Platelet function was assessed by PFA-100. Increased platelet reactivity is reflected by decreases in closure time.

Platelet function, blood platelet count and different coagulation parameters (i.e. aPTt, PT and plasma factor levels) were determined. The PFA-100 closure time reflects platelet aggregation in a shear stress-dependent way. No association between closure time and sex, type of diabetes or age was observed. As represented in figure 9, the closure time was associated positively with mtDNA content in peripheral blood (r = 0.30; p = 0.003). The PFA-100 closure time increases – by 30.9 s (95% CI, 10.9 to 50.9) – with each doubling in mtDNA content (table 8). The number of blood platelets per microliter of blood was significantly higher in women than in men ($293x10^3$ vs. $253x10^3$; p = <0.001). An association between mtDNA and platelet count was not found (data not shown). Of the measured coagulation parameters (i.e. aPTt, PT and plasma factor levels), aPTt and plasma factor VIII were correlated with the relative amount of mtDNA (figure 9). Each increase in mtDNA content was associated with an increase in aPTt (r = 0.23; p = <0.001) and a decrease in plasma factor VIII level (r = -0.22; p = <0.001). Forcing studied covariates into the models, including sex, age, type of diabetes, BMI, glycated hemoglobin, use of statins, outdoor temperature and socioeconomic status, did not alter the reported findings significantly. *These results validate the use of mtDNA content in circulating peripheral leukocytes as an indicator of a person's thrombotic state.*

3.8.   Effect-Modification

Studies have pointed to reduced susceptibility to the effects of air pollution in those that take statins [39-40]. Therefore the interaction term of mtDNA content in blood by statin use was tested. The interaction term of distance from residence to major road and mtDNA content by statin use did not reach statistical significance (p = 0.46). There was also no effect-modification by sex (p = 0.72 for interaction), age (p = 0.42), type of diabetes (p = 0.20) and BMI (p = 0.23) on the association between distance and mtDNA content. The interaction term of modeled PM_{10} at participant's residence and mtDNA content was also tested. However, the interaction term did not reach statistical significance in any of the models (p > 0.20).

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Table 8. Estimated change of different inflammatory and thrombotic parameters for a twofold increase in relative mitochondrial copy number in peripheral blood cells (based on a model with log relative mitochondrial copy number).

Endpoint	Model	Change	95% CI	*p*-value
Inflammatory				
Total Blood Leukocyte Count / µl	A	-1000	-1408 to -592	<0.001
	B	-912	-1319 to -504	<0.001
	C	-852	-1280 to -424	<0.001
Neutrophil Count / µl	A	-775	-1067 to -483	<0.001
	B	-672	-963 to -381	<0.001
	C	-653	-960 to -346	<0.001
Monocyte Count / µl	A	-78.7	-118.1 to -39.4	<0.001
	B	-61.0	-99.4 to -22.6	0.002
	C	-50.2	-90.2 to -10.3	0.014
Plasma hs-CRP Level (%)	A	-15.4	-36.1 to 12.0	0.24
	B	-14.3	-35.1 to 13.1	0.28
	C	-15.9	-36.8 to 12.1	0.24
Thrombotic				
PFA-100 Closure Time (s)	A	30.9	10.9 to 50.9	0.003
	B	33.4	12.9 to 53.8	0.002
	C	38.1	18.4 to 57.8	<0.001
Activated Partial Thromboplastin Time (s)	A	2.02	1.00 to 3.03	<0.001
	B	1.77	0.75 to 2.78	<0.001
	C	1.69	0.61 to 2.76	0.002
Plasma Factor VIII Level (ng/ml)	A	-20.4	-31.1 to -9.77	<0.001
	B	-13.5	-23.6 to -3.45	0.009
	C	-10.4	-20.9 to 0.11	0.054

Estimates calculated for a twofold increase in relative mitochondrial copy number in peripheral blood cells (based on a model with log relative mitochondrial copy number). CI = confidence interval.
Model A: Unadjusted
Model B: Adjusted for age, sex and type of diabetes
Model C: Adjusted for age, sex, type of diabetes, BMI, glycated hemoglobin, use of statins, outdoor temperature and socioeconomic status
For numbers see figure 3.

