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Summary

The environment encompasses the totality of the conditions we live in ranging from environmental pollution to social conditions and lifestyle features. Such environmental factors are already long-time acknowledged to be involved in all aspects of health and disease. Nevertheless, it remains largely unknown how they can impact biological systems. While environmental factors may not immediately trigger the manifestation of adverse health outcomes, they may provoke reactions at the molecular level. Such adaptations at the molecular level can initiate / be part of / be a consequence of a cascade of cellular events that may be triggered by the environmental stressor. They may result in adaptations of physiological functions and they may as such ultimately affect health. Molecular alterations hereby may provide insights in the mechanism underlying exposure-related health effects and may point out the toxicological potential of environmental factors. As such, the identification of early molecular signs of biological effects remains pivotal in understanding the health consequences of the diversity of environmental factors. Population-based studies investigate human risks in a real life context. Therefore, population-based studies as used in this doctoral dissertation generate along with toxicological approaches the ultimate evidence to evaluate causative mechanisms and risks of a broad range of environmental factors.

The specific objectives of this dissertation were to investigate:

- the association between particulate matter exposure and salivary extracellular miR-146a and miR-222, which were previously reported to be responsive to particulate matter exposure, in primary school children (chapter 2).
- the association between exposure to toxic metals, organochlorines and perfluorinated compounds and placental mitochondrial DNA (mtDNA) content using a multipollutant approach in newborns (chapter 3).
- the association between exposure to toxic metals, organochlorines and perfluorinated compounds and biomarkers of aging using a multipollutant approach in adults (chapter 4).
- the association between neonatal levels of the metabolic hormones insulin and leptin, which reflect the metabolic status of the newborn, and cord blood mtDNA content (chapter 5).

- the association between indicators of physical (in)activity, including BMI, self-reported screen time and out-of-school sports activities, and salivary extracellular miR-146a and miR-222 expression, which are involved in cardiometabolic health, in primary school children (chapter 6).

In this PhD project we specifically studied molecular markers that can be indicative of early biological effects and that were valuable due to their dynamic nature and their relevance for the mechanism underlying possible health effects.

Extracellular miRNAs embody a mode of intercellular communication implemented in various physiological as well as pathological processes. We observed associations between extracellular miRNA levels in children's saliva and ultrafine particulate matter exposure (chapter 2) and screen time (chapter 6). These findings contribute to evidence on (i) the novel hypothesis suggesting involvement of extracellular miRNAs in communication of cellular burden of particulate matter exposure, which may explain systemic effects upon exposure; (ii) the involvement of extracellular miR-222 and miR-146a in cardiometabolic processes; (iii) the vulnerability of children for environmental effects, that may be translated to adverse health outcomes in later life, as indicated by changes in extracellular miRNA levels.

Maintenance of mitochondrial function, which can be exemplified by mtDNA content, and telomere length is pivotal for normal cellular functioning. We showed that placental mtDNA content (chapter 3), as well as mtDNA content and telomere length in adults (chapter 4) are associated with environmental pollutant exposure. Our findings promote the understanding of (i) the particular sensitivity of mitochondria and telomeres to environmental pollutants, even at low concentrations; (ii) the influence of exposure to multiple pollutants that should be studied in a multipollutant context. Additionally we found that neonatal mtDNA content was associated with neonatal levels of metabolic hormones (chapter 5). This contributes to the current insights in the relation between metabolic disturbances and altered mitochondrial function by exemplifying that a link may already be established from birth onwards.

Nederlandstalige samenvatting

De omgeving of het milieu omvat het geheel van de omstandigheden waarin we ons bevinden, gaande van milieuvervuiling tot sociale omstandigheden en leefstijl. Het is reeds lang geweten dat dergelijke omgevingsfactoren betrokken zijn bij zeer veel aspecten van gezondheid en ziekte. Desondanks is het voor de meeste omgevingsfactoren nog steeds niet geweten op welke manier ze biologische functies en gezondheid kunnen verstoren. De verstoring van de gezondheid komt meestal pas later tot uiting en dus niet gelijktijdig met de blootstelling of kort erna. Omgevingsblootstellingen kunnen echter wel sneller moleculaire veranderingen teweegbrengen die de fysiologische processen kunnen verstoren. Dergelijke moleculaire effecten kunnen de oorzaak of het gevolg zijn van, of deel uitmaken van, een reeks cellulaire processen die tot stand komen als een gevolg van de omgevingsfactoren. Op deze manier kunnen de moleculaire effecten bijdragen aan een verandering in fysiologische systemen, en uiteindelijk leiden tot gezondheidsproblemen. Moleculaire veranderingen kunnen zo de kennis verrijken over de werkingsmechanismen waarmee omgevingsfactoren zorgen voor gezondheidsproblemen en over hun toxicologisch potentieel. Het identificeren van vroegtijdige moleculaire veranderingen blijft dus uitermate belangrijk om de gezondheidsrisico's van de verschillende omgevingsfactoren te herkennen en te begrijpen. Observationele studies onderzoeken risicofactoren voor de mens zoals ze in het echte leven voorkomen. Populatieonderzoeken, zoals gebruikt in dit doctoraatsonderzoek, vormen samen met toxicologische studies dan ook het ultieme wetenschappelijke bewijs om de onderliggende mechanismen en gezondheidsrisico's van omgevingsfactoren te evalueren.

De specifieke doelstellingen van deze thesis waren het onderzoeken van:

- de associatie tussen fijn stof blootstelling en extracellulaire miRNAs in speeksel bij kinderen, meer specifiek miR-146a en miR-222 die reeds door andere studies bij volwassenen in verband werden gebracht met fijn stof blootstelling (hoofdstuk 2).
- de associatie tussen blootstelling aan toxische metalen, organische chloorverbindingen en perfluors en de mitochondriale DNA (mtDNA) inhoud van de placenta bij pasgeborenen, aan de hand van een multipollutie benadering (hoofdstuk 3).

- de associatie tussen blootstelling aan toxische metalen, organische chloorverbindingen en perfluors en moleculaire merkers voor veroudering, meer specifiek de mtDNA inhoud en telomeerlengte, bij volwassenen, aan de hand van een multipollutie benadering (hoofdstuk 4).
- de associatie tussen het gehalte van de metabole hormonen insuline en leptine, die de metabole toestand van de pasgeborene weerspiegelen, en de mtDNA inhoud van het navelstrengbloed (hoofdstuk 5).
- de associatie tussen indicatoren voor fysieke (in)activiteit, waaronder BMI en de gerapporteerde scherm-tijd en buitenschoolse sportactiviteiten, en extracellulaire miRNAs in speeksel bij kinderen, meer specifiek miR-146a en miR-222 die gelinkt zijn aan de cardiometabole gezondheid (hoofdstuk 6).

We onderzochten moleculaire merkers die bijzonder bruikbaar zijn omwille van hun dynamische karakter, wat aangeeft dat ze kunnen reageren op omgevingsfactoren, en hun belang in het werkingsmechanisme voor mogelijke gezondheidseffecten.

Extracellulaire miRNAs maken deel uit van een vorm van communicatie tussen cellen, die is beschreven voor zowel normale fysiologische alsook pathologische processen in ons lichaam. We toonden een associatie tussen extracellulaire miRNAs in speeksel van kinderen en de blootstelling aan ultrafijn stof (hoofdstuk 2) en scherm-tijd (hoofdstuk 6) aan. Deze resultaten dragen bij aan de kennis over (i) de recente hypothese die stelt dat extracellulaire miRNAs betrokken zijn bij communicatie tussen cellen m.b.t. gevolgen van fijn stof blootstelling; (ii) de betrokkenheid van extracellulair miR-222 en miR-146a voor cardiometabole gezondheidsuitkomsten; (iii) de bijzondere gevoeligheid van kinderen voor omgevingsfactoren, hier weergegeven door de veranderingen in extracellulaire miRNAs die mogelijks vertaald kunnen worden naar ongunstige gezondheidsuitkomsten later in het leven.

Behoud van mitochondriale functie en telomeerlengte is van groot belang voor normaal functioneren van de cel. We toonden dat de mtDNA inhoud van de placenta (hoofdstuk 3), alsook de mtDNA inhoud en telomeerlengte bij volwassenen (hoofdstuk 4) geassocieerd zijn met milieuvervuilende stoffen. Onze bevindingen dragen bij aan de inzichten in (i) de bijzondere gevoeligheid van de mitochondriën en telomeren voor vervuilende stoffen, zelfs bij lage concentraties;

(ii) de invloed van gelijktijdige blootstelling aan milieuvervuilende stoffen die onderzocht zou moeten worden volgens een multipollutie benadering. Bovendien toonden we aan dat de mtDNA inhoud geassocieerd was met de concentraties van metabole hormonen bij pasgeborenen (hoofdstuk 5). Dit draagt bij aan de huidige kennis over de relatie tussen metabole balans en de mitochondriale functie, door aan te tonen dat een verband tussen beiden reeds van bij de geboorte aanwezig is.

Chapter 1

General introduction

Since 2003, the majority of the human genome, the complete set of genes encoded in the 23 chromosome pairs of our DNA, is mapped by the human genome project [1]. This highly contributed to the understanding of the genome. Since then, many advancements have been made in the field of genetics and genomics, for example in further elucidating the onset and development of some complex diseases [2]. However, apart from genetics, the environment plays an equal important role in the development of personal traits or various aspects of human health and disease. The interplay between our genes and the environment was already acknowledged by scientists around 1930 [3]. According to the World Health Organization (WHO), a quarter of the global burden of disease is attributed to environmental factors [4]. However, the mechanisms of environmental effects on the molecular and biological functions remain largely unknown.

Environmental factors that may influence biological functions or disease risks are considered exposures and are quite diverse. They consist out of physical exposures (e.g. temperature), chemical exposure (e.g. toxic metals, pesticides), biological exposures (e.g. viral infections), behavioural exposures (e.g. physical activity, social status) or life events (e.g. loss of a job). The exposome concept was introduced due to the diversity of exposures encountered throughout the life course, and due to the need for accurate quantifications of these exposures to keep up with the advancements made in the field of genomics. In parallel to the genome, the exposome was defined as the totality of environmental exposures, including lifestyle factors, throughout the life course, already starting from the prenatal period onwards [5].

Environmental factors can impact biological functions and health at various stages of life, but effects are not always manifested concurrently with the exposure. In the general population the majority of exposures occurs in low doses and will not result in (acute) health effects. Nevertheless, the exposures can induce some molecular responses that may contribute to alter the risk for chronic disease development [6]. Therefore it is exceedingly important to identify early clues indicating the biological effects. Biomolecular markers describe alterations on the cellular level, and can be indicative of the presence or of the extent of an exposure or of health effects. Hence, such markers can point out a possible risk for human health [7]. In this respect, environmental molecular epidemiology employs

molecular markers to identify (early) effects of exposure to environmental factors on cellular function, which may herald the linking of exposure to adverse health outcomes. This is particularly relevant to understand how environmental factors might contribute to disease mechanisms, and to identify early signs of such an effect even at low exposure levels.

ENVIRONMENTAL FACTORS

Vulnerable time windows for environmental factors

The time period in life during which we encounter certain environmental factors can be critical for the impact hereof. A wealth of evidence indicates that early-life environmental factors or adversities may shape health throughout the life course, which is known as the developmental origin of health and disease [8]. During stages of life that are characterized by development and growth, humans are particularly vulnerable for the health effects associated with exposure to environmental factors. Thus, the time period from conception to adulthood reflects an important time window for the environmental stressors to impact human health [9]. Firstly, the prenatal period is an important period during which environmental factors can impact the susceptibility to disease in later stages of life [8]. During the *in utero* period, foetal exposure is defined by maternal exposures. Many substances, such as environmental chemicals (e.g. metals or alcohol) or pharmaceuticals (e.g. non-steroidal anti-inflammatory drugs), can be passed on to the foetus, since they are able to cross the placental barrier [10,11]. Environmental factors to which the mother is exposed can also affect the developing foetus in an indirect manner by affecting placental function and maternal biological functions or health. In early life, breastfeeding can also be involved in the transfer of environmental exposures from the mother to the newborn, especially for lipophilic substances [12]. Because of their higher exposure in proportion to body weight, children are disproportionally exposed compared to adults. For example, children consume more food and beverages in proportion to their body weight and have a higher breathing frequency thus also consume more air. Although they live in the same environment as adults, the exposure routes can be different due to their activities (e.g. playing outside) and child-specific behaviours (e.g. crawling of toddlers) [13]. Furthermore, both the prenatal period and childhood are characterized by a continuous growth. During the foetal period organogenesis takes place. Next,

during childhood the body, the reproductive and immune system are still immature and further developing. Therefore the immature detoxification systems and metabolism will operate differently than in adults [14]. Accordingly, children can have relatively higher exposures than adults, and the exposures can have a higher impact [9]. Hence, 33% of childhood diseases can be assigned to environmental factors, as estimated by the WHO [4]. Although newborns and children represent a more vulnerable subgroup of the population, investigating the impact of environmental factors in other age groups remains of great significance as well.

Exposure to environmental pollutants

In the Western world, the 20th century was a period of major innovation in industry and advances in technology. Along with the industrial revolution, the development of chemicals for manufacturing products was accelerating. Ever since, the number of chemicals keeps rising, with up to 85,000 different chemicals nowadays in the Toxic Substances Control Act (TSCA) Chemical Substance Inventory [15]. However, studies on the impact of the many chemicals could not keep up with the pace of the technological innovations. As such, many chemicals were extensively produced and only afterwards the consequences for human health were manifested and deciphered. Until now, environmental pollutant exposures and their effects remain for a large extent unknown and require much further study.

Particulate matter

Particulate matter (PM) is an ubiquitously complex mixture of small particles and liquid droplets, suspended in the air and contributing to air pollution. Apart from natural sources such as wildfires, sea salt or windblown dust, anthropogenic activities are important sources of PM, mainly those involved in combustion processes of wood or fossil fuels. PM has a variable composition with respect to the components involved (e.g. nitrates, black carbon, metals, allergens etc.), but can be categorized according to the size of the particles. As such, PM_{10} and $PM_{2.5}$ are defined as particles with an aerodynamic diameter respectively ≤10 µm or ≤2.5 µm, whereas ultrafine particles (UFP) have an aerodynamic diameter in the nanosize range, thus ≤0.1 µm.

Upon exposure, the inhaled particles can enter the body. The deposition of the particles in the respiratory system is determined by their size, with the smallest

particles penetrating the body more deeply. UFPs are able to enter the blood stream and can be translocated throughout the body [16,17]. The toxic potential and biological effects of the particles may be attributed to their origin, composition and size [18-21]. Exposure to PM is associated with an increased respiratory and cardiovascular morbidity and mortality. Systemic inflammation and oxidative stress are considered key hallmarks in the mechanisms hereof [22]. However, until now the exact mechanism underlying PM-induced health effects remains unknown. Finally, PM is the component of air pollution that is most harmful for human health [23] and is considered an IARC group 1 carcinogen for humans [24], based on the wealth of evidence relating PM exposure to the lung cancer risk [25]. Accordingly, air quality guidelines for PM concentrations (i.e. thresholds for the annual mean concentration) are established by the WHO at 20 µg/m³ for PM_{10} and 10 µg/m³ for $PM_{2.5}$, and by the European Commission at 40 µg/m³ for PM_{10} and 25 µg/m³ for $PM_{2.5}$. It is of note that up to now, no thresholds are identified below which no adverse effects were observed. Additionally, there are no guidelines for UFP concentrations as a consequence of insufficient evidence linking UFP exposure to adverse health outcomes.

Organochlorines

Organochlorines or chlorinated hydrocarbons are a diverse class of chemicals that is characterized by the presence of one or more covalently bound chlorine atoms [26]. Well known examples are dichlorodiphenyltrichloroethane (DDT), lindane, and polychlorinated biphenyls (PCBs). In the past, from the 1940s until the 1960s, these organic compounds were commonly used as insecticides and pesticides. In the period of 1930 until 1980, PCBs were mainly employed as coolants and lubricants in transformers or capacitors of electrical devices because of their insulating capacity.

Organochlorine compounds belong to the class of persistent organic pollutants (POPs), which are characterized by their high persistence in the environment. They are not easily broken down, they are present in the soil and bio-accumulate in the food chain [26]. Therefore, a ban for some compounds was already introduced in the 1980s in some countries. In 2004, the usage of important organochlorines, among which chlordane, DTT, hexachlorobenzene, and PCBs, was banned in Europe [27]. Nevertheless, these pollutants still pose a threat for human health due

to their persistence in the environment. For example, due to their lipophilic character these compounds can be found in foodstuffs with a high fat content and accumulate in adipose tissue [28]. In some parts of the world DDT is still used for malaria control and illegal use as an insecticide is suspected in some developing countries. Furthermore, the compounds may still leach into the environment by exposure to products still containing them (e.g. old electrical devices that still contain PCBs), or as by-products (e.g. dioxins) in the production of other organochlorines.

Purposefully, pesticides have a toxic function as they are applied to kill, reduce or repel organisms (e.g. insects, fungi or weeds) to protect and preserve crops or to control disease (e.g. malaria). As a consequence, they may also be harmful for human health, the environment and ecosystems [29]. Indeed, usage of organochlorine pesticides was linked to adverse health outcomes. For example, DDT was linked to neurobehavioral, reproductive and developmental health effects [30]. IARC classified PCBs as group 1 (carcinogenic to humans), other organochlorines such as DDT and lindane were classified as group 2B (possibly carcinogenic to humans) [24].