4.   Discussion

4.1.   Mitochondrial DNA Content and Exposure Markers

The key finding of this human study of diabetic subjects is that mtDNA copy number in peripheral blood leukocytes, which is an established marker of mitochondria damage and malfunctioning, is associated with surrogate markers for exposure to traffic-related air pollution (i.e. distance from residence to a major road and modeled month average PM_{10} at the participant's home address). This association could not be explained by age, sex, type of diabetes, BMI, glycated hemoglobin, use of statins, outdoor temperature or socioeconomic status.

4.1.1. Mitochondrial DNA content and exposure markers in healthy subjects

A recent study on foundry workers exposed to a wide range of PM levels has already shown associations of blood mtDNA copy number with exposures to airborne PM. In this cohort, mtDNA copy number was higher on the 4th day than the one of the 1st day of the same work week, but this increase was inversely associated with the average personal exposure level between the two mtDNA copy number measurements [20].

The mitochondrial genome is more susceptible to ROS-induced damage when compared with the nuclear genome, and increases in mtDNA copy number in response to oxidative damage result from a cellular response that compensates for dysfunctional mitochondria [23]. The vulnerability of mtDNA to oxidative damage could be due to many factors including: the absence of histones or DNA-binding proteins, a limited basic repair mechanism, genes consisting only of exons without introns, and replicating rapidly without a significant proofreading system [19]. Therefore, the positive correlation between mtDNA content and PM [20] or cigarette smoke exposure [19, 41] in healthy subjects could be seen as a cellular response to the increased oxidative stress. This feedback response in cells challenged with ROS has important consequences. Cells, with adequate antioxidant capacity and good quality of mitochondria, increase their mitochondrial abundance to meet higher energy demands. The increased mitochondrial energy production is used to repair ROS-induced cell damage. However, cells with compromised antioxidant activity increase the abundance of defective mitochondria bearing impaired respiratory chain or mutated mtDNA. This causes excess ROS production and further oxidative DNA damage, which can eventually lead to cellular senescence or apoptosis [42].

4.1.2. Mitochondrial DNA content and exposure markers in diabetic subjects

In this study of diabetic subjects, a negative correlation between the relative content in mtDNA and exposure to traffic-related air pollution was observed. This observation suggests that exposure to particulate air pollution in persons with diabetes modulates mtDNA replication in a negative manner. It is well accepted that mtDNA replication does not coincidence with the cell cycle and occurs independently of nuclear DNA replication. However, the nucleus plays an important role in regulating mtDNA copy number since all *trans*-acting factors associated with mtDNA replication are encoded by nuclear DNA [43].

The most important proteins that function in mtDNA replication are listed in table 9. DNA polymerase γ (POLG) is the only known DNA polymerase in animal cell mitochondria. The components of human POLG (i.e. conserved polymerase and exonuclease domains) possess all activities necessary to carry out mtDNA replication and repair to some extent. Two other proteins that function in mtDNA replication, mitochondrial single-stranded DNA-binding protein (mtSSBP) and Twinkle, work together to achieve helix destabilization during replication.

Table 9. Proteins that function in mtDNA replication.

Protein	Function
POLG	mtDNA replication (polymerase activity) and repair (exonuclease activity)
mtSSBP	Destabilization helix during replication (helicase activity)
Twinkle	Augmentation processivity and fidelity of POLG
PGC-1α	Initiation mtDNA replication Induction expression NRF-1, NRF-2 and Tfam
NRF-1	Coordination expression of mitochondrial genes
NRF-2	Induction expression Tfam and mTFB
Tfam	Regulation replication of the mitochondrial genome
mTFB	Regulation protein binding at the D-loop of mtDNA

DNA polymerase γ (POLG), mitochondrial single-stranded DNA-binding protein (mtSSBP), peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), nuclear respiratory factor-1 and 2 (NRF-1 and NRF-2), and mitochondrial transcription factor A (Tfam) and B (mTFB).

Initiation of mtDNA replication occurs within the D-loop, the *cis*-regulatory region of the mitochondrial genome, after activation of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α). This nuclear transcriptional coactivator regulates the expression of nuclear respiratory factors (such as nuclear respiratory factor-1 and 2 (NRF-1 and NRF-2)) and mitochondrial transcription factors (such as mitochondrial transcription factor A (Tfam) and B (mTFB)). The latter two proteins are also cooperatively transcribed by both NRF-1 and NRF-2. These factors are then imported into the mitochondrial matrix, act on the promotors within the D-loop region of mtDNA, and regulate replication of the mitochondrial genome [43-44].

Hence, it is possible that particulate air pollution in persons with diabetes modulates mtDNA replication by altering expression levels of transcription factors (e.g. NRF-1, NRF-2, Tfam and mTFB) and regulators (e.g. PGC-1α) that function in mtDNA replication. Many signaling pathways have been shown to be responsive to oxidative stress elicited by excess production of ROS. Both activating protein-1 (AP-1) and NF-κB are the transcription factors long established to respond to oxidative stress. However, very few direct links have been established between the transcriptional activity of AP-1 or NF-κB and expression of proteins that function in mtDNA replication [42].

4.1.3. Redox control of mitochondrial DNA replication

It has been suggested that mitochondria are important in the activation of NF-κB, a protein that plays a central role in the regulation of many of the genes involved in cellular defense mechanisms, pathogen defenses, immunological responses, and the expression of cytokines and cell-adhesion molecules. The ROS generated in the mitochondrial respiratory chain have been proposed as being the intermediate messengers involved in the activation of NF-κB. Given that ROS can both promote and inhibit NF-κB transcriptional activation, it is possible that mitochondrial oxidant production in response to stimuli can ultimately induce totally different responses in normal and dysfunctional mitochondria [45].

PGC-1α has emerged as a key protein that can regulate mtDNA content and has been referred as the 'master regulator' of mtDNA replication. This coactivator appears to be part of a redox-sensitive pathway similar to the ROS-sensitive transcription factor NF-κB. In fact, the human PGC-1α promoter contains an NF- κB binding site, which suggests NF-κB may also regulate the expression of PGC-1α [46].

Thus, it is hypothesized that mitochondrial oxidant production in response to traffic-related air pollution inhibits NF-κB transcriptional activation in diabetic patients, leading to reduced expression levels of proteins that function in mtDNA replication (figure 10 - right). Whereas peripheral blood cells of health subjects, with good quality of mitochondria, possibly activate both NF-κB and mtDNA replication in response to ROS (figure 10 - left). Reestablishment of the balance between oxidative stress and antioxidant defenses in mitochondria will be crucial for normalization of mitochondrial function as well as preventing diabetes complications. Normalizing mitochondrial ROS levels by overexpressing MnSOD in cultured bovine aortic endothelial cells prevented high glucose-induced activation of at least three different pathological pathways related to late diabetic complications [47].
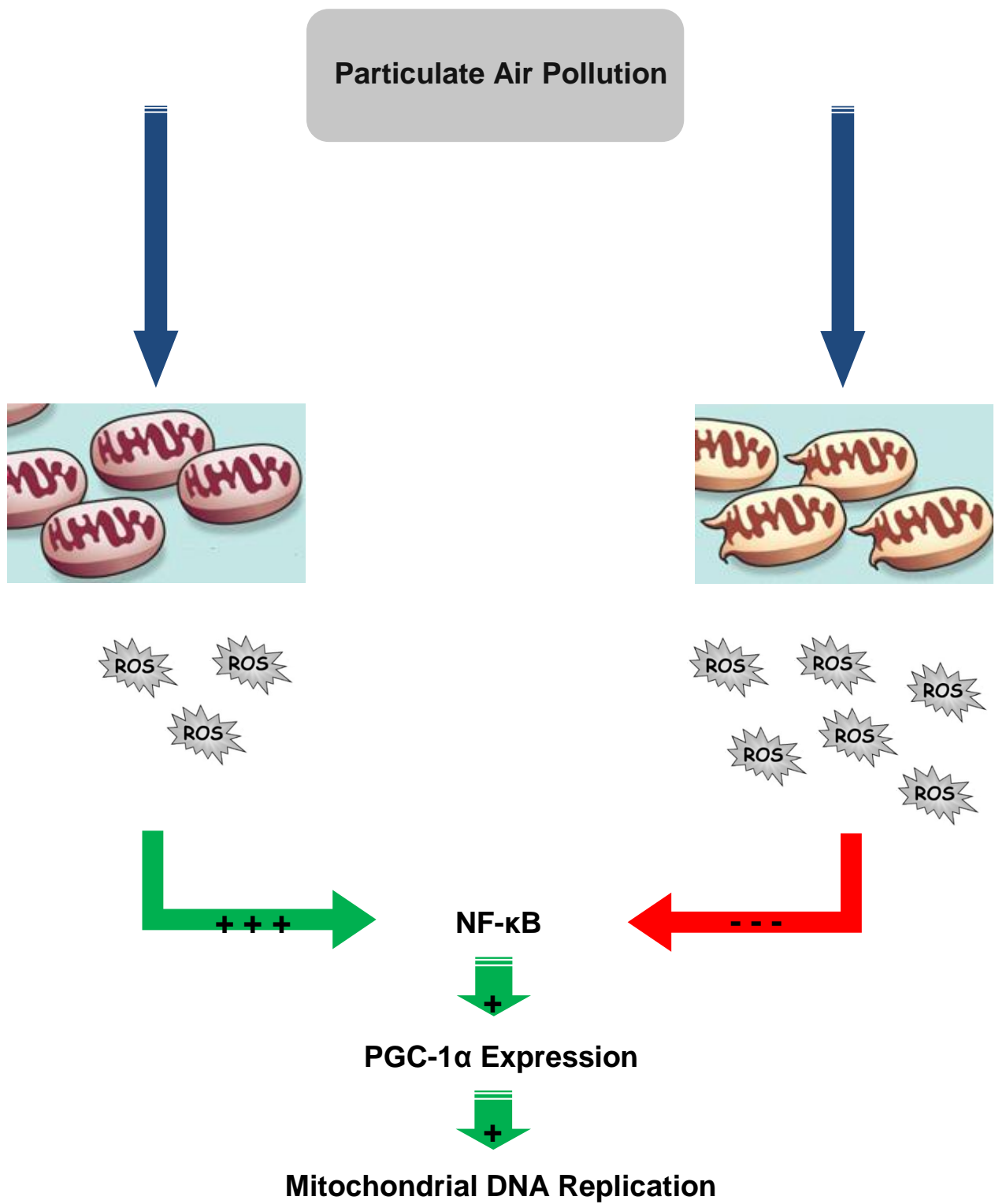
Figure 10. Signal transduction pathway involved in mtDNA replication. Mitochondrial oxidant production in response to traffic-related air pollution activates NF-κB in healthy subjects, leading to increased expression levels of proteins that function in mtDNA replication (left). This can be seen as a compensatory response in cells challenged with mild oxidative stress. However, excess production of ROS by dysfunctional mitochondria in persons with diabetes may result in severe oxidative damage and perturb the stress response, leading to a decrease in mtDNA content (right).