Perfluoroalkyl substances (PFAS)

Perfluorinated compounds are structurally characterised by the presence of carbon-fluoride (C-F) bonds, which are very strong and stable chemical bonds granting the unique properties of these chemicals, and absence of carbon-hydrogen (C-H) bonds. Due to their chemical and thermal stability and both hydrophobic and lipophobic nature of the fluorine moiety, PFAS are used as surfactants for repelling of stains, water or grease in consumer products [31,32]. It is of note that PFAS are manmade and not occurring naturally. Well-known products containing PFAS are non-sticking frying pans with Teflon, stain-repellent treatments for clothes or furniture and firefighting foams.

While PFAS have been used since the 1950s, it is only recently known that these chemicals can be released from the products that contain them into the environment [33]. Once released into the environment, these compounds remain very stable and are difficult to break down, therefore tending to accumulate and persisting for a long time. As such, exposure might occur through dietary uptake via consumption of contaminated foodstuffs (e.g. via uptake of PFAS in the

soil/water, via leaching of PFAS present in packaging or via use of non-sticking cookware), or via non-food products [34]. PFAS were defined as candidate POPs by the Stockholm Convention in 2009 based on their persistence in the environment and since 2010, the European Commission adopted the recommendation to monitor PFAS in food [35]. The production of perfluorooctanoic sulfonic acid (PFOS) was phased out by its main manufacturer 'The 3M Company' since 2002 [36].

Because of their surface activity and amphiphilic nature, PFAS behave differently from other POPs. Unlike other POPs, PFAS are rather hydrophilic and will bind to proteins, with the majority of PFAS binding to serum albumin [37]. Animal studies indicated a wide range of toxic effects upon PFAS exposure, including immunotoxicity, endocrine effects, carcinogenic and developmental effects [34]. However, studies on health effects in humans are scarce. There are indications of thyroid disorders, certain types of cancer and immunotoxicity linked to PFAS exposure from human epidemiological studies [37]. However, the biological mechanisms underlying possible health effects are currently not known. Unfortunately, although there are more than 4,500 different PFAS [38], most studies investigating health effects mainly focus on two most known PFAS: PFOS and perfluorooctanoic acid (PFOA). This limits the insights in the general human toxicity of PFAS exposure. Thus, adverse health effects of PFAS in humans remain to be investigated in depth [39]. Despite limited evidence in humans, PFOA was recently classified as possibly carcinogenic to humans (group 2B) [24].

Toxic metals

Metals are naturally occurring in the earth's crust and found in volcano eruptions, ore aggregates, rocks or petroleum. Atmospheric deposition, aquatic dispersion to soil and water (ground and surface), resuspension of sediments and weathering of rocks may entail a natural metal contamination of air, water and soil in our living environment. Anthropogenic activities may also significantly contribute to metal contamination of the environment either by primary production of metals and alloys or their uses for consumer products [40]. Ambient air may be polluted by metals through combustion of fossil fuels, incineration of metal-containing waste, and emissions from secondary metal scrap smelters. Metal-laden effluents and dust fall from industrial processes may enrich specific toxic metals in water supplies and agricultural land areas contaminating drinking water, vegetables and

crops grown on those soils. Domestic exposure to toxic metals has also been reported to cause health problems: for example use of lead pipes for canalisation of soft drinking water [41]. Iatrogenic exposure and toxicity have been described for metals used in a medical context: for example parenteral nutrition may result in manganese intoxication [42], antimony is used in parasite drugs [43] and arsenic and mercury salts were previously beneficial in treatment of syphilis [44]. Some metals were/are intensively used in agriculture as pesticides (e.g. lead arsenate [45] or manganese-containing fungicides maneb and mancozeb [46]). Tobacco smoke is an important source of toxic metals, especially cadmium [47].

Toxic metals are a group of metallic elements that can be harmful for human health. Some of these elements (e.g. copper, iron, manganese, selenium, and zinc) are essential for correct human metabolism and are under homeostatic control, but when they exceed a tolerable level they may entail toxic manifestations. For other metals such as arsenic, cadmium, mercury, and lead no biological function beneficial for the human organism is known, and even at low biological concentrations they may lead to toxic effects [48]. Adverse systemic outcomes associated with metal intoxication are diverse and usually differ depending on the metal and the absorbed dose [49]. Furthermore, IARC evaluated the carcinogenic evidence on toxic metals. As such, inorganic arsenic, beryllium, cadmium, hexavalent chromium and nickel are classified as group 1 (carcinogenic to humans); lead belongs to group 2B (possibly carcinogenic to humans); trivalent chromium, inorganic mercury and selenium were classified in group 3 (not classifiable as to its carcinogenicity to humans) [24].

Since ancient times, humans exploit metals for domestic, artisanal, and industrial purposes involving mining, smelting and refinery of metal ores. Industrial activities brought about distinct occupational diseases, mostly due to excessive inhalation exposure in nonferrous and chemical industry. Additionally they caused environmental pollution with widespread public health consequences associated with inhalation and ingestion of toxic metals, for example as has been documented for cadmium by the Cadmibel study [50]. This longitudinal investigation from 1985 to 2005 of a population cohort living in the vicinity of zinc/cadmium smelters in the Noorderkempen (Belgium) revealed renal effects [51] and increased calciuria [52], osteoporosis and bone fractures [53], and lung cancer [54] associated with urinary

cadmium (a surrogate for the body burden of cadmium) and cadmium in soil or vegetables. Currently metal exposures decreased over the last decades or were banned from certain consumer products (e.g. lead tetraethyl in gasoline), which markedly decreased concentrations of biomarkers of exposure to these metals (e.g. blood lead). In spite of reduced exposure levels, biological effects are not ruled out and the toxicity mechanism of metals on the molecular level remains to be elucidated.

Single pollutant vs. multipollutant approach

Humans are continuously exposed to a wide range of environmental pollutants at the same time. The effects from exposures in a mixture may differ from the effects of isolated exposures, as co-exposures may have a synergistic, additive or even an antagonistic effect. As a consequence, the interaction between exposures in the body may culminate in other or more pronounced biological effects than toxicity mechanisms of individual exposures.

Epidemiological studies mainly investigate(d) individual exposures, addressing the associations between individual pollutants and their effects. Until now, such studies have contributed extensively to advances in human health, as they were the basis for the legal framework that is currently available for many environmental factors. As a consequence, the extent of the exposure to many harmful pollutants has been reduced through the availability of guidelines. However, with low-dose exposures it may become difficult to interpret individual effects [55]. Contrary to the guidelines set to lower human exposure to pollutants, evidence of low-dose effects is suggesting that safe exposure levels may not exist and low-dose exposures should not be neglected [56,57].

In light of the abundance of environmental pollutants mankind is exposed to and the increasing evidence of effects associated with low-dose exposures in the general population, there is a rising concern on the effects of mixtures [57]. The European Commission and EFSA recognised this and launched initiatives to further harmonise and optimise the risk assessment of multiple exposure in humans, for example by setting up frameworks to assess combined toxicities of exposures. Multiple exposures of an individual have specific correlation structures, that may limit the statistical separation of their effects [58]. As such, identifying the relevant exposure from such a mixture was an enduring problem in the field of

epidemiology. However, more recently, due to statistical advancements, it is possible to model associations between environmental exposures and biological outcomes, while accounting for other co-exposures [59-61]. Nevertheless, single pollutant studies remain equally important and should be conducted in parallel to multipollutant studies. They remain exceedingly important to identify environmental pollutants with high impact on public health and the need of a legislative framework for such toxic exposures, particularly in light of the further growing number of chemicals and the increasing insights on the impact of environmental pollutants as well as lifestyle factors on human health.

Metabolic health indicators

It is generally accepted that the prevalence of overweight and obesity are worldwide increasing in all age groups, and are major risk factors for a number of chronic diseases, including diabetes, cardiovascular disease and ultimately the metabolic syndrome [62]. In 2015, the prevalence of obesity was 5% in children and 12% in adults [63]. Contrary, the metabolic health status of an individual comprises more than solely the obesity status. It additionally evaluates the presence of other important risk factors, such as waist circumference or criteria for insulin resistance [64]. The metabolic health status may be a better predictor for adverse health outcomes [65]. Lifestyle interventions are first-line options to ameliorate the metabolic health and reduce the risk for adverse health outcomes. On the other hand, a wide range of lifestyle factors may influence the metabolic health.

Physical (in)activity and sedentary behaviour

Over the last decades, decreasing trends in activity and increasing trends in sedentary behaviour have evolved in parallel to each other and are globally becoming a major public health problem. The WHO estimated that up to 31% of the subjects older than 15 years of age are insufficiently active [66]. Physical inactivity contributes in the global burden of coronary heart disease, type 2 diabetes, breast cancer and colon cancer, and premature mortality [67]. The public health importance of physical inactivity might be comparable to that of smoking [68]. On the other hand, physical activity is associated with health promotion. The WHO has established guidelines for physical activity for children (5 to 17 years old), adults (18 to 64 years old) and elderly (older than 65) [69]. In order to be sufficiently active, children must engage at least 60 minutes per week in

moderate-to-vigorous intensity physical activities. For adults, 150 minutes of moderate intensity aerobic physical activity per week or 75 minutes of vigorous intensity aerobic physical activity is recommended. In elderly, the recommendations may vary according to the mobility, physical ability and health condition [69].

In parallel to physical inactivity, the trends of increasing sedentary behaviour are of considerable public health importance. By definition, physical inactivity is the failure to meet the activity guidelines, and may be linked to indicators of sedentary behaviour. Yet, sedentary behaviour is defined as any waking activity with a low energy expenditure (≤ 1.5 metabolic equivalents, METs) while being in a sitting, reclining or lying position [70]. Examples of such sedentary behaviour are sitting behind a computer or tablet, sitting in meetings, commuting, watching television, etc. Although sedentary behaviour is normal, high portions of sedentary behaviour are associated with an increased risk for chronic diseases and all-cause mortality, which may be partially attenuated by increasing physical activity levels [71].

Hormones as indicators of the metabolic status

Hormones are part of the endocrine system and crucial in signalling for important physiological functions. The release of hormones can occur in response to either alterations in humoral stimuli (i.e. changes of substances in extracellular fluids such as blood), hormonal stimuli or neural stimuli. Metabolic hormones play a role in the regulation of metabolism and the energy balance.

Insulin is produced by β-cells of the pancreatic islets of Langerhans in response to glucose concentrations, as a consequence of intake of carbohydrates in food. Although glucose is the prime stimulus for insulin secretion, other stimuli may modify the response. Upon increased glucose levels, the release of insulin facilitates the uptake of glucose by cells to convert it to cellular energy. Furthermore, insulin stimulates the glycogen synthesis and formation of fatty acids and triglycerides, which are important energy reserves. Thus, insulin plays a pivotal role in the regulation of cellular energy [72]. In insulin resistance the body is less efficient to respond to the actions of insulin, resulting in a higher need of insulin production and release to maintain glucose levels within a normal range. However, at some point insulin production may become insufficient to cope with the insulin resistance and will fail to keep glucose levels within a healthy range.

As a consequence, a state of hyperinsulinemia and hyperglycaemia arises. This may impact many organ systems in various ways according to the dependence and actions of insulin, resulting in a wide range of pathological effects. Insulin resistance is an important hallmark of type 2 diabetes. Overweight or obesity, high-calorie or high-carbohydrate diet and physical inactivity are among the factors assumed to contribute to insulin resistance [72]. This further emphasizes relation of insulin (and insulin resistance) with the metabolic health status.

Leptin is mainly produced by adipocytes in function of energy stores or body fat mass, food intake and glucose uptake. Leptin serves as a negative feedback mechanism to the hypothalamus, a part of the brain that regulates food intake and body weight, in order to indicate satiety and to inhibit further food intake. Hereby, leptin plays an important role in the central regulation of the energy homeostasis, mainly on the long-term (in response to fat reserves) but also on a short-term (in response to food intake) [73]. In relation to the body's fat reserves, obese subjects have higher circulating leptin levels, indicating a decreased sensitivity to leptin [74]. It is hypothesized that leptin resistance can develop due to (chronic) overeating, due to defective transport to the brain across the brain-blood-barrier, due to problems with the leptin receptors in the hypothalamus, or due to deficiencies with downstream mediators. However, exact causes of leptin resistance are currently not know [75]. Mutations in the leptin gene on both alleles will result in a complete leptin deficiency and obesity as a consequence, however this is rather rare. Furthermore, it is still unclear if obesity may contribute to leptin resistance or if it's the other way around that leptin resistance promotes obesity [76].

MOLECULAR EFFECT MARKERS

Molecular alterations are on the path of exposure and exposure-related health outcomes. On the one hand, exposure does not necessarily result in an adverse health effect, but molecular effects are perhaps not excluded. On the other hand, adverse health effects are not necessarily manifested concomitantly with the exposure. Identification of molecular alterations contributes to the understanding of the mechanistic basis of pathways linking exposures to health outcomes.

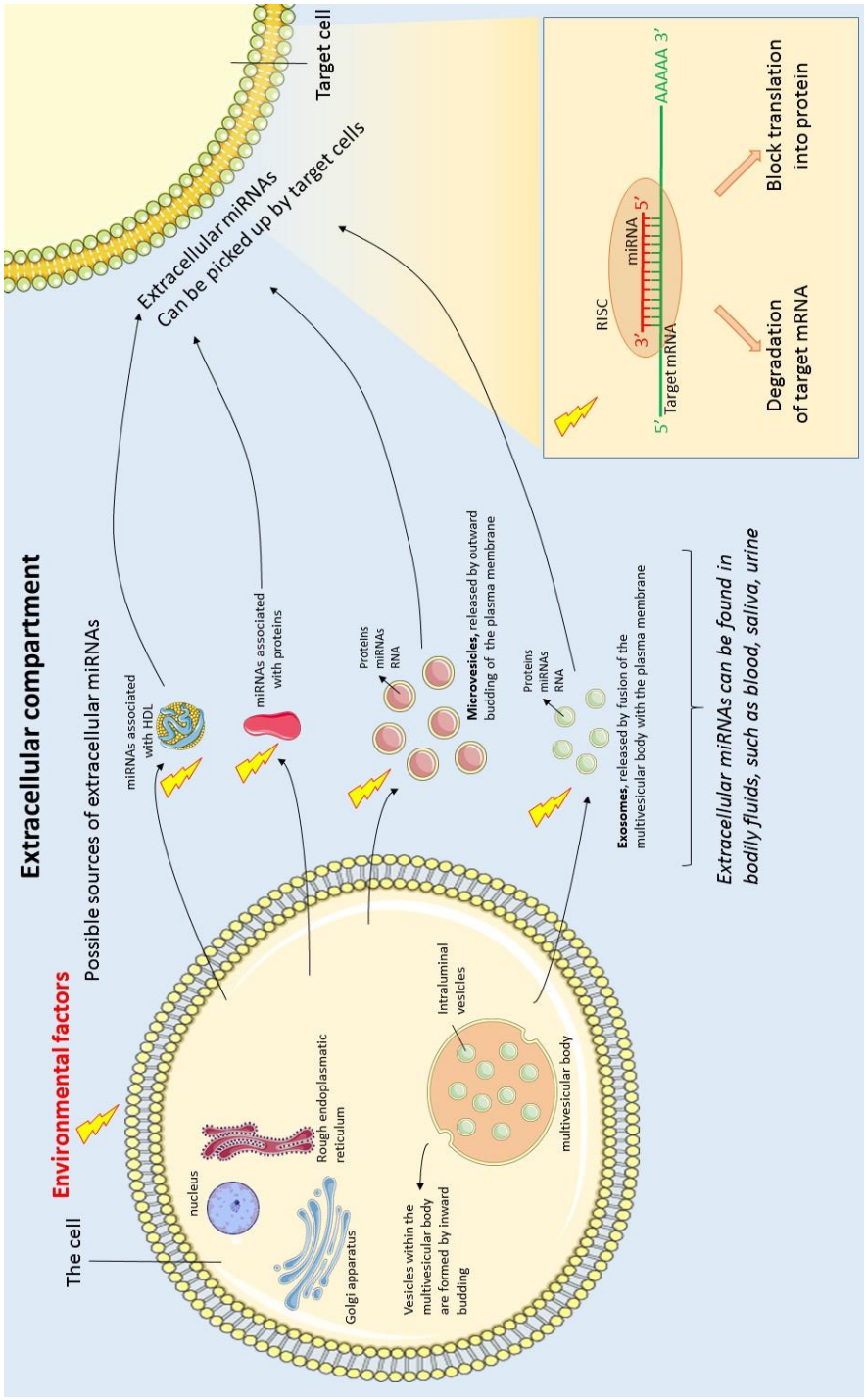

Figure 1: Conceptual presentation of the hypothesis that environmental factors may impact extracellular miRNAs. Environmental factors may induce alterations in the expression ofmRNA and non-coding RNAs such as miRNAs. On the one hand, this may impact the phenotype of the cell. On the other hand, this may alter the release of miRNAs into the extracellular environment complexed with (lipo)proteins or present in vesicles. These extracellular miRNAs may be picked up by recipient cells, in which they may induce alterations in the phenotype by targeting of mRNAs.