4.1.4. Turnover of the circulating peripheral leukocytes

Practical and ethical considerations limit the types of tissues available for mtDNA assessment in humans. Therefore, mtDNA content has predominantly been studied in easy available peripheral white blood cells (WBCs). It must be emphasized that these cells have a rapid turnover. A normal differential WBC count shows about 5-10% monocytes, 20-40% lymphocytes and 40-75% granulocytes. Human monocytes and granulocytes have a short circulatory half-life (hours-days). On the other hand, the time spent in the circulation by lymphocytes is more variable. About 75% of lymphocytes are long-lived T-cells with half-lives of a few months up to a year. The remaining 25% are T-cells, B-cells and null cells that have half-lives of a few hours to about 5 days [48].

The findings of the present study suggest that the correlations between PM exposure and relative mtDNA copy number were the result of subchronic exposure to traffic-related air pollution. As monocytes and granulocytes represent recent exposure only, the lymphocyte subpopulation contributes most to the measured variability in blood mtDNA content in response to particulate air pollution. A separation of the different WBC-subpopulations might be necessary to obtain a more reliable estimate of exposure-induced effects on mtDNA content.

4.2.   Mitochondrial DNA Content and Different Endpoints

Another key finding of this study is that blood mtDNA content within an a priori susceptible population of diabetic patients is inversely correlated with known biomarkers of inflammation (e.g. blood cell counts) and thrombosis (e.g. platelet reactivity). This association could not be explained by age, sex, type of diabetes, BMI, glycated hemoglobin, use of statins, outdoor temperature or socioeconomic status.

4.2.1. Mitochondrial DNA content and biomarkers of inflammation

Recent and chronic exposures to PM have been associated with markers of systemic inflammation, seen as an increase in blood leukocyte counts, in persons with diabetes [16]. In the present study an inverse correlation is found between blood mtDNA content and both subchronic exposure to particulate air pollution and blood leukocyte counts. *This suggests that mitochondrial dysfunction, reflected in content alterations of mtDNA that lead to a reduced relative mtDNA copy number in peripheral blood, represents a biological effect along the path linking particulate air pollution to proinflammatory changes, such as increases in blood leukocyte counts.*

It has been shown that inflammation is involved in the development of atherosclerosis [49]. Exposure to PM leading to systemic inflammation might therefore also play a role in the development of atherosclerosis. In rabbits, a study reported that repeated exposure to PM_{10} was associated with both systemic inflammation and the progression of the atherosclerotic process [50]. Chronic inflammation is more prominent in type 2 diabetes than in persons with type 1 diabetes. According to this, the relative mtDNA copy number was found to be significantly lower in persons with type 2 diabetes than in those with type 1 diabetes. However, no evidence of a higher sensitivity to air pollution-induced effects on mtDNA content in persons with type 2 diabetes compared with their type 1 counterparts was found. This can be explained by the well-controlled glycated hemoglobin levels (arithmetic mean = 7.5%) in the present study population. Furthermore, insulin use in persons with type 2 diabetes was high (90%).

4.2.2. Mitochondrial DNA content and biomarkers of thrombosis

Thrombotic status is also documented because a negative association between platelet reactivity and mtDNA content in peripheral blood was found. Platelet reactivity, measured *ex-vivo* with the PFA, allows a quantitative measure of platelet aggregation as the time required to close a small aperture in a biological active membrane by relevant stimuli. This study shows that the closure time correlated positively with mtDNA content. A twofold increase in relative mitochondrial copy number in peripheral blood was associated with an increase in the PFA closure time of 30.1 s. This is comparable with the effect of a daily intake of 75 mg aspirin during 2 weeks. This caused an increase in the median PFA closure time of 30 s in 10 healthy individuals [51].

Currently, it is well recognized that thrombosis underlies most acute complications of atherosclerosis, such acute myocardial infarction. Exposure to PM has been associated with prothrombotic effects on platelet function in a presumably more susceptible population of diabetic patients [16]. The results of this study showed a negative association between mtDNA content in peripheral leukocytes and both subchronic exposure to traffic-related air pollution and platelet reactivity. *This suggests that mitochondrial dysfunction, reflected in content alterations of mtDNA that lead to a reduced relative mtDNA copy number in peripheral blood, represents a biological effect along the path linking traffic-related air pollution to prothrombotic changes, such as prothrombotic changes on platelet function.*

Finally, it is assumed that decreases in blood mtDNA content in response to particulate air pollution in this study population do not induce mitochondrial respiratory chain dysfunction. To induce mitochondrial respiratory chain dysfunction, mtDNA damage of more than 80% is necessary [52]. In the present study, the estimated reduction of mtDNA content for an interquartile range increase in month average modeled PM_{10} was only 11.7%.

4.3. Study Limitations

This study has limitations. Causality cannot be established by observational studies. Statistical analyses used in observational studies can only identify associations. The relative mtDNA copy number was measured on buffy coat from peripheral blood, which contains both blood leukocytes and platelets. Platelets have been shown to account for a large proportion of mtDNA content in blood DNA [38]. However, this is unlikely to have introduced bias, since no association was found between relative mtDNA copy number and blood platelet count. No significant differences were found in exposure (proximity to major roads or PM_{10}) between the different socioeconomic classes. The exposure markers used here (i.e. distance from residence to a major road and modeled background level PM_{10} at the participant's home address) are surrogates for exposure to traffic-related air pollution. Although this study has focused on PM, a potential role of gaseous pollutants associated with traffic air pollution (such as sulfur dioxide, carbon monoxide, nitrogen oxides and ozone) cannot be excluded. This study involved an a priori susceptible population of patients, because diabetic subjects are more sensitive to the deleterious effects of particulate air pollution [31]. This means that conclusions of the study results not necessarily apply to healthy subjects.

5. Conclusion and Synthesis

In summary, in the present human study of diabetic patients, the possible use of relative mtDNA copy number in peripheral leukocytes as a marker for exposure to particulate air pollution is validated. Given that inflammation and thrombosis are main mechanism by which PM air pollutants affect cardiovascular health, the potential correlation with known biomarkers of inflammation and thrombosis is also investigated.