Extracellular miRNAs

MicroRNAs (miRNAs) are a class of non-coding short RNAs, of approximately 22 nucleotides. miRNAs are ubiquitously expressed and have an important role in the regulation of gene expression at the post-transcriptional level. By means of complementary base pairing miRNAs are able to bind to gene transcripts (i.e. messenger RNA, mRNA) resulting in the degradation of the mRNA and/or constraining the translation of the mRNA to a protein. In this way, one miRNA may bind to multiple distinct mRNAs, and one mRNA can be targeted by various miRNAs. While this mechanism of posttranscriptional gene expression regulation is involved in maintenance of physiological functions, it is also implicated in the aetiology of various disorders, including cardiovascular [77] and metabolic disorders [78]. The expression of miRNAs is very dynamic, hereby enabling fast responses to changing physiological needs or environmental stressors. As a consequence, miRNAs are meaningful indicators of early biological effects of a wide range of environmental stimuli [79].

miRNAs are not only present intracellular, but may also be secreted into the extracellular compartment. There are suggestions that extracellular miRNAs are simply cellular by-products without specific function. However, increasing evidence supports the hypothesis of selective secretion of specific miRNAs for communicative purposes [80]. Hence, miRNAs may facilitate communication between cells on a short or longer distance. Uptake of the extracellular miRNAs by recipient cells may modulate gene regulation in this cell and as such adapt a range of processes. This mechanism is currently the subject of many studies in molecular research, and appears to be implicated in the disease mechanisms of a wide range of disorders, among which cardiovascular disease and cancer [81]. Extracellular miRNAs remain protected from degradation by RNAses in the extracellular environment by their association with vesicles or proteins. Extracellular miRNAs are associated with three types of extracellular vesicles: (i) exosomes are small vesicles (30-100 nm diameter) that are formed by inward budding of the multivesicular body, that releases the exosomes into the extracellular environment upon connection with the cellular membrane and outward budding; (ii) microvesicles represent a group of vesicles that are released by means of budding from the cell and are typically larger than exosomes [80]; (iii)

apoptotic bodies, which have no communicative purpose, can also contain miRNAs. Furthermore, miRNAs can be associated with high-density lipoproteins (HDL) [81]. Yet the majority of extracellular miRNAs in circulation appears to be joined with an Ago2-protein complex, which is also involved in miRNA synthesis [82]. Accordingly, extracellular miRNAs, which may be implicated in various pathophysiological mechanisms, are present in bodily fluids, such as saliva, urine, blood and breast milk. This warrants their potential as candidate biomarkers for a wide range of health outcomes, including cardiovascular disease [83], cancer [84] or neurological outcomes [85]. Furthermore, extracellular miRNAs may be the missing-link between environmental factors and health outcomes indicating their potential value as biomarkers of effect as exemplified in Figure 1.

Mitochondrial DNA content

The mitochondria are considered the powerhouses of the cell, since the generation of cellular energy, ATP, takes place in this organelle. Mitochondria have a unique structure, consisting of a double-membrane system, an intermembrane space and the matrix inside of the inner membrane. The inner membrane of the mitochondria is convoluted and characterized by infoldings which form the cristae. These unique features all have a functional purpose in the process of oxidative phosphorylation. Furthermore, mitochondria have their own genome, which is different from the nuclear genome. The mitochondrial genome is circular, approximately 16.5 kilobase pairs long and maternally inherited, it has 37 genes which code for 13 proteins involved in electron transport and oxidative phosphorylation, and proteins required for their translation. Nevertheless, the nuclear DNA encodes the majority of proteins needed for oxidative phosphorylation. The mitochondria contain multiple copies of their genome that are organized as nucleoids, which are DNA-protein complexes anchored to the inner membrane inside the matrix. Furthermore, a cell may have many mitochondria, resulting in up to thousands of mtDNA copies per cell dependent on the cell type [86].

Like the nuclear DNA, the mitochondrial DNA (mtDNA) encounters mutations and damaging stressors. However, compared to the nuclear genome, the mitochondrial genome has a reduced repair capacity for DNA damage [87]. For example, mtDNA lacks nucleotide excision repair systems, which removes DNA lesions or DNA adducts, and mismatch repair is limited. Moreover, the mtDNA

lacks histones, which may protect DNA against mutations. In addition, the mitochondria are a primary source of reactive oxygen species (ROS) as by-products of oxidative phosphorylation. As a consequence, mtDNA is particularly vulnerable for oxidative stress and is mainly affected by oxidative damage. Thus, the mitochondrial genome is much more vulnerable for mtDNA damage compared to the nuclear genome [88]. Since mtDNA encodes proteins needed for essential mitochondrial functioning, damage to the mtDNA may impact mitochondrial function. However, substantial DNA damage might be required before effects on mitochondrial function are established, since there are multiple mtDNA copies that may compensate for this [89].

mtDNA is maintained by dynamic interaction between mtDNA degradation and mtDNA replication and mitochondrial biogenesis [90]. The amount of mtDNA in a cell, or the mtDNA content may represent the mitochondrial function [91]. It is hypothesized that the mtDNA copy number may be increased in function of initial stages of DNA damage, in order to cope with functional losses due damaged mtDNA. When a critical point of mtDNA damage is exceeded, functional losses may not be compensated by increases in mtDNA abundance and dysfunctional mtDNA might be removed, as such resulting in a reduced mtDNA copy number [92]. Furthermore, it is hypothesized that mtDNA content may be regulated in function of the cellular energy needed to accommodate the oxidative phosphorylation requirements. Alterations in the mtDNA copy number may play a role in the risk for disease, since changes and variation in mtDNA abundance are observed in a wide range of disorders [93,94].

Apart from endogenous sources of ROS, exogenous factors may contribute to a state of increased oxidative stress. Some environmental pollutants may contribute in the formation of adducts on the mtDNA, may accumulate in the mitochondria, or may be metabolically activated by mitochondrial cytochrome P450. Hence, the mtDNA can be a (secondary) target in the toxicity mechanisms of environmental factors of which alterations in the mtDNA abundance can be indicative [95].

Telomere length

Telomeres are ribonucleoprotein complexes consisting of repetitions of the 5'-TTAGGG-3' nucleotide sequence at the end of the chromosomes. Telomeres protect the chromosome structure from losses of genetic information during replication, and from end-to-end fusing with other chromosomes [96]. During replication of DNA in the course of cell division, the double-stranded DNA will be opened at a A-T rich regions to initiate the DNA replication process, hereby creating a replication fork that will be opened further throughout the replication process (e.g. comparable to a zipper). Then the DNA polymerase elongates the DNA in a '5 to '3 direction (i.e. on the newly formed strand) starting from a RNA primer which is formed and bound to the template strand. For one strand of the DNA, the elongation occurs in the same direction as the opening of the replication fork (i.e. the leading strand), thus one RNA primer is sufficient. For the other strand, elongation occurs in the opposite direction (i.e. the lagging strand), and multiple RNA primers are needed when the replication fork further opens, hereby creating Okazaki fragments. At the very end of the lagging strand, the last RNA primer cannot be replaced by DNA after removal of the RNA primer and thus the very ends of the chromosomes are not replicated. This end-replication problem causes telomeres to shorten as a consequence of cell divisions [97]. Telomere length may as such shorten until critical lengths, which will ultimately induce cellular senescence and apoptosis [98]. Nevertheless, mechanisms to preserve telomere length exist. The enzyme telomerase is a reverse transcriptase that appends telomeric repeats to the end of telomeres to compensate reductions in the telomere length, but is not present in all cell types. Furthermore, sheltrin, which is a telomere-specific protein complex, prevents the activation of DNA damage responses and accommodates telomerase recruitment. Additionally, the structural organisation of the terminal ends of the telomeres into a T-loop is regulated by the sheltrin complex and contributes to the maintenance of the telomeres [99].

Telomere length represents the life span of the cells, but is also connected to chronological aging of an individual. Reduction of telomere length is associated with chronological aging and is estimated to a loss of 25-30 base pairs per year [100]. The telomere length is associated with age-related disorders such as atherosclerosis, and longevity [101]. Due to their high guanosine content and

inability to repair single-strand breaks, telomeres are particularly vulnerable to oxidative stress [102]. Damaged nucleotides within telomeres inhibit the proceeding of the replication fork and may as such contribute to telomere attrition [103]. Telomere length is prone to environmental influences, which may interfere with processes of telomere length maintenance or may induce oxidative stress. However, some environmental factors may contribute to telomere shortening, whereas others may be associated with telomere elongation. It was postulated that telomere length can be influenced by environmental factors already from early life onwards. Hereby telomere length may (i) be involved in the mechanism of exposure-related health outcomes and (ii) be an pivotal biomarker to assess the biological impact of a wide range of exposures throughout the life course [104].

AIMS OF THE DOCTORAL THESIS

Insight in the mechanisms of the health effects associated with exposure is needed to understand the (early) biological effects of environmental factors and life style. However, little is known about (i) biomolecular changes in relation to particulate matter air pollution exposure in children, (ii) the identification of environmental chemicals from a set of co-exposures that impact biological systems, as well as (iii) molecular effects of metabolic stressors in early life.

In this doctoral dissertation, the biological impact of environmental factors is explored on different molecular levels in neonates, children and adults. We focussed on the effects of (ultra)fine PM, environmental chemicals (including organochlorines, perfluorinated compounds and toxic metals), metabolic hormones and physical (in)activity. mtDNA content, telomere length and saliva extracellular miR-146a and miR-222 were used to evaluate early molecular effects.

The specific objectives of this doctoral dissertation were as follows:

1. To investigate the association between exposure to **fine and ultrafine PM** and saliva **extracellular miRNAs** in primary school children (Chapter 2).
2. To investigate the association between exposure to **environmental chemicals** and **placental mtDNA content** in newborns adopting a multipollutant approach (Chapter 3).
3. To investigate the association between exposure to **environmental chemicals** and **biomarkers of ageing** in an adult population using a multipollutant approach(Chapter 4).
4. To investigate the association between cord blood **metabolic hormones** and **cord blood mtDNA content** in newborns (Chapter 5).
5. To investigate the association between indicators of **physical (in)activity** and sedentary behaviour and saliva **extracellular miRNAs** (Chapter 6).

In order to reach these objectives, three observational studies were employed. In the COGNAC study, a panel study of primary school children, we investigated the effects of (ultra)fine PM exposure (Chapter 2) and physical (in)activity (Chapter 6) on saliva extracellular miRNAs. In the third cycle of the Flemish Environment and Health Study (FLEHS III 2012-2015), we investigated the effects of environmental chemical exposure (Chapter 3) and metabolic hormones (Chapter 5) on mtDNA content in newborns. In the FLEHS III adult study, we investigated the association between exposure to environmental chemicals and markers of biological ageing (Chapter 4).

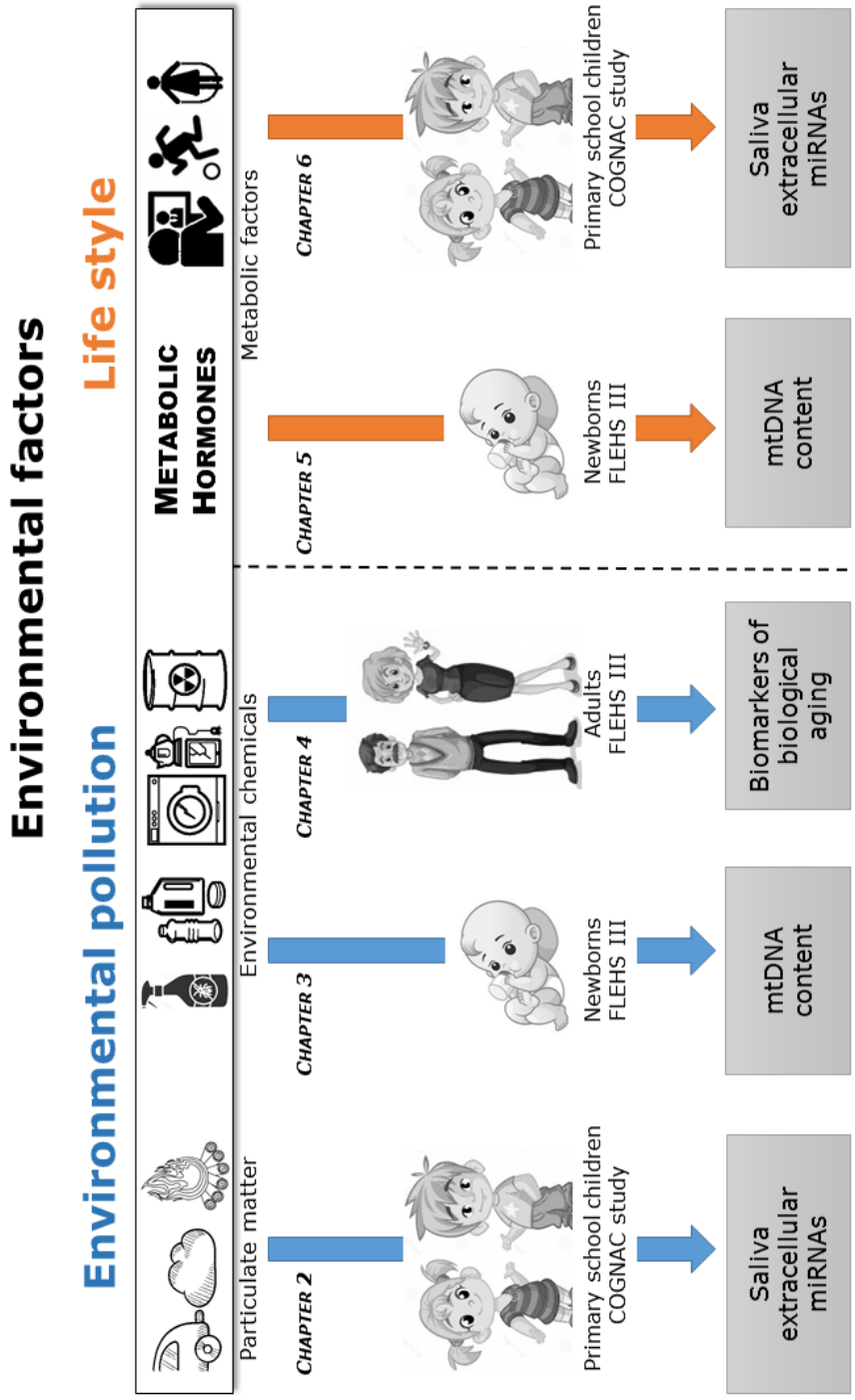

Figure 2: Conceptual overview of the objectives in this doctoral dissertation.

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Chapter 2

Recent exposure to ultrafine particles in school children alters miR-222 expression in the extracellular fraction of saliva

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Vriens A. *et al.* Environmental Health (2016)

ABSTRACT

Background: Ultrafine particles (<100 nm) are ubiquitous present in the air and may contribute to adverse cardiovascular effects. Exposure to air pollutants can alter miRNA expression, which can affect downstream signaling pathways. miRNAs are present both in the intracellular and extracellular environment. In adults, miR-222 and miR-146a were identified as associated with particulate matter exposure. However, there is little evidence of molecular effects of ambient air pollution in children. This study examined whether exposure to fine and ultrafine particulate matter (PM) is associated with changes in the extracellular content of miR-222 and miR-146a of children.

Methods: Saliva was collected from 80 children at two different time points, circa 11 weeks apart and stabilized for RNA preservation. The extracellular fraction of saliva was obtained by means of differential centrifugation and ultracentrifugation. Expression levels of miR-222 and miR-146a were profiled by qPCR. We regressed the extracellular miRNA expression against recent exposure to ultrafine and fine particles measured at the school site using mixed models, while accounting for sex, age, BMI, passive smoking, maternal education, hours of television use, time of the day and day of the week.

Results: Exposure to ultrafine particles (UFP) at the school site was positively associated with miR-222 expression in the extracellular fraction in saliva. For each IQR increase in particles in the class room (+8504 particles/cm³) or playground (+28776 particles/cm³), miR-222 was, respectively 23.5% (95% CI: 3.5, 41.1; p=0.021) or 29.9% (95% CI: 10.6, 49.1; p=0.0027) higher. No associations were found between miR-222 and $PM_{2.5}$ and between miR-146a and recent exposure to fine and ultrafine particles.

Conclusions: Our results suggest a possible epigenetic mechanism via which cells respond rapidly to small particles, as exemplified by miR-222 changes in the extracellular fraction of saliva.

BACKGROUND

Exposure to particulate matter (PM) is associated with adverse health outcomes such as an increased morbidity and mortality due to cardiovascular, respiratory and carcinogenic events [1-3]. The particles with an aerodynamic diameter smaller than 2.5 µm ($PM_{2.5}$) are especially harmful since they can penetrate the body more deeply [4,5]. It has been shown that inhaled ultrafine particles (UFP) with a diameter less than 0.1 µm can reach the circulation and are able to penetrate target organs other than the respiratory system [5-7], even crossing the blood-brain-barrier [8].

Micro-RNAs (miRNA) transfer signals to regulate gene expression at the posttranscriptional level and fine-tune the translation of mRNAs into proteins, both in health and disease [9]. They regulate cellular reactions in response to environmental insults and have been described as being responsive to PM exposure in humans [10-14]. The joining of the miRNAs with a carrier protects them from degradation in the extracellular environment. Additionally, miRNA mediated pathways operating via extracellular vehicles, such as microvesicles and protein complexes represent a potent cell-to-cell communication [15,16] and can be influenced by PM exposure [11].