The key finding of this study is that relative mtDNA copy number, which is an established marker of mitochondria damage and malfunctioning, is inversely correlated with subchronic exposure to particulate air pollution in a susceptible target population. Another finding is the negative association between the relative copy number of mtDNA in circulating peripheral leukocytes and known biomarkers of inflammation and thrombosis. This indicates that this novel biomarker gives a good indication of a person's inflammatory and thrombotic status. These findings suggest that mitochondrial dysfunction, reflected in lowered mtDNA copy numbers in peripheral blood, represents a potential biological pathway linking particulate air pollution to proinflammatory and prothrombotic changes. These study results in persons with diabetes have important implications for understanding the biological mechanisms of air pollution on cardiovascular health, because both a prothrombotic tendency and system inflammation play an important role in atherosclerosis and cardiovascular disease.

This is the first study reporting the association between PM exposure and blood mtDNA content in an a priori susceptible population of diabetic patients. The results of this study add new information to previous *in-vitro* experiments. These experiments demonstrated that particulate pollutants generate oxidative stress and cause changes in mitochondrial morphology, including mitochondrial swelling and a loss of cristae [53].

Future research is warranted to better elucidate the pathways that might link particulate air pollution with content alterations of mtDNA. The main focus should be on transcription factors (e.g. NRF-1, NRF-2, Tfam and mTFB) and regulators (e.g. PGC-1α) that function in mtDNA replication. Also, further studies are required to validate the use of other age-related biomolecular markers (e.g. DNA methylation content and telomere length) for evaluating the relation between particulate air pollution and cardiovascular health (54-55).

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7. Appendix

7.1. Appendix 1: Isolation of Genomic DNA from Buffy Coat Samples

Magmax Multi-Sample Kit	*4413021*	Isopropanol
1 Deep Well Tip Comb	*4388487*	Vortex
7 Deep Well Plates	*4388476*	Plate Shaker
1 Standard Plate	*4388475*	

Preparation

- Prepare **DNA Binding Bead Mix** *under ice-cold conditions*

Component	Volume
DNA Binding Beads	16 µl
Nuclease-free Water	4 µl
Total (DNA Binding Bead Mix)	20 µl

- Prepare **RNase A mix** *under ice-cold conditions*

Component	Volume
RNase A	5 µl
Nuclease-free Water	95 µl
Total (RNase A mix)	100 µl

- Select the **4413021 DW Tissues** protocol

Disrupt the samples

- Aspirate the **plasma** and mix the red blood cells and leukocytes

- Prepare a homogenous **Buffy Coat Lysate**

Component	Volume
Buffy Coat Layer	75 µl
Multi-Sample DNA Lysis Buffer	225 µl
Buffy Coat Lysate	300 µl

Perform the DNA extraction and elution

- Transfer **300 µl Buffy Coat Lysate** to a well of a Deep Well Plate

    o Add 90 µl **Isopropanol** (100%)
      ▪ Seal the plate
      ▪ Shake for 3 minutes on a Plate Shaker (speed 9)

    o Add 20 µl of prepared **DNA Binding Bead Mix**
      ▪ Seal the plate
      ▪ Shake for 3 minutes on a Plate Shaker (speed 9)

- Prepare the plates for the Magnetic Particle Processor

Position	Plate Type	Reagent (Step I)	Volume	Reagent (Step II)	Volume
1	Deep Well Plate	Sample			
2	Deep Well Plate	Wash Buffer 1	150 µl		
3	Deep Well Plate	Wash Buffer 2	150 µl		
4	Deep Well Plate	RNase A mix	100 µl	DNA Lysis Buffer Isopropanol	100 µl 120 µl
5	Deep Well Plate	Wash Buffer 2	150 µl		
6	Deep Well Plate	Wash Buffer 2	150 µl		
7	Standard Plate	DNA Elution Buffer 1	50 µl	DNA Elution Buffer 2	50 µl
8	Deep Well Plate Tip Comb				

- Load the Magnetic Particle Processor and start the **4413021 DW Tissues** protocol

Start Protocol	16 Minutes	Add DNA Lysis Buffer Isopropanol	22 Minutes	Add DNA Elution Buffer 2	6 Minutes	Protocol Finished

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Richting: **master in de biomedische wetenschappen-milieu en gezondheid**
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