The present study investigates the association between children's extracellular miRNAs in saliva and recent $PM_{2.5}$ and UFP exposure. Saliva contains both buccal epithelial cells and leukocytes [17] as such it can be a non-invasive alternative, preferred to blood samples [18,19]. Indeed, expression profiles of extracellular non-coding RNAs in saliva are similar to other fluids [20]. Furthermore, extracellular miRNAs are present in saliva [21] and saliva can be used to detect alterations at the molecular level in association with an external stressor [22]. We evaluate the expression of miR-222, which has a function in cell cycle and vascular biology as well as miR-146a which plays an important role in inflammation. Both have been shown responsive to PM exposure on a cellular level in adults [10,12,13]. To our knowledge, this is the first study investigating the effects of acute air pollution exposure on extracellular miRNA expression in saliva of children.

METHODS

Study population

This study was part of the COGNAC (COGNition and Air pollution in Children) study, a panel study which comprised three study visits. Between 2011 and 2013, we invited children (grades three to six) from three primary schools in Flanders (Belgium) to participate in the study. The parents of participating children filled out a questionnaire including information about the current and previous residential addresses, the socioeconomic status of the family, and the smoking behavior of the family members. In total the COGNAC study included 334 children recruited from three primary schools. For this specific within the COGNAC children cohort, 80 children from two schools were randomly selected from the overall cohort. Among the 80 children, three sibling pairs and one twin pair was included. Each child collected saliva samples at two of the three study visits, which were on average 11 weeks apart (first in November and second in January or the first week of February).

In order to rule out intra-individual diurnal variation, the three repetitive study visits were always scheduled on the same time during the day and the same day of the week. The examinations took place between November and February on Monday, Tuesday, Thursday, and Friday between 8:30 a.m. and 2:10 p.m.. All parents provided written informed consent for participation and oral assent of the children was renewed at each clinical examination. The COGNAC study was approved by the medical ethics committee of Hasselt University and the Eastern-Limburg Hospital, Belgium and informed consent for participation in the study was given by the children's parents.

Air quality assessment

Measurements of air pollutants at school

We used portable devices to measure ultrafine particles (UFPs) with a diameter 10-300 nm (Aerasense NanoTracer; Phillips, Eindhoven, The Netherlands), and particulate matter (PM with diameter < 2.5 μm) (AEROCET 531; MetOne Instruments Inc., Grants Pass, Oregon, US) in the school and at the playground on the examination days as part of the field work. The measurements were performed in the morning (9-12 a.m.). For each child, the measured outdoor

pollution levels of the 10 minute recess when children were at the school playground, before the study visit were used. Thus, outdoor exposure levels reflect ambient air pollution during the last time that the child was outside, which was approximately one hour before saliva donation.

Modeled $PM_{2.5}$ concentrations at home address

We used a spatial temporal interpolation method to model the daily residential exposure levels (μg/m³) of $PM_{2.5}$, at each child's home address. This method takes into account land cover data obtained from satellite images (CORINE land cover data set) [23] and pollution data of fixed monitoring stations in combination with a dispersion model [24]. The model calculates the daily interpolated exposure concentrations in a high resolution receptor grid based on information from the Belgian telemetric air quality networks, point sources, and line sources. Overall model performance was evaluated by leave-one-out cross-validation. Validation statistics of the interpolation tool gave a spatial temporal explained variance of more than 0.80 for $PM_{2.5}$ [25]. We used this model to estimate the residential exposure on the day (lag 0) of the examination, the day (lag 1) before the examination and two days (lag 2) before the examination as well as the average exposure of the 48 hours before the examinations.

Since the parameters for fine and ultrafine PM were measured or interpolated in a exposure window ranging from hours to two days before sampling, they reflect recent exposure.

Molecular measurements

Sample collection

Before sampling, subjects refrained at least 30 minutes from eating, drinking or hygienic procedures. Additionally, they rinsed three times with tap water to avoid contamination of the samples by food residues. Subjects had to collect 2 ml of whole saliva into the Oragene® RNA self-collecting kit (DNA Genotek Inc., Kanata, Ontario, Canada). The samples were immediately afterwards stabilized for RNA preservation. Within 6 hours after sampling, samples were stored at -20 °C until further analysis.

Isolation of extracellular miRNAs

Extracellular miRNA in saliva were isolated by differential centrifugation and ultracentrifugation of the samples. The protocol for isolation of the extracellular fraction was adapted from Théry et al [26], as such that it combined the sample processing, required for RNA stability when working with the DNA Genotek containers. After thawing, the Oragene® containers (DNA Genotek Inc., Kanata, Ontario, Canada) were incubated at 50 °C for one hour. Then, 1 ml aliquots were incubated at 90 °C for 15 minutes. To pellet the debris in the saliva, 40 µl of neutralizer solution (DNA Genotek Inc., Kanata, Ontario, Canada) was added to the samples and centrifuged at 1 500 x *g* for 10 minutes. The supernatant was collected and centrifuged at 16 000 x *g* for 20 minutes. Next, the supernatant was ultracentrifuged at 160 000 x *g* for one hour (Optima LE-80K Ultracentrifuge and ti70 fixed angle rotor; Beckman; Analis, Suarlée, Belgium). Polyallomer tubes for ultracentrifugation (Beckman; Analis, Suarlée, Belgium) were pre-treated with RNA*Zap* (Life Technologies, Gent, Belgium) to eliminate RNAse activity. Afterwards, the pellet was resuspended in 1x PBS (pH 7.4) and ultracentrifuged at 160 000 x *g* for one hour. The vesicle-containing pellets were resuspended in RNAse-free water and stored at -80 °C. The composition of the extracellular fraction and size distribution of the vesicles were evaluated using nanoparticle trafficking analysis in a subset of the samples (Nanosight Ltd.; Amesbury, UK) (Supplemental information).

miRNAs and larger RNA species were isolated using the miRNeasy mini kit (Qiagen; Valencia, California, USA) following the manufacturer's instructions. After homogenization, the samples were spiked with 250 fmol *C. elegans miR-39* for normalization of the expression data [27,28]. Total RNA and miRNA yield of the samples was quantified using Qubit assays (respectively Qubit br RNA assay and Qubit miRNA assay; Life Technologies; Ghent, Belgium). Furthermore, presence of miRNA was evaluated using small RNA Bioanalyzer (Agilent 2100; Agilent Technologies, Amstelveen, The Netherlands).

miRNA expression analysis

miR-222 and miR-146a were quantified using a two-step real-time PCR (RT-qPCR) with Taqman miRNA assays (Life Technologies). Reverse transcription was performed using 125 ng of total RNA input using looped primers (Megaplex RT primers human pool A & Taqman microRNA RT kit; Life Technologies) on a PCR gradient thermal cycler (TC-5000; Techne, Burlington, NJ, USA). cDNA synthesis ran 40 cycles of two minutes at 16 °C, one minute at 42 °C and one second at 50 °C; the reaction was inactivated at 85 °C for five minutes. cDNA samples were stored at -20 °C until qPCR analysis. Products of the reverse transcription were mixed with reagents of the Taqman miRNA assay and the Taqman Fast Advanced mastermix (Life Technologies) for quantification of the miRNAs. qPCR was carried out on ABI 7900HT sequence detection system (Applied Biosystems; Life Technologies) and thermal cycling was for 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and one minute at 60 °C. Primer efficiencies of the Taqman assays were 108% and 101% for miR-222 and miR-146a, respectively. Efficiency of the *cel-miR-39* assay was 102%. All runs were carried out in triplicate and with a no-template control (NTC) on 384-well plates with three inter run calibrators (IRC). Raw qPCR data were analyzed using the SDS Relative Quantification Software (version 2.3; Applied Biosystems). Cq values were transformed to a relative quantity against the external spike-in miRNA in qbase+ software (Biogazelle; Zwijnaarde, Belgium).

Statistical analysis

Statistical analyses were carried out using SAS software (version 9.4; SAS Institute Inc., Cary, NC, USA). miRNA expression data were $\log_{10}$-transformed to obtain normal distribution of the data. The association between the air pollutants and extracellular miRNA expression data was assessed using a mixed model taking in account possible confounders as well as the repeated measures by school and subject. Models were constructed based on *a priori* selected covariates including sex, age (continuous), BMI (continuous), exposure to passive tobacco smoke (categorical: yes/no), maternal education (categorical: low/high), time and day of examination, the hours/week spend watching TV (continuous), extracellular RNA concentration and school. High maternal education levels were defined as college or university. Time of the day was evaluated categorically (before 10 a.m.,

between 10 a.m. and 12 a.m. or after 12 a.m.) as well as day of the week. Q-Q plots of the residuals were used to test the assumptions of the models. All effect estimates were calculated as the percent change in extracellular miRNA expression associated with an IQR increment in the air pollutant concentration.

RESULTS

The study group of 80 participating children comprised 43 girls (53.8 %). The characteristics are given in Table 1. Briefly, age averaged 10.4 years (range 8.0 – 12.8) and BMI averaged 17.0 kg/m² (range 12.7 – 23.4). Repeated measures were carried out for each individual. As such, the miRNAs were quantified in two samples that were collected at different time points.

Table 1: Characteristics of the study population (n=80)

Variable	Mean ± SD or n (%)
Boys	37 (46.3%)
Age, years	10.44 ± 0.97
BMI, kg/m²	17.01 ± 2.42
TV watching, hours/week	9.32 ± 5.46
Exposure to tobacco smoke	9 (11.2%)
Maternal education	
Low	26 (32.5%)
High	54 (67.5%)

Mean ± SD; frequency (%) SD = standard deviation; BMI = body mass index. High maternal education was defined as college or university.

Table 2 gives an overview of the recent exposure parameters for fine and ultrafine particles, which were monitored at the school site, both indoor and outdoor. Daily average $PM_{2.5}$ exposures at the school site were obtained by interpolation based on the school addresses. Indoor concentrations of UFP were on average 10300 particles/cm³ and $PM_{2.5}$ averaged 4.6 µg/m³ in the examination room. At the playground, UFP was on average 32100 particles/cm³ and $PM_{2.5}$ was on average 16.6 µg/m³. Daily $PM_{2.5}$ at the day of the study visit averaged 24.2 µg/m³.

We applied pollutant-specific mixed models to estimate the association of miR-222 and miR-146a expression levels and exposure to UFP or $PM_{2.5}$ and in the extracellular fraction of saliva (Table 3), while adjusting for the *a priori* selected covariates: sex, age, BMI, exposure to passive smoking, maternal education level, time and day of examination, time/week spent watching TV and the extracellular RNA concentrations. Because of the repeated measures design of the study, sampling and exposure measurements from two different time points were used to increase statistical power.

Recent UFP exposure was significantly associated with an increase in extracellular miR-222 expression in the saliva (Figure 1). An IQR increment in indoor UFP concentration (+ 8504 particles/cm^3) was associated with a 23.5% increase (95% confidence interval (CI): 3.5, 41.1) in extracellular miR-222 levels (p=0.021). Similarly, an IQR increment in outdoor UFP concentration (+ 28776 particles/cm^3) was associated with a 29.9% increase (95% CI: 10.6, 49.1) in miR-222 expression in saliva extracellular fraction (p=0.0027). Daily average $PM_{2.5}$ levels at the residence during the day of the study visit and the two days before the study visit were not associated with extracellular miR-222 (Table 3). The mixed models did not show any association between air pollution exposure and miR-146a (Table 3).

Table 2: Recent exposure to fine ($PM_{2.5}$) and ultrafine (UFP) particles at the school site

Pollutant indicator	Mean (SD)	P25	P50	P75	P90
Indoor at school during the examination					
UFP, #/cm³	10304 (6115)	5577	8208	14081	18852
$PM_{2.5}$, µg/m³	4.6 (3.6)	2.2	3.4	5.0	10.0
Outdoor at school during the examination					
UFP, #/cm³	32134 (21572)	15842	24162	44618	67838
$PM_{2.5}$, µg/m³	16.6 (16.7)	7.2	11.5	17.5	44.5
Modeled daily residential $PM_{2.5}$					
Day of the examination (lag 0), µg/m³	22 (15.5)	11	22.6	26.3	42.5
Day before the examination (lag 1), µg/m³	19 (18.3)	7.4	11.6	24.2	40.1
Two days before the examination (lag 2), µg/m³	18.6 (20.6)	6.7	11.8	18.4	68.9
Average of 48h before the examination, µg/m³	18.8 (18.7)	7.9	12.2	20.8	57.8

SD= standard deviation; P25= 25th percentile; P50= median; P75=75th percentile; P90= 90th percentile

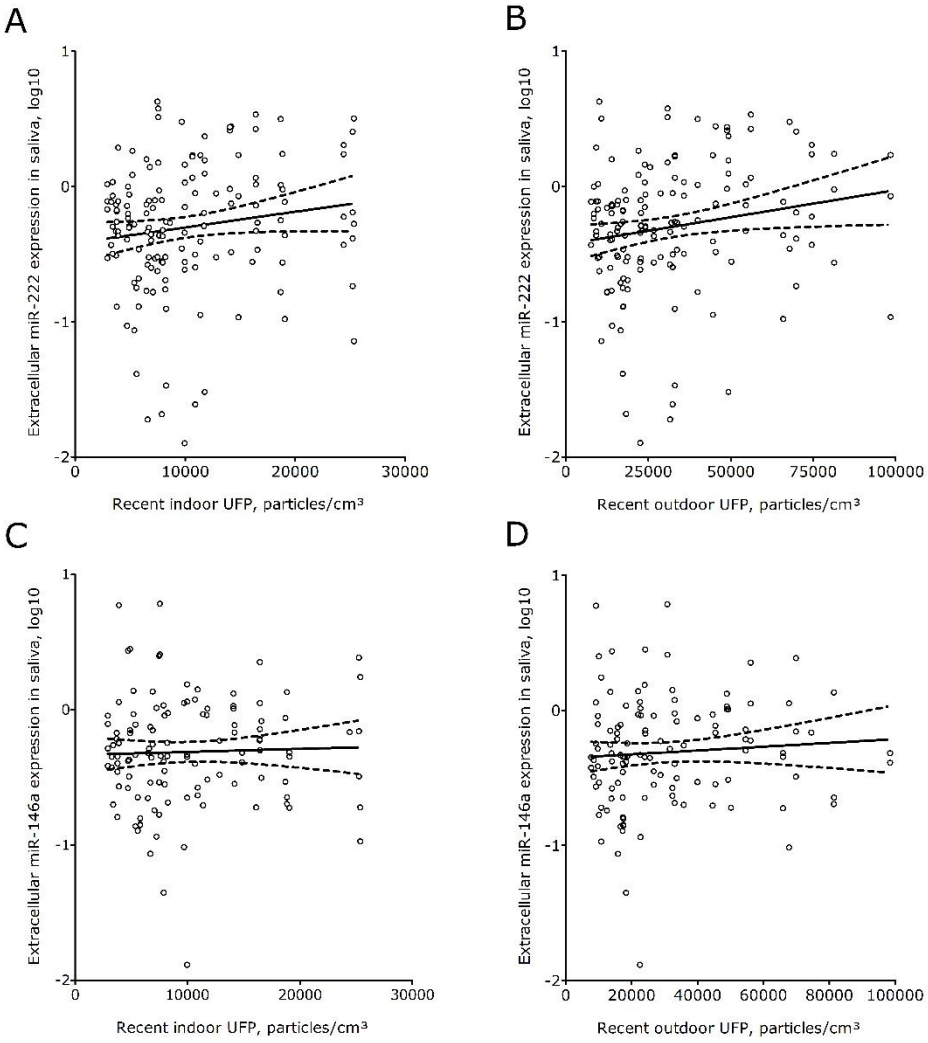

Figure 1: Correlation plot between recent UFP exposure and miR-222 and miR-146a. Plotting the miR-222 expression levels from the salivary extracellular fraction for the recent UFP exposure shows a positive association with both indoor (A) and outdoor (B) UFP.

Table 3: The association between extracellular miRNA expression and recent exposure to fine ($PM_{2.5}$) and ultrafine (UFP) particles

		miR-222		miR-146a	
Pollution indicator	**IQR**	**Estimate (95% CI)**	**p-value**	**Estimate (95% CI)**	**p-value**
Indoor at school during the examinations					
UFP, #/cm³	8504	23.5 (3.5, 41.1)	0.021	4.2 (-9.4, 17.8)	0.54
$PM_{2.5}$, µg/m³	2.8	4.7 (-9.1, 19.3)	0.50	4.9 (-4.6, 14.8)	0.31
Outdoor at school during the examinations					
UFP, #/cm³	28776	29.9 (10.6, 49.1)	0.0027	10.6 (n/a)	0.13
$PM_{2.5}$, µg/m³	10.33	-2.3 (-11.5, 7.1)	0.63	-1.4 (-7.4, 4.6)	0.65
Modelled daily residential $PM_{2.5}$					
Day of the examination (lag 0), µg/m³	15.3	8.20 (-5.8, 22.4)	0.26	-1.6 (-11.6, 8.5)	0.76
Day before the examination (lag 1), µg/m³	16.8	6.92 (-6.8, 20.8)	0.33	-4.9 (-15, 5.1)	0.34
Two days before the examination (lag 2), µg/m³	11.7	3.96 (-5.4, 13.4)	0.41	-2.4 (-9.2, 4.4)	0.48
Average of 48h before the examination, µg/m³	12.9	5.31 (-5.7, 16.4)	0.35	-3.5 (-11.5, 4.5)	0.39

Estimated effects sizes are adjusted for school, time of the day, day of the week, age (continuous), gender, BMI (continuous), passive smoking, maternal education level, the hours TV /week and RNA content of the extracellular fraction. All estimates are represented as the % change in miRNA expression for an IQR increase in exposure to the pollutant. $P<0.05$ was considered as a significant association.

DISCUSSION

miRNAs are responsive to external stimuli, including PM [10-13] and contribute to cardiovascular disease development and progression [29-31]. Here, we focused on the extracellular levels of miR-222 as well as miR-146a, since both miRNAs have been shown responsive to PM exposure at the cellular level [10,12,13]. Furthermore, these miRNAs have been linked with the initiation and progression of atherosclerosis via endothelial dysfunction and inflammation [30,32]. Air pollution is ubiquitous and ultrafine particles (<100 nm) translocate from the lung into the system and may contribute to adverse cardiovascular effects. In our repeated measure design, we found that miR-222 expression in saliva of children between 8-12 years of age was positively associated with exposure to ultrafine particles at school. Up-regulation of miR-222 is associated with vascular damage via decreased endothelial progenitor cell differentiation [33-36] and increased vascular smooth muscle cell cycling [37], as it targets c-Kit and its ligand stem cell factor [33] and p27kip1 [38]. miR-222 was reported to target eNOS in an indirect fashion [39] via direct inhibition of the transcription factor ets-1, which regulates eNOS expression [40,41]. Additionally, since p27kip1 is an important regulator of cell cycle arrest, miR-222 has an oncogenic character [38]. This observation in the salivary extracellular fraction of children is consistent with observations in blood miRNA expression in steel workers [10]. An increased miR-222 expression in white blood cells (WBC) after three days of work was observed compared with baseline [10]. Additionally, controlled O_3 exposure induced an increase in miR-222 expression in human sputum, which is a proxy for the respiratory system [42]. On the other hand, Motta and colleagues did not find an association between PM exposure and miR-222 expression in WBC in a subset of the steel workers population[12]. Furthermore, an inverse association between leukocyte miR-222 and 7-day exposure to $PM_{2.5}$ and sulfate has been observed in the Normative Aging Study [13]. The latter two studies were performed in a population with different characteristics and exposure levels and in the case of Motta et al., a different technique (miRNA microarray) was used. Our results add to the evidence of a possible epigenetic mechanism via which cells are influenced by UFP or can respond to UFP, not only in heavily exposed adults, but also in children.

We did not find any significant changes in salivary extracellular miR-146a abundance in association with acute changes in UFP or $PM_{2.5}$ exposure. Similarly, Bollati reported no association between PM and cellular miR-146a expression in three days post exposure samples of steelworkers compared with baseline, though miR-146a expression was inversely associated with PM metal compounds cadmium and lead [10]. However, Motta *et al.* performed miRNA microarray analysis on WBC in a subset of the steel plant population and found an up-regulation of miR-146a after three days exposure [12]. A study by Fossati found an inverse association between miR-146a expression in WBC and $PM_{2.5}$ and black carbon exposure (7-days moving average) [13]. Possibly, miR-146a expression was not affected in our population or potential changes in miR-146a expression on a cellular level might not be reflected into the extracellular fraction of miRNAs. It should be clear from the above that the number of studies dealing with air pollution exposure and miRNA expression are limited and that maybe because of the different study designs, the results are hard to compare.

Extracellular miR-222 levels in saliva were significantly associated only with UFP, the smallest fraction of PM. However, using daily average $PM_{2.5}$ exposure at home based on a land-use dispersion model showed a trend similar to the recent exposure to UFP analyses. A study by Nemmar and colleagues, showed that upon inhalation, UFP can already be detected in circulation after one minute and in extrapulmonary organs within 5 minutes [5]. Extracellular miR-222 is significantly associated with PM when considering the exposure shortly before the measurements.

miRNAs in our study originate either from microvesicles or aggregated proteins which can be present in saliva. Differential ultracentrifugation as used in this study cannot separate vesicles from proteins [43]. However, to gain information about the content of the samples, we determined the size of the nanoparticles in a subset of the saliva samples by means of nanoparticle tracking analysis (Supplemental information). The particles with a size of 100 to 600 nanometer were most abundant (see supplemental figure), these can include exosomes, microvesicles or aggregated proteins [43]. In the future, studying specific fractions might further elucidate our results. The role of extracellular miRNAs and the microvesicles or protein complexes that bear them is not studied often in relation to air pollution.

However, the limited evidence that is available, supports a role of extracellular mediators in the cardiovascular disease mechanisms after PM exposure. Using an experimental setup in mice, exposure to traffic-related PM led to an increased abundance of microvesicles with thrombogenic potential in plasma [44]. In a diabetic population, acute changes in $PM_{2.5}$ were associated with a decrease of procoagulant microvesicles present in plasma [45]. Similarly, a study by Frampton and colleagues showed an increase in microparticles which expressed tissue factor after 2 and 5 days of $PM_{2.5}$ exposure in diabetes type 2 patients [46].

Blood is a widely preferred biofluid for use in observational studies, nevertheless it can be challenging to collect blood samples in a population of children. Saliva on the other hand is minimally invasive, contains a diverse range of proteins, RNAs and miRNAs and may actually reflect an individual's physiological condition [18,19,47]. Saliva may not only be used for diagnostic purposes, but it is also promising as indicator of local and systemic health. The overlap in altered salivary transcriptome and the transcriptome of tumor-tissue and the salivary glands, indicates that salivary glands might be affected by tumor-specific factors and as such might affect the salivary transcriptome [48].

The strengths of the present study are the repeated measures, which allow to control for intraindividual variation and a higher statistical power. Furthermore, recent exposure parameters were assessed at the school of the study participants and reflect at least to some extent a personal exposure since children spend much time at school. Our study is among the first to explore the effect of air pollution exposure on miRNA expression in children. Children are especially vulnerable because their body is not fully developed and cannot adequately cope with the toxic exposure to PM. Children tend to be more active and have a higher ventilation rate, therefore more particles can be deposited in their body [49,50]. A limitation of this study is the explorative character of salivary extracellular miRNAs, as we choose to study miRNAs that were reported to be responsive in adult populations. Early studies on miRNA expression and PM exposure in humans highlighted the importance of miR-222 and miR-146a [10,12-14]. These findings are supported by recent publications as well [51,52]. However, studies using untargeted approaches indicated the involvement of wider range of miRNAs in the effects PM

[53,54]. Also, no targets of the miRNAs were profiled to indicate a downstream effect of altered miRNA expression.

CONCLUSION

The present study provides evidence of an epigenetic response to UFP by alterations in the saliva extracellular miRNA abundance. Children had a higher expression of saliva extracellular miR-222 on days with higher ambient concentrations of ultrafine particulates (diameter smaller than 300 nm), compared to days with lower exposure. This rapid response was not observed with exposure parameters of larger particles, suggesting that UFP exposure is particularly relevant in the process of rapid adaptations of the extracellular miRNA content. However, the health consequences of altered extracellular expression levels miRNAs in response to air toxins remain to be elucidated.

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SUPPLEMENTAL INFORMATION

Characterization of extracellular fraction present in saliva of children

Nanoparticle Tracking analysis

A subset of ten random samples was analyzed in order to determine the size distribution of the particles in the extracellular fraction. Nanoparticle Tracking Analysis (NTA) is carried out on extracellular particles diluted in PBS, using NanoSight LM10-HS (Nanosight Ltd., Amesbury, UK) equipped with a 405 nm laser and a CMOS camera system. Selected samples for NTA were analyzed in triplicate for 60 seconds. The temperature was monitored during the measurements. Data was analyzed with the NTA software (version 3.0), which generated three outputs with the size and concentration of the particles, which were averaged.

The presence of larger particles and the high quantity of particles in the samples resulted in high scattering, which causes great differences in the repeated NTA measurements. As such, large standard errors were observed between the measurements (Figure S1). NTA results also indicate an abundance of bigger particles in the samples, with predominance of particles of 200 nm and 500 nm in diameter (Figure S1). Due to the presence of large particles, the detection threshold was put at 4 and the camera level at 10. As such, the registration of the smallest particles (< 100 nm) can be withheld and as such they could not be observed. The NTA results indicate that the vast majority of the present vesicles is smaller than 600 nm (Figure S1).

We are aware that the fraction we yielded upon ultracentrifugation might contain a variety of structures that can harbor the miRNAs. Since the origin of the miRNAs was not further determined, we prefer to refer to it as extracellular miRNAs from saliva.

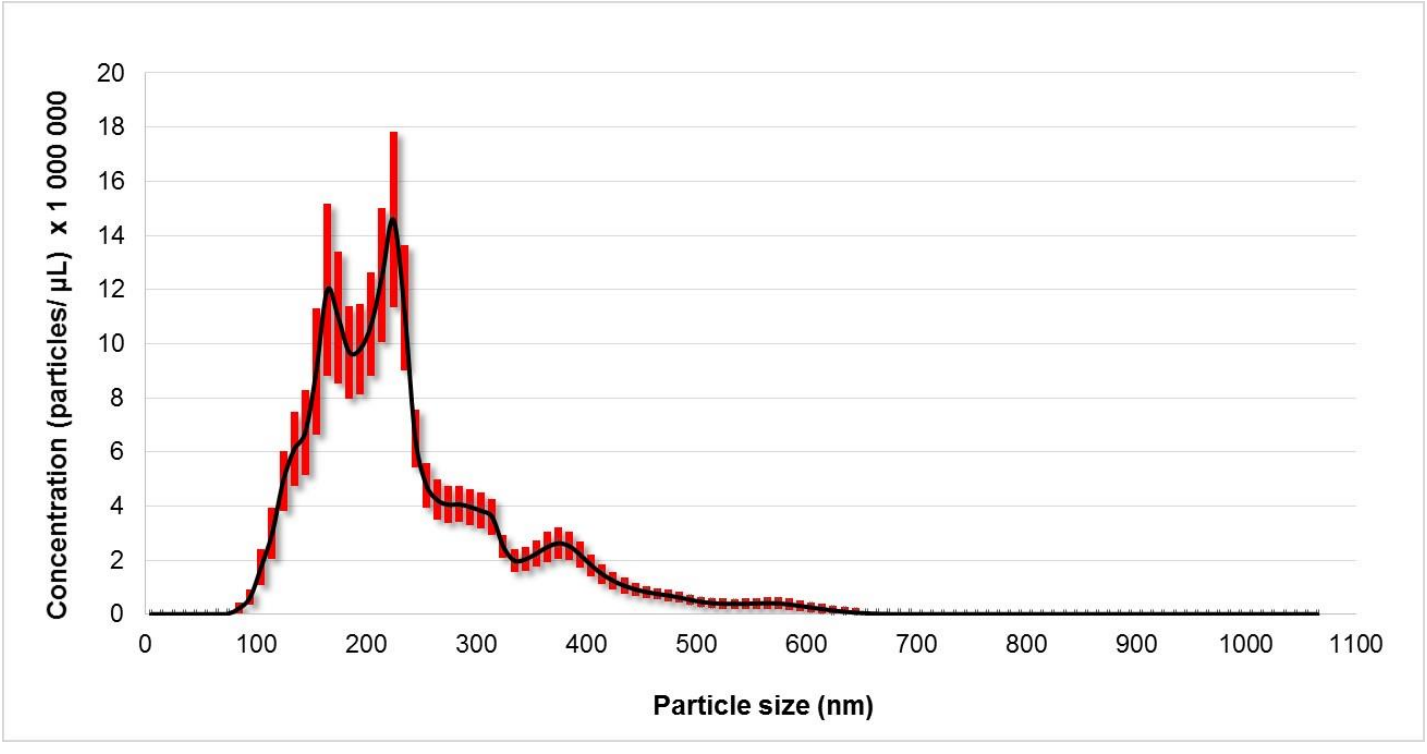

Figure S1: Size distribution structures in the extracellular fraction of saliva. The size distribution of the particles present in the extracellular fraction of saliva was estimated by Nanoparticle Tracking Analysis (NTA). The frequency for each particle size was obtained by averaging the concentrations from eight random samples. The concentrations were plotted against the particle size. Variation among the samples is represented by the red bars, reporting the standard error of the mean.

Table S1: Correlation between on-site measurements and interpolation data at the school

On-site measurement during the study	Daily mean on the day of the study	r	p-value
UFP	$PM_{2.5}$	0.52	<0.001
$PM_{2.5}$	$PM_{2.5}$	0.64	<0.001

UFP= ultrafine particles, $PM_{2.5}$ = PM with an aerodynamic diameter less than 2.5 µm, r=Spearman correlation coefficient

Chapter 3

Neonatal exposure to environmental pollutants and placental mitochondrial DNA content: a multipollutant approach

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Vriens A. *et al.* Environment International (2017)

ABSTRACT

Background: Placental mitochondrial DNA (mtDNA) content can be indicative of oxidative damage to the placenta during fetal development and is responsive to external stressors. *In utero* exposure to environmental pollutants that may influence placental mtDNA needs further exploration. We evaluated if placental mtDNA content is altered by environmental pollution in newborns and identified pollutants independently associated to alterations in placental mtDNA content.

Methods: mtDNA content was measured in placental tissue of 233 newborns. Four perfluoroalkyl compounds and nine organochlorine compounds were quantified in cord blood plasma samples and six toxic metals in whole cord blood. We first applied a LASSO (least absolute shrinkage and selection operator) penalized regression model to identify independent associations between environmental pollutants and placental mtDNA content, without penalization of several covariates. Then adjusted estimates were obtained using an ordinary least squares (OLS) regression model evaluating the pollutants' association with placental mtDNA content, adjusted for several covariates.

Results: Based on LASSO penalized regression, oxychlordane, p,p'-dichlorodiphenyldichloroethylene, β-hexachlorocyclohexane, perfluorononanoic acid, arsenic, cadmium and thallium were identified to be independently associated with placental mtDNA content. The OLS model showed a higher placental mtDNA content of 2.71% (95% CI: 0.3, 5.2; p=0.03) and 1.41% (0.1, 2.8; p=0.04) for a 25% concentration increase of respectively cord blood β-hexachlorocyclohexane and arsenic. For a similar concentration increase of cord blood thallium, a 4.88% lower placental mtDNA content (95% CI: -9.1, -0.5; p=0.03) was observed.

Conclusion: In a multipollutant approach, low fetal exposure levels of environmental pollutants might compromise placental mitochondrial function as exemplified in this study by alterations in mtDNA content.

BACKGROUND

Fetuses and newborns are particularly vulnerable to exogenous compounds. Since their detoxification system is not fully developed, exposures can interfere with birth outcomes [1], but can also modify an individual's susceptibility to adult disease in later life [2]. The placenta is a transient organ during gestation involved in the implantation of the early embryo, produces hormones to facilitate the fetal growth and exchanges nutrients, gases and waste products between the developing fetus and the mother. Nevertheless, the placenta does not embody a perfect barrier to harmful substances and transplacental migration of for example toxic metals [3,4], organochlorine compounds [3,5,6] and perfluoroalkyl compounds [3,7,8] has been described.

An important limitation in the current understanding of early life environmental exposures is that exposure to multiple pollutants is rarely taken into account simultaneously and mechanisms activated at low levels of exposure are still largely undefined. Mitochondria are prone to damage induced by reactive oxygen species (ROS) and can compensate genomic insults by altering their abundance, as such they can react very quickly to exposures [9]. Mitochondrial physiological states determined by mitochondrial DNA (mtDNA) copy number can sense environmental perturbations, and might be indicative of disease mechanisms [10-12]. Environmental stressors, such as particulate matter in air pollution, can alter placental mtDNA content [13] , and its potential role to mediate birth weight has been suggested [14]. Oxidative stress is not only involved in metal toxicity [15], but is also proposed as a possible mechanism of toxicity of organochlorines [16,17] and perfluoroalkyl compounds [18,19].

The Flemish Environment and Health Study (FLEHS) was initiated to establish updated reference values of pollutants and to study the impact of exposure to these pollutants on human health. In this framework, we examined the association between environmental exposure to toxic metals, organochlorines and perfluoroalkyl compounds and placental mitochondrial DNA content. Environmental exposure was represented by the biomarkers quantified in cord blood (i.e. arsenic (As), cadmium (Cd), copper (Cu), manganese (Mn), lead (Pb) and thallium (Tl), polychlorinated biphenyl (PCB) -138, -153, -180,

hexachlorobenzene (HCB), oxychlordane (OXC), β-hexachlorocyclohexane (β-HCH), transnonachlor (TN), p,p'-dichlorodiphenyldichloroethylene (p,p'-DDE), p,p'-dichlorodiphenyltrichloroethane (p,p'-DDT) and perfluorooctane sulfonate (PFOS), perfluorooctanoate (PFOA), perfluorohexane sulfonic acid (PFHxS), perfluorononanoic acid (PFNA) and perfluorobutanesulfonic acid (PFBS). We independently selected exposures using shrinkage-based regression to identify independent effects of the most predictive exposures for placental mtDNA content.

METHODS

Study population and recruitment

This study was a part of the third cycle of FLEHS, which recruited mother-newborn pairs in six hospitals in Flanders, Belgium to obtain a representative sample of the population. All women giving birth in one of the participating hospitals could enter the study, if they lived at least five years in Flanders and were able to fill out an extensive Dutch questionnaire. On a subset of the population we determined the recruitment rate, which was 18.7% of the deliveries. Respectively 8.3% and 1.6% of the mothers did not meet the language requirements and 5-year residency.

In total, 281 mother-newborn pairs were recruited from November 2013 to November 2014. Of these 281 pairs, 250 had placental biopsies available of which five were excluded because of caesarean section, six did not have a quantification of the organochlorine compounds and an additional three had no cord blood perfluoroalkyl compounds measurement available. Three newborns lacked information on socio-economic status. The remaining 233 subjects were included in the current study (Supplemental figure S1). The medical ethical committee of University of Antwerp and University Hospital of Antwerp as well as the local ethical committee of each participating hospital approved the study. All subjects in the study gave informed consent to participate and filled out a questionnaire during their stay in the hospital maternity. The questionnaire addressed the general health status of the mother (e.g. weight management, infections, illness or complications during the pregnancy, allergy), life-style (smoking and alcohol use during pregnancy), socio-economic status (e.g. occupation, education), household composition and housing conditions and dietary patterns.

Sample collection

Umbilical cord blood was collected immediately after delivery using polypropylene Na-EDTA tubes, which were previously tested for metal contamination. Until the biopsies were taken placentas were kept in the delivery room and stored at 4°C. Villous tissue, which is protected by the chorioamniotic membrane, was sampled at the fetal side of the fresh placenta. One biopsy was taken 4 cm from the umbilical cord, via a standardized protocol. Afterwards, the biopsies were stored at -80 °C until DNA isolation.

Measurement of biomarkers of exposure

The selection of pollutants was based on (i) health impact, (ii) current policy relevance, (iii) potential for remediation and (iv) feasibility. As such, chemicals with known historical pollution in Flanders, Belgium (metals, organochlorines) as well as more recent emerging chemicals (perfluoroalkyl compounds) were included.

The toxic metal pollutants As, Cd, Cu, Pb, Mn and Tl were determined in whole cord blood samples after acid digestion. The measurements were carried out using high resolution inductively coupled plasma – mass spectrometry (HR-ICP-MS) as described by Schroijen *et al.* [20]. The limit of detection (LOD) in the blood samples was 0.09 µg/L for As, 0.0097 µg/L for Cd, 0.49 µg/L for Cu, 0.22 µg/L for Pb, 0.15 µg/L for Mn and 0.48 µg/L for Tl.

The following organochlorine compounds were quantified in cord blood plasma samples: PCB congeners 138, 153, and 180, p,p'-DDE, p,p-DDT, HCB, OXC, TN and β-HCH. The quantification was carried out following earlier described protocols by Dirtu *et al.* [21]. The limit of quantification (LOQ, which corresponds to LOD*3.33) is 2 ng/L for each PCB congener, 20 ng/L for p,p'-DDE, 10 ng/L for p,p'-DDT and HCB, 2 ng/L for OXC and TN and 5 ng/L for β-HCH. Organochlorine concentrations were normalized to blood lipid concentrations, which were calculated using the following formula: total lipids = 50.49 + 1.32*(total cholesterol + triglycerides) [90].

The perfluoroalkyl compounds PFOS, PFOA, PFHxS, PFNA and PFBS were determined in cord blood plasma. The measurements were carried out based on

the protocol described by Midasch *et al.* [7,22]. The LOQ for PFOS, PFOA, PFHxS and PFBS was 0.2 µg/L and 0.1 µg/L for PFNA.

Samples with a concentration below the LOD or LOQ were assigned LOD/2 or LOQ/2 as concentration. At least 74% of the measurements were above the respective LOD or LOQ for all exposures except for p,p'-DDT, for TN and PFBS. For p,p'-DDT 18% of the measurements was above the LOQ, for TN 44% was above the LOQ and PFBS was for all samples below the LOQ. p,p'-DDT and TN were considered binary (above or below the LOD) in the analyses, while PFBS was omitted from the analyses.

Measurement of mtDNA content

Excess blood was removed from the placental biopsies, the samples were homogenized and DNA was isolated using the QIAamp DNA mini kit (Qiagen), which uses silica-membrane columns.

Mitochondrial DNA content (mtDNA) in placental tissue was measured by determining the ratio of two mitochondrial gene copy numbers (*MTF3212/R3319* and *MT-ND1*) to a single –copy nuclear control gene (*36b4*) using a real-time quantitative polymerase chain reaction (qPCR). qPCR reactions were carried out on a 384-well plate on the 7900HT Fast Real-Time PCR System (Applied Biosystems) in a 10 µl volume containing: 5 µl QuantiTect SYBR Green (Qiagen) mastermix, 0.3 µl of respectively forward and reverse primers (300 nM) and 1.9 µl RNAse-free water and 6 ng DNA diluted in 2.5 µl RNAse-free water. Primer sequences for mitochondrial genes are reported elsewhere [13]. All reactions were carried out in triplicate, no-template controls and six interrun calibrators were included in each qPCR run. The thermal cycling profile for the three transcripts was 10 minutes at 95°C for activation of the polymerase enzyme and initial denaturation, followed by 40 cycles of 15 seconds at 94°C for denaturation and 70 seconds at 58°C for annealing and extension. After thermal cycling, the raw data were collected and processed using SDS 2.3 software (Applied Biosystems). The cycle quantification (Cq) values were normalized relatively to the *36b4* gene using qBase+ software (Biogazelle, Ghent, Belgium) taking into account the run-to-run differences [23]. mtDNA content levels were not influenced by the storage time and plate effects (Supplemental figure S2).

Statistical analysis

Placental mtDNA content and all exposure biomarkers were log10-transformed in order to reduce skewness of the data. A correlation heatmap between all biomarkers of exposure was constructed to investigate the collinearity of the data. For As, we evaluated differences in cord blood As levels according to the reported consumption of fish and seafood.

Population characteristics are represented as mean (SD) for continuous variables and number (%) for categorical variables. The unadjusted associations for the characteristics and placental mtDNA content were determined using linear regression.

The relevant biomarkers of environmental exposures were selected using least absolute shrinkage and selection operator (LASSO) penalized regression analysis [24]. This method allows selecting the important predictors and also estimates the regression coefficient. However, it should be noted that the effect estimates of LASSO are biased as a consequence of the penalty.

$$\tilde{\beta} = \arg min_{\beta} \{ \sum_{i=1}^{N} \left(y_i - \beta_0 - \sum_{j=1}^{p} x_{ij}\beta_j \right)^2 + \lambda \sum_{j=1}^{p} |\beta_j| \}$$

with n= sample size and p=number of parameters

The higher the penalty parameter λ, the higher the penalization on the variables, thus a more sparse model will be selected, with more coefficients shrank to zero. The optimal value of the penalty parameter λ was determined using 10-fold cross-validation. The LASSO model was tested over a grid of λ values and the λ where the mean squared error [25] was within one standard error of the minimum MSE of prediction from 100 times repeated cross-validation was chosen. A set of covariates was chosen *a priori* based on (i) a known relation with placental mtDNA content [13,26,27] or (ii) the assumption that the variable might capture confounding factors (i.e. education for social-economic status). The covariates of interest contained the continuous variables gestational age (weeks), maternal pre-pregnancy BMI, maternal age, and the categorical variables newborn's sex, parity (three categories: none, one, two or more), smoking during pregnancy (two categories: no and yes), season of birth (four categories) and highest educational

level within the household (three categories: low, middle and high). The set of *a priori* selected covariates was entered in the LASSO analysis without penalization, in order to adjust for potential confounders. The independent variables (i.e. the exposures and covariates) were standardized prior to fitting the LASSO regression model.

We applied multiple ordinary least squares (OLS) regression models to estimate the adjusted effects of the exposures, which were selected by LASSO. The assumptions of the OLS regression models were checked using the Q-Q plots of the residuals (normality) and residual by predicted plots (homoscedasticity). The variance inflation factors (VIFs) of the regressors were calculated to check for multicollinearity (provided in supplemental table S2). We performed sensitivity analyses (Supplemental table S3) without any covariates in the models (unadjusted model), including solely newborn-related characteristics (model 1), and including maternal covariates (model 2) to study the influence of possible confounding factors and to models including fish (model 3) and seafood consumption (model 4). Data cleaning was performed in SAS software (version 9.4, SAS Institute Inc, Cary, NC, USA), penalized regression (*glmnet* package) and the OLS regression (*stats* package) were carried out in RStudio software (R version 3.3.1).

RESULTS

Study population

The characteristics of the mother-newborn pairs in this study are listed in Table 1. The participating mothers were on average (SD) 30 (4) years old. The mothers had an average pre-pregnancy BMI of 24 (4) kg/m² and 11% (n=25) of the mothers reported smoking during the pregnancy. Fish and seafood intake are major exposure routes for As, 40% of the mothers reported to eat fish less than once in a week, 26% reported weekly fish consumption and 35% reported to eat fish more than once in a week. 37% of the women reported never to eat seafood, and 21% and 42% reported to eat seafood monthly or more than once in a month. The newborns, including 121 boys (52%), had a mean gestational age of 39 (1.3) weeks. On average, the birth weight was 3462 (437) grams.

Biomarkers of environmental exposure

The concentrations of environmental pollutants in cord blood are shown in Table 2 and Figure 1 depicts their correlation, after log-transformation. Pollutants of the following categories showed high correlation: (i) PCBs (correlation coefficients (R) ranged from 0.84 to 0.95); (ii) organochlorine compounds (R ranged from 0.19 to 0.39), with exception of p,p'-DDT and (iii) perfluoroalkyl compounds (R ranged from 0.4 to 0.63). PCBs showed high correlation with organochlorine compounds (R ranged from 0.34 to 0.64), except with HCB (R=0.1) and p,p'-DDT (R=0.07). The perfluoroalkyl compounds were significantly associated with the organochlorines (correlation coefficients range from 0.11 to 0.44). Cd levels were significantly correlated with cord blood Cu, Pb and Mn with correlation coefficients of respectively 0.44, 0.21 and 0.28. Mn and Cu were also correlated (R= 0.42). The levels of the biomarkers of exposures by tertiles of the placental mtDNA content are depicted in Supplemental table S1.

Cord As levels were significantly higher in mothers that reported to eat fish more than once a week (p=0.008), compared to less than once a week. Mothers who reported never to eat seafood showed significantly higher (p=0.003) cord As levels than mothers that reported to eat seafood more than once a month.

Table 1: Characteristics of the mother-newborn pairs (n=233) and their association with placental mtDNA content

Characteristic	Mean ± SD or n (%)	Estimate (95% CI)	p-value
Maternal			
Age, years	30.3 ± 4.3	3.32 (-2.37, 9.09)	0.25
Parity			0.91
0	104 (45%)	0.44 (-13.64, 16.82)	0.95
1	80 (34%)	-2.37 (-16.65, 14.36)	0.77
≥ 2	49 (21%)	Ref	/
Pre-pregnancy BMI, kg/m²	24.0 ± 4.2	6.97 (1.18, 12.83)	0.018
< 18.5	5 (2%)	-26.34 (-50.12, 8.78)	0.12
18.5 – 25	154 (66%)	Ref	/
25 – 30	50 (21%)	18.06 (2.68, 35.76)	0.02
> 30	24 (10%)	-6.51 (-22.6, 12.9)	0.48
Net weight gain, kg [a]	13.3 ± 4.4	-0.82 (-6.58, 5.03)	0.78
Smoking during pregnancy	25 (11%)	-14.57 (-28.86, 2.06)	0.092
Education [b]			0.61
Low	17 (7%)	11.92 (-10.44, 39.85)	0.32
Middle	67 (29%)	1.67 (-10.55, 15.55)	0.80
High	149 (64%)	Ref	/
Fish consumption [a]			0.70
< Weekly	91 (40%)	-5.03 (-16.97, 8.61)	0.45
Weekly	59 (26%)	-5.03 (-18.29, 10.38)	0.50
> Weekly	80 (35%)	Ref	/
Seafood consumption [c]			0.17
Never	84 (37%)	-5.65 (-17.15, 7.46)	0.38
Monthly	49 (21%)	-13.68 (-25.91, 0.58)	0.059
> Monthly	96 (42%)	Ref	/

Table 1 (continued): Characteristics of the mother-newborn pairs (n=233) and their association with placental mtDNA content

Characteristic	Mean ± SD or n (%)	Estimate (95% CI)	p-value
Newborn			
Sex, male	121 (52%)	9.05 (-2.66, 22.17)	0.13
Gestational age, weeks	39.4 ± 1.3	-6.79 (-12.19, -1.15)	0.019
Birth weight, g	3462 ± 437	-5.90 (-11.57, -0.24)	0.041
Season of birth			0.005
Winter	92 (39%)	Ref	/
Spring	74 (32%)	19.76 (4.87, 36.76)	0.008
Summer	50 (21%)	25.30 (7.91, 45.49)	0.003
Autumn	17 (7%)	-1.99 (21.70, 22.67)	0.86
Cord blood lipids, mg/dL	187 ± 31.5	/	/

a: n=230, b=Low education was defined as lower high school, middle education was defined as higher high school and high education level was defined as a college or university degree, c= n=229. All estimates are represented as the relative percentage change in mtDNA content, in case of continuous variables it is the effect size for a SD increase in the characteristic.

Table 2: Characteristics of the biomarkers of environmental pollutants, quantified in umbilical cord blood (n=233)

	Mean (SD)	Median	P5	P95	%> LOD or LOQ [a]
Arsenic, µg/L	1.19 (1.84)	0.63	0.15	4.03	100%
Cadmium, µg/L	0.02 (0.01)	0.02	0.01	0.04	98.7%
Copper, µg/L	564 (97.0)	573	398	729	100%
Manganese, µg/L	31.5 (11.1)	29.6	16.7	53.0	100%
Lead, µg/L	7.09 (3.40)	6.07	3.27	12.3	100%
Thallium, ng/l	19.4 (5.77)	18.1	12.3	30.7	100%
PCB138, ng/g fat	12.2 (6.6)	10.9	3.98	24.7	100%
PCB153, ng/g fat	19.4 (10.6)	17.3	6.35	40.2	100%
PCB180, ng/g fat	10.4 (6.83)	8.46	2.99	22.2	100%
β-HCH, ng/g fat	4.55 (3.81)	3.80	<LOQ	10.1	76.0%
p,p'-DDE, ng/g fat	86.2 (111)	54.3	21.80	293	100%
p,p'-DDT, ng/g fat	/	/	/	/	18.0%
Hexachlorobenzene, ng/g fat	15.2 (11)	14.7	<LOQ	34.9	78.5%
Oxychlordane, ng/g fat	1.66 (1.08)	1.53	<LOQ	3.32	74.7%
Transnonachlor, ng/g fat	/	/	/	/	45.1%
PFHxS, µg/L	0.42 (0.25)	0.38	<LOQ	0.91	86.3%
PFNA, µg/L	0.25 (0.18)	0.21	<LOQ	0.52	90.1%
PFOA, µg/L	1.36 (0.75)	1.27	0.52	2.46	100%
PFOS, µg/L	1.39 (1.02)	1.12	0.42	3.28	99.6%

[a] For the trace elements (As, Cd, Mn, Pb and Tl) the limit of detection (LOD) was reported, whereas for the organochlorines and perfluoroalkyl compounds the limit of quantification (LOQ) was reported.

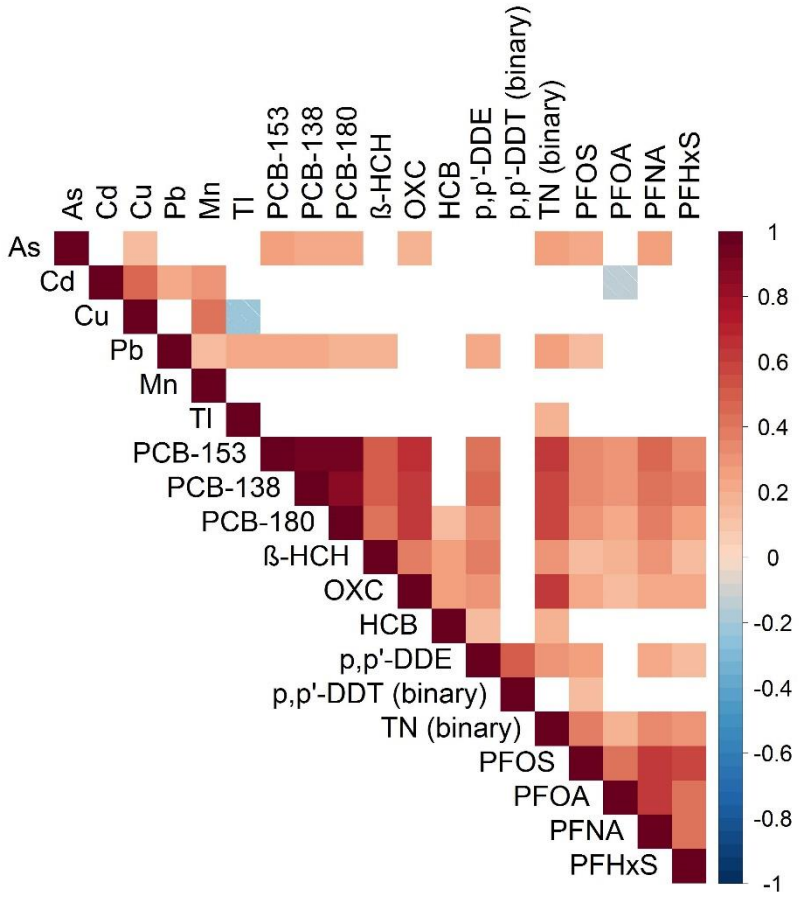

Figure 1: Correlation map of the biomarkers of environmental pollutants (log10). Only significant ($p<0.05$) correlations are represented in the plot, insignificant correlations were left blank. Abbreviations: PCB= polychlorinated biphenyl; β-HCH= β-hexachlorocyclohexane, p,p'-DDE= dichlorodiphenyldichloroethylene; p,p'-DDT= dichlorodiphenyltrichloroethane; PFBS= perfluorobutanesulfonic acid; PFHxS= perfluorohexanesulfonic acid; PFNA= perfluorononanoic acid; PFOA= perfluorooctanoic acid; PFOS= perfluorooctanesulfonic acid

Multipollutant model for placental mtDNA content

Selection of the exposure biomarkers by LASSO regression analysis was applied for the placental mtDNA content without penalization of the following covariates: newborn's sex, gestational age, maternal age, pre-pregnancy BMI, parity, education level, smoking during pregnancy and season of birth. Cross-validation selected the λ with the lowest MSE and the highest λ within one SE of the minimum MSE (Figure 2A, respectively indicated by the full red line and dashed red line). For highest λ within 1 SE of the minimum MSE (dashed red line in Figure 2B), OXC, β-HCH, p,p'-DDE, PFNA, As, Cd and Tl, were selected as important predictors for placental mtDNA content, with non-zero coefficients.

We used an OLS regression model to estimate adjusted regression coefficients for the selected pollutants, taking into account the possible confounders as well as the other pollutants selected by LASSO (Table 3). The VIFs (Supplemental table S2) ranged from 1.05 to 2.19, indicating that the correlation between the variables was moderate and did not bias the regression coefficients. In this multi-pollutant model, cord blood levels of β-HCH, As and Tl were significantly associated with placental mtDNA content (Table 3).

An increment of 25% in the mean cord blood As and β-HCH levels were respectively associated with a 1.41% higher (95% CI: 0.07, 2.77) placental mtDNA content (p=0.04), and a 2.71% higher (95% CI: 0.25, 5.23) placental mtDNA content (p=0.03). The estimated effect of 25% higher cord blood Tl levels corresponded to 4.88% lower (95% CI: -9.09, -048) placental mtDNA content (p=0.03). Sensitivity analyses in which we additionally adjusted for the reported consumption of seafood and fish (Supplemental table S3) provided similar results.

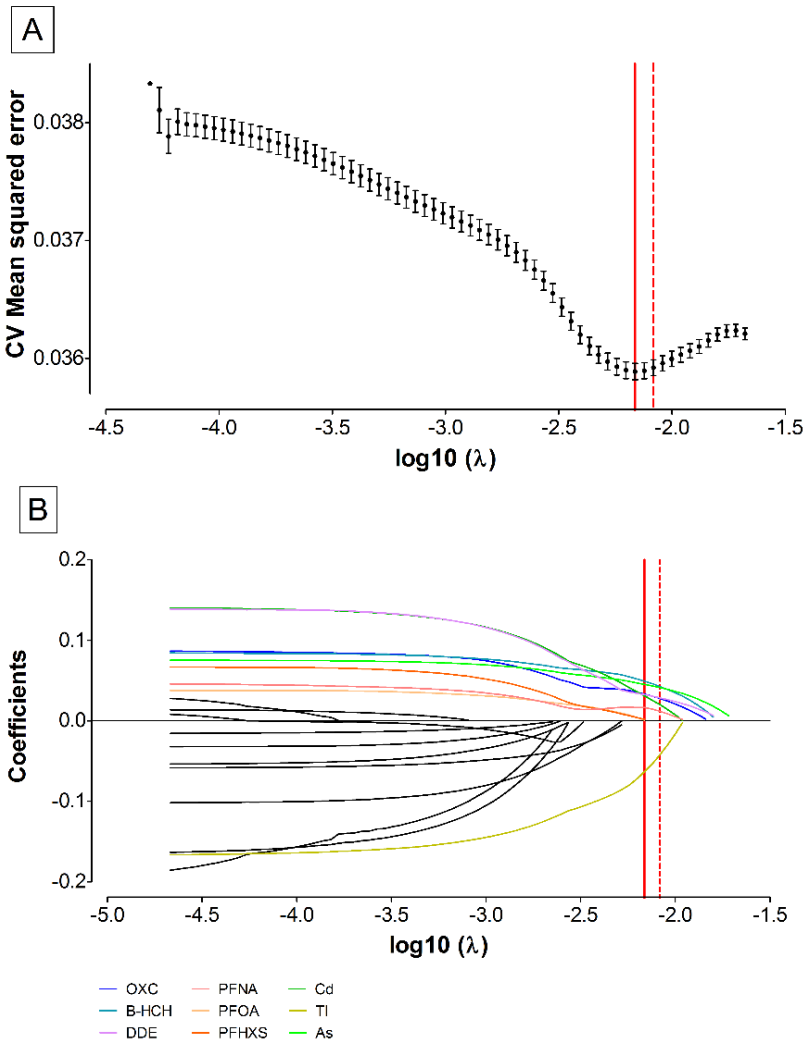

Figure 2: [A] The prediction error of the LASSO regression model in function of the penalty parameter (log10 λ). [B] The LASSO solution path, with the coefficient profiles for biomarkers of environmental pollution as a function of the penalty parameter (log10 λ). Increasing values for λ, pose a more stringent penalty on the regression coefficients, shrinking more coefficients to zero. The horizontal red line depicts the cross-validated optimum of λ (minimum MSE), the dashed red line depicts the highest value of λ where the MSE was within one standard error (SE) of the minimum MSE. To improve the comprehensibility of the graph, forced variables were not included in the plot. OXC, β-HCH, p,p'-DDE, PFNA, As, Cd and Tl were selected at the λ indicated by the dashed line (highest value of λ where the MSE was within one standard error (SE) of the minimum MSE) and PFOA and PFHxS were additionally selected λ indicated by the red line (minimum MSE).

Of the covariates accounted for in the model, gestational age, maternal pre-pregnancy BMI, maternal smoking, and season of birth were significant predictors of placental mtDNA content. A 1-week increase in gestational age was associated with a 4.69% decrease (95% CI: -8.75, -0.45; p=0.03) in placental mtDNA content. 1 kg/m² higher maternal pre-pregnancy BMI was associated with 1.52% higher (95% CI: 0.11, 2.95; p=0.03) placental mtDNA content. Placental mtDNA content of non-smoking mothers was 24.71% (95% CI: 3.07, 50.90; p=0.02) higher than placental mtDNA content of mothers who reported to smoke during pregnancy.

Table 3: The estimated effects of environmental pollutants on placental mtDNA content

Pollutant	Estimated effect (95% CI)	p-value
Oxychlordane	1.03 (-1.29, 3.41)	0.39
β-hexachlorocyclohexane	2.71 (0.25, 5.23)	0.0307
p,p'-DDE	0.99 (-0.80, 2.80)	0.28
Arsenic	1.41 (0.07, 2.77)	0.0389
Cadmium	2.51 (-0.64, 5.75)	0.12
Thallium	-4.88 (-9.09, -0.48)	0.0303
Perfluorononanoic acid	1.07 (-0.94, 3.12)	0.30

The estimated effects of the pollutants were determined using a multiple OLS regression model. The estimated effects are represented as a percent (%) change in placental mtDNA content for a 25% increment in mean concentration of the environmental pollutants in cord blood. The model was adjusted for sex, gestational age, season of birth, maternal age, maternal pre-pregnancy BMI, smoking during pregnancy, parity and education. Gestational age, maternal pre-pregnancy BMI, smoking during pregnancy and season of birth were significantly associated with placental mtDNA content in our model.

DISCUSSION

We studied cord blood biomarkers of exposure to toxic metals, organochlorines and perfluoroalkyl compounds and their relation with placental mtDNA content. We applied LASSO regression, a multipollutant penalized regression approach, to study independent effects of environmental exposures in association to placental mtDNA content of newborns. Cord blood OXC, β-HCH, p,p'-DDE, PFNA, As, Cd and Tl were independently selected, and cord blood levels of β-HCH, As and Tl were significantly associated to mtDNA in a multipollutant unpenalized regression model.

We identified mainly positive correlations between the exposure levels within metals, organochlorine and perfluoroalkyl compounds. Exposure to organochlorines and perfluoroalkyl compounds in our newborns tended to cluster and these exposures may therefore occur simultaneously. Only between Tl and Cu and between Cd and PFOA we observed inverse correlations.

mtDNA content is considered as a marker of oxidative stress and mitochondrial function. Variation in the mitochondrial abundance, mtDNA content and expression of respiratory genes are associated with alteration in intracellular ROS [28]. It is suggested that the increase in mtDNA content with increasing ROS, is a compensatory mechanism, as mitochondrial function might be impaired or mtDNA might be damaged [9,29,30]. Persistent or more severe oxidative stress is suggested to alter the rate of the mtDNA replication, contributing to a decrease in mtDNA content and function [28,31]. Yet, it remains unclear how the mtDNA content is regulated under oxidative stress-responses of different environmental stimuli. Up to now, studies in adults reported higher mtDNA content in relation to exposure to pesticides [32], higher mtDNA content in an occupational exposed group to polycyclic aromatic hydrocarbons (PAH) [33] and lower mtDNA content in association with PAH in house dust [33,34], higher mtDNA content with benzene exposure [35,36] and lower mtDNA content in association with particulate matter [37,38] as well as higher mtDNA content [39]. In newborns, lower placental [13,14] and lower cord blood [40] mtDNA content have been reported in relation to prenatal ambient air pollution. From the available studies, it becomes clear that the direction of mtDNA content alterations can vary for different exposures, but also discrepancies in the findings for one exposure (for PAH and PM) can be observed. This is also observed by

studies investigating mtDNA content in relation to As exposure [41-44]. It is also suggested that responses in the mtDNA content can vary according to the dose and time-window of the exposure [37]. We found a positive association between As and β-HCH and mtDNA content, and an inverse association with Tl and mtDNA content. All three pollutants have been described to interfere with the oxidative balance and/or mitochondrial function, as exemplified by studies listed in Table S4. We hypothesize that differences in the mitochondrial toxicity mechanism, the dose and time-window of the exposures may account for the inverse association with Tl, compared to a positive association for As and β-HCH. Yet, future studies are needed to elucidate discrepant findings on mtDNA content for different environmental pollutants.

Since the placenta is a highly metabolically active organ that enables active transport mechanisms, it has a high energy demand [45]. The functioning of the placenta is important for fetal growth and development as it ensures transport of nutrients, hormones, gases and waste products between the mother and the fetus. Therefore, defective functioning of the placenta is detrimental for fetal health and implemented in many pregnancy disorders [46,47]. β-HCH [6,48,49], As [50,51] and Tl [52] are among the pollutants that are reported to cross the placenta, and previous studies highlighted adverse effects in newborns associated with exposure to these pollutants. Maternal exposure to As during pregnancy is associated with an increased risk for congenital heart anomalies [53], for birth defects [54], adverse effects on fetal growth [55-57] and the fetal immune system [58]. High maternal Tl and β-HCH exposure were associated with an increased risk for low birth weight [49,59-61]. Also, mothers of preterm born neonates had higher exposure levels of β-HCH, compared to controls [62]. Disturbance of the oxidative balance is involved in a variety of placenta-related pregnancy complications and birth outcomes [63], is hypothesized to play an important role in the mechanism of action of many pollutants and is a key feature in the regulation of mtDNA content. The mechanisms for adverse fetal outcomes in relation to exposure to these pollutants are still unknown. Our study provides new insights in the possible molecular mechanism underlying the adverse birth effects associated with exposure to these pollutants. Future studies may elucidate the mechanism underlying our observation, and focus on molecular mechanisms underlying environmental pollution effects on placental function as changes in mtDNA content might be a

biological mechanism involved in exposure-related health outcomes [64]. Previously, placental mtDNA content was significantly associated with birth weight [14]. However, in our population we only observed a univariate association (p=0.04), which lost significance after controlling for gestational age (p=0.22). Furthermore, future studies should examine mtDNA content in association with combined exposures in older study populations. Several studies showed positive associations between mtDNA and telomere length [65,66], early changes in mtDNA content may lead to different aging phenotypes later in life.

Inhalation of particles containing these elements and consumption of food and drinking water are important routes of exposure for As and Tl. As is naturally occurring in soil and is present in water and air. Coal-burning and smelting processes are important sources of Tl, which is present in air and soil [67,68]. The use of HCH as an insecticide is prohibited, however, the compound (as well as other POPs) persist in the environment and contaminates soils [69], crops grown on contaminated soil can take up these elements by the roots. Since β-HCH accumulates in fatty tissues and it is slowly metabolized, cord blood levels reflect the *in utero* period, serum levels of this pollutant have an elimination half-life of 7 years and as such also might reflect maternal long term exposure [70]. On the other hand, cord blood As and Tl have an elimination half-life of 2 to 4 days and reflect recent exposure. Nevertheless, if the exposure to these pollutants occurs regularly, instead of intermittent, the levels might reflect the exposure on a longer term. Compared to biomonitoring programs or studies in other countries, the pollutant levels of the present study are lower or comparable in magnitude (as summarized in Table S5). Compared to the 2008-2009 biomonitoring campaign [71], Tl and As were slightly higher in the present samples of 2014. Arsenic levels were comparable in magnitude to the levels reported by Rahbar *et al.* [72] and Wang *et al.* [73]. Compared to a Chinese population-based study, the cord blood levels for As and Tl were lower in our study [74]. β-HCH levels in cord blood were lower compared to studies in France [75], China [76], Slovakia [77], Spain [78] and Mexico [79] and comparable to a study in Korea [80]. Despite the low concentrations of β-HCH, Tl and As in our population, we were able to identify associations with placental mtDNA content, suggesting that mitochondria are sensitive to low exposure levels. This emphasizes the applicability of the mitochondria to function as a

environmental sensor for revealing early molecular signs, even at low exposure levels.

This study has some limitations. Our study sampled a biopsy from the fetal side of the placenta, which contains different cell types including cytotrophoblasts and syncytiotrophoblasts. We have no information on the distribution of these cells in our biopsy and were not able to take this into account. Future studies are needed to investigate mtDNA content (responses) of placental cell types. Although we accounted for possible confounding factors, we cannot exclude that the effects of the pollutants on placenta mtDNA content are possibly affected by residual confounding. Furthermore, we had observations with undetectable levels of some pollutants for which we assigned values using a single imputation technique.

The use of penalized regression to select the relevant pollutants is highly relevant within the exposome concept to identify important exposures [81-83] and is a major strength of the study. Yet, the studies using penalized regression to consider the relevant pollutants are limited [25,84-86]. This allows collinearity of multiple exposures entered in one model, in contrary to least squares regression which is sensitive to collinearity. LASSO penalized regression will shrink certain pollutants to zero, so only a subset of the pollutants will contribute to our outcome. However, LASSO regression will select independent pollutants that are most predictive of the outcome, while possibly also others that are correlated to this selected pollutant might be predictors of the outcome. Elastic net regression can select sets of collinear exposures but accordingly is less sparse. We opt for a more sparse model with fewer non-zero pollutants to screen for the most relevant exposures, for which LASSO regression with stronger sparsity was more appropriate compared to elastic net. Hence, it cannot be excluded that exposures were not selected because of a correlated exposure. As a consequence, LASSO might as well select false discoveries, particularly early on the LASSO path. A simulation study indicated that compared to other techniques for assessing the effects of multiple pollutants, LASSO regression is less affected by false discoveries [82] and may be suited for a screening study as ours. We quantified the environmental pollutants in cord blood, since this reflects more accurately the exposure of the newborns during gestation, compared to quantifications in maternal blood. Yet, there are only few studies reporting the concentrations of environmental pollutants in cord

blood. A specific strength in this study is that we can compare the current pollutant levels with the concentrations measured in previous FLEHS biomonitoring programs [87-89], in which a decline of environmental pollutants in newborns in Flanders, among which PCBs, HCB, p,p'-DDE, Pb, Cd, PFOS and PFOA, over time is reported [90]. Even with low exposure levels, we identified significant associations with mtDNA.

CONCLUSION

We show in a multi-pollutant context, independent effects of cord blood As, Tl and β-HCH on placental mitochondrial DNA content, pointing towards the important role of mitochondria as target of multiple pollutants at low fetal concentrations.

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SUPPLEMENTAL INFORMATION

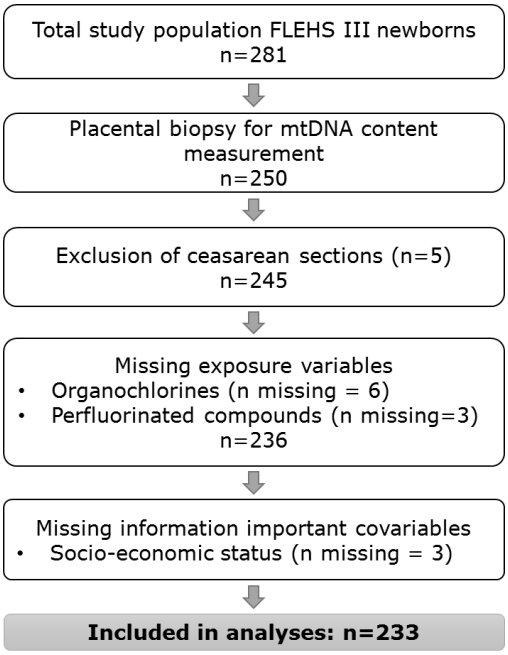

Figure S1: Flowchart illustrating the selection procedure of study participants from the FLEHS III birth cohort for the present study (n=233).

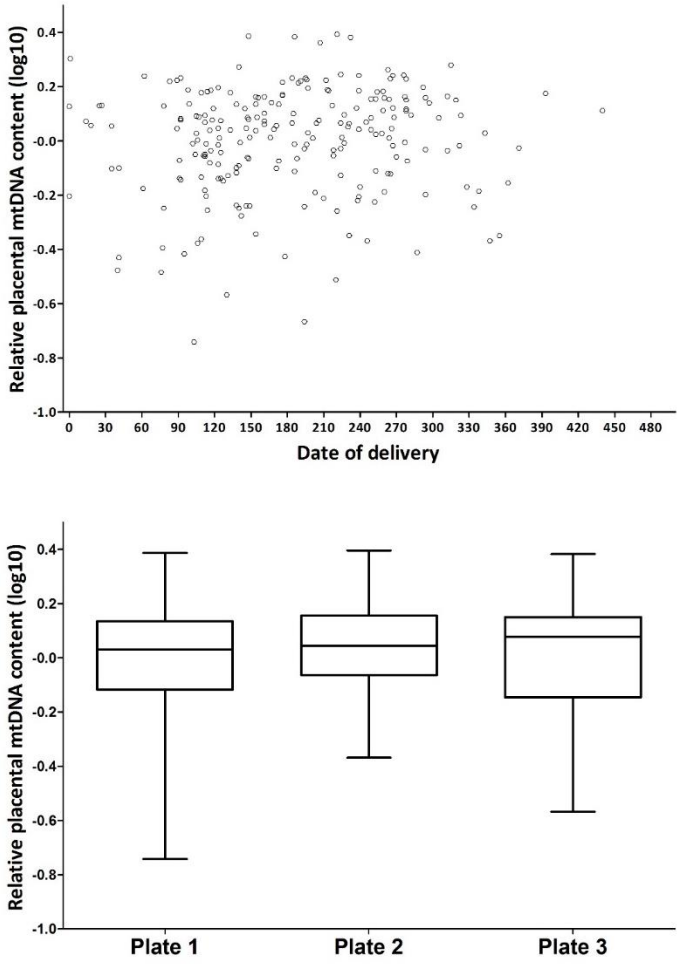

Figure S2: Quality control of mtDNA content to assess the influence of storage time and plate effects on the mtDNA content. The upper figure shows the placental mtDNA content in function of the time, which is represented by the date of delivery with 0 being the date of the oldest sample and 30 being 30 days later than the oldest sample until to 440 days later when the last sample was collected. There is no correlation or pattern visible in this plot, indicating the mtDNA content measurement is not influenced by the storage time. The lower figure shows the distribution (max, Q1, median, Q3 and min) of the mtDNA content by the plates on which they are measured with qPCR. There is no difference in the mtDNA content associated with the plates on which they're measured, indicating no batch effects.

Table S1: Exposure levels by tertiles of placental mtDNA content

	T1 mtDNA content	T2 mtDNA content	T3 mtDNA content
As (µg/L)	0.513 (0.677)	0.794 (0.963)	0.741 (1.10)
Cd (µg/L)	0.020 (0.0081)	0.019 (0.0107)	0.022 (0.0099)
Cu (µg/L)	562 (97.8)	537 (156)	575 (89.7)
Mn (µg/L)	30.9 (13.5)	27.5 (14.1)	30.9 (15.6)
Pb (µg/L)	6.46 (3.16)	6.17 (4.44)	6.61 (3.68)
Tl (µg/L)	18.6 (6.22)	18.2 (6.28)	18.6 (7.59)
PCB138 (ng/g fat)	10.0 (9.82)	10.2 (6.60)	11.2 (11.3)
PCB153 (ng/g fat)	16.2 (12.2)	16.2 (12.2)	17.4 (16.8)
PCB180 (ng/g fat)	8.32 (7.47)	8.32 (5.97)	9.12 (8.87)
β-HCH (ng/g fat)	3.24 (3.96)	3.98 (3.42)	3.63 (2.80)
p,p'-DDE (ng/g fat)	55.0 (53.3)	61.7 (51.6)	66.1 (48.5)
HCB (ng/g fat)	10.5 (14.2)	11.2 (14.1)	11.5 (12.7)
OXC (ng/g fat)	1.26 (1.15)	1.38 (1.21)	1.48 (1.39)
PFHxS (µg/L)	0.324 (0.28)	0.331 (0.27)	0.398 (0.33)
PFNA (µg/L)	0.178 (0.16)	4.79 (0.19)	0.224 (0.16)
PFOA (µg/L)	1.12 (0.65)	1.23 (0.78)	1.26 (0.66)
PFOS (µg/L)	1.07 (0.92)	1.12 (0.82)	1.20 (0.97)
p,p'-DDT, binary	14 (19%)	15 (19%)	13 (17%)
TN, binary	33 (43%)	34 (44%)	38 (49%)

The exposure levels are represented as geometric mean (IQR) a: binary exposures (0= below LOD, 1= above LOD) are represented as the number (%) above the LOD. T1= first tertile, T2= second tertile, T3= third tertile.

Table S2: The variance inflation factors (VIF) of the regressors in the ordinary least squares regression model

	Variance inflation factor
Newborn's sex	1.047
Gestational age	1.066
Parity	1.647
Maternal age	2.189
Pre-pregnancy BMI	1.240
Smoking during pregnancy	1.236
Season of birth	1.476
Education	1.540
Oxychlordane	1.512
B-HCH	1.907
P,p-DDE	1.246
Arsenic	1.275
Cadmium	1.133
Thallium	1.118
PFNA	1.312

The multiple ordinary least squares regression model includes the pollutants selected by LASSO when using λ with a MSE within one SE of the minimum MSE and the *a priori* selected covariates.

Table S3: Sensitivity analyses

Pollutant	Estimated effect (95% CI)	p-value
Oxychlordane		
Unadjusted	1.38 (-0.90, 3.71)	0.24
Model 1	0.47 (-1.81, 2.80)	0.69
Model 2	0.91 (-1.48, 3.36)	0.46
Model 3	1.09 (-1.30, 3.54)	0.38
Model 4	0.99 (-1.41, 3.45)	0.42
β-hexachlorocyclohexane		
Unadjusted	0.86 (-1.31, 3.09)	0.44
Model 1	2.56 (0.29, 4.89)	0.027
Model 2	2.73 (0.22, 5.31)	0.033
Model 3	2.84 (0.31, 5.44)	0.028
Model 4	2.77 (0.19, 5.41)	0.035
p,p'-DDE		
Unadjusted	1.16 (-0.73, 3.07)	0.23
Model 1	0.99 (-0.84, 2.85)	0.29
Model 2	1.05 (-0.78, 2.92)	0.26
Model 3	0.82 (-1.02, 2.69)	0.38
Model 4	1.06 (-0.78, 2.92)	0.26
Arsenic		
Unadjusted	1.20 (-0.13, 2.55)	0.08
Model 1	1.02 (-0.27, 2.31)	0.12
Model 2	1.13 (-0.17, 2.44)	0.09
Model 3	1.31 (-0.06, 2.69)	0.06
Model 4	1.45 (0.04, 2.87)	0.044
Cadmium		
Unadjusted	0.94 (-2.20, 4.19)	0.56
Model 1	2.01 (-1.19, 5.32)	0.22
Model 2	2.59 (-0.65, 5.93)	0.12
Model 3	2.17 (-1.08, 5.53)	0.19
Model 4	2.61 (-0.67, 5.99)	0.12
Thallium		
Unadjusted	-2.57 (-6.96, 2.02)	0.27
Model 1	-3.97 (-8.18, 0.43)	0.08
Model 2	-4.93 (-9.17, -0.49)	0.03
Model 3	-4.87 (-9.10, -0.45)	0.032
Model 4	-4.85 (-9.12, -0.39)	0.034
Perfluorononanoic acid		
Unadjusted	0.26 (-1.78, 2.35)	0.80
Model 1	0.91 (-1.10, 2.95)	0.38
Model 2	1.09 (-0.95, 3.17)	0.30
Model 3	1.07 (-1.01, 3.19)	0.31
Model 4	1.05 (-1.02, 3.16)	0.32

Unadjusted model contain only the selected exposure variables. Model 1 = adjusted for the newborn-related covariates: sex, gestational age and season of birth; model 2= adjusted for newborn-related and maternal covariates and maternal age, parity and maternal pre-pregnancy BMI; model 3 = all covariates of the main model + seafood consumption, model 4= all covariates of the main model + fish consumption. The estimated effects of the pollutants were determined using a multiple OLS regression model. The estimated effects are represented as a percent (%) change in placental mtDNA content for a 25% increment in mean concentration of the environmental pollutants in cord blood.

Table S4: Studies linking exposure to β-HCH, As and Tl to disturbance of the oxidative balance or altered mitochondrial function

	Type of study	Reference
β-hexachlorocylcohexane		
Increased ROS production and apoptosis	In vitro study	[1]
Increased expression of inflammatory genes	Observational study	[2]
Polymorphism in xenobiotic-metabolizing genes modifies associations with birth weight	Observational study	[3]
Mitochondrial function	Experimental animal study	[4,5]
General, toxicity mechanism	Review	[6]
Arsenic		
Mitochondrial oxidative stress & dysfunction	Review *	[7]
DNA damage and disruption of repair mechanisms	Review *	[8]
Decreased mtDNA content & altered mitochondrial function	In vitro study	[9]
Decreased mtDNA content & altered mitochondrial function	In vitro study	[10]
Increased mtDNA content	Case control study + in vitro study	[11]
Increased mtDNA content, particularly in subjects with less efficient As metabolism	Observational study	[12]
Thallium		
Increased hydrogen peroxide formation	In vitro study	[13]
Increased oxidative DNA damage	Observational study	[14]
Disturbance of the oxidative balance	Experimental animal study	[15,16]
Mitochondrial swelling	In vitro	[17-22]
Alterations in mitochondrial morphology	Experimental animal study	[23]
General, toxicity mechanism	Review	[24]

* There is extensive evidence for involvement of oxidative stress and targeting the mitochondria in the toxicity mechanism of arsenic, we therefore included solely reviews on this topic.

Table S5: Cord blood levels of arsenic, thallium and β-hexachlorocyclohexane in other studies

Country	Measure of central tendency	Reported values	Ref
β-Hexachlorocyclohexane			
Belgium [a]	Mean ± SD	4.55 ± 3.81 ng/g fat	/
France	Median, maximum	11.27 ng/g fat, 108.3 ng/g fat	[25]
China	Median, maximum	35.29 ng/g fat, 261.3 ng/g fat	[26]
Slovakia	Mean ± SD	11.3 ± 10.4 ng/g fat	[27]
Spain	Geometric mean (95% CI)	20 ng/g fat (17 - 23)	[28]
Mexico	Mean ± SD	28 ± 5.4 mg/kg fat	[29]
Korea	Median (IQR)	7.5 ng/g fat (5.3 – 10)	[30]
Arsenic			
Belgium [a]	Mean ± SD	1.19 ± 1.83 µg/l	/
Belgium [b]	Geometric mean (95% CI)	0.56 µg/l (0.49 – 0.65)	[31]
Jamaica	Mean ± SD	1.0 ± 0.9 µg/l	[32]
China	Median	2.84 µg/l	[33]
China	Mean ± SD	7.21 ± 5.73 µg/l	[34]
Thallium			
Belgium [a]	Mean ± SD	0.019 ± 0.0058 µg/l	/
Belgium [b]	Geometric mean (95% CI)	0.017 µg/l (0.016 – 0.018)	[31]
China	Mean ± SD	0.41 ± 0.33 µg/l	[34]

a: Present study, FLEHS biomonitoring 2014; b: FLEHS biomonitoring 2008-2009

Table S6: Supporting information with the LASSO path

	log10 λ	**Order of appearance**
Arsenic	-1.719	1
Cadmium	-1.961	4
Copper	-2.567	8
Manganese	-2.607	9
Lead	-2.284	6
Thallium	-1.961	4
PCB138	-3.739	12
PCB153	-2.648	10
PCB180	-2.486	7
β-HCH	-1.799	2
p,p'-DDE	-1.799	2
p,p'-DDT	-2.284	6
Hexachlorobenzene	-2.648	10
Oxychlordane	-1.840	3
Transnonachlor	-3.092	11
PFHxS	-2.163	5
PFNA	-1.961	4
PFOA	-2.163	5
PFOS	-2.567	8

We provided the penalization parameter log10 λ to indicate at which point in the LASSO path the estimate for the pollutant will be > 0 and the pollutant will thus enter the model. The order of appearance indicates the order of entering the model, showing that arsenic entered the model first and transnonachlor was the last pollutant to enter the model.

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Chapter 4

Exposure to environmental pollutants and their association with biomarkers of aging: a multipollutant approach

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ABSTRACT

Background: Various environmental factors contribute to the development of age-related disorders, such as cardiovascular diseases and cancer. Mitochondrial DNA (mtDNA) content and telomere length are putative aging markers and are sensitive to environmental stressors, including pollutants, and may be on the path linking exposure to adverse health outcomes. Our objective was to identify, from a set of environmental exposures, which exposure is associated to leukocyte mtDNA content and telomere length in adults.

Methods: This study includes 175 adults from 50 to 65 years old from the cross-sectional Flemish Environment and Health study (FLEHS III 2012-2015), of whom leukocyte telomere length and mtDNA content were determined using qPCR. The levels of exposure of metals (Sb, As, Cd, Cu, Hg, Ni, Tl), organohalogens (PCB-138, PCB-153, PCB-180, HCB, β-HCH, OXC, CN, TN, p,p'-DDE, p,p'-DDT, BDE-153) and perfluorinated compounds (PFHxS, PFNA, PFOA, PFOS) were measured. We investigated leukocyte mtDNA content and telomere length in association with these environmental pollutants by performing sparse partial least squares regression analyses to assess the multipollutant associations and by conducting ordinary least squares regression to evaluate single pollutant associations.

Results: We included biomarkers of exposure that were detectable in at least 85% of the study population and showed a specific correlation structure. While accounting for possible confounders and (confounding) co-exposures, we identified that urinary Cd, serum HCB and PFOS exposure were positively associated with mtDNA content, while urinary Cu and serum PFHxS exposure were inversely associated with mtDNA content. Urinary Sb and Hg exposure were positively associated with leukocyte telomere length, while urinary Cu and serum PFOA showed an inverse association.

Conclusion: Our findings support the hypothesis that environmental pollutants interact with molecular hallmarks of aging.

BACKGROUND

Throughout their lifespan, humans are exposed to a wide range of environmental pollutants, including metals, persistent organic pollutants and many other contaminants and often correlations exist among these exposures [1]. As such, correlated co-exposures might confound single pollutant associations, which can be precluded by studying multipollutant associations that evaluate a larger set of environmental pollutants at the same time [2-4].

Telomeres are ribonucleoprotein structures, consisting of TTAGGG tandem repeats interacting with proteins to form a nucleoprotein cap at the end of chromosomes [5]. During DNA replication, the distal ends of chromosomes on lagging strands cannot be fully replicated, which is known as the end-replication problem, resulting in a shortening of telomeres with each cell division [6]. Telomere length represents therefore the replicative capacity of cells. Consequently, telomere length is linked to longevity and age-related diseases, such as cardiovascular disorders and cancer, and is regarded as a marker of biological aging and disease-susceptibility [7,8].

Mitochondria are organelles pivotal for cellular energy, yet on this account they also contribute to a great extent of endogenous production of reactive oxygen species (ROS) in the cell. The mitochondrial DNA (mtDNA) lacks several protective measures, such as repair systems or histones, making it particularly vulnerable to oxidative mtDNA damage [9]. mtDNA damage may affect mitochondrial function and mtDNA copy number can be indicative hereof [10]. During aging, mitochondrial function and mtDNA integrity decline [11], possibly caused by ROS [12]. Aging is associated with an accumulation of mtDNA damage, and is linked with alterations in the mtDNA content [11].

Telomere length and mtDNA content are considered biomarkers of biological aging and are linked to age-related diseases [13]. Furthermore, both have been implemented in molecular epidemiology as cellular sensors for environmental stressors on the basis of their sensitivity to oxidative DNA damage and inflammation [14,15]. Exposure to some environmental pollutants can be associated with the (premature) onset of age-related diseases. However, molecular markers of biological aging may be affected prior to the manifestation of such diseases and

can be involved in the mechanism underlying exposure-related health outcomes. Nevertheless, the impact of exposure to multiple pollutants on mtDNA content and telomere length remains unknown. We have investigated a set of biomarkers of environmental exposures, which can be detrimental for human health, and their association with biomarkers of biological aging using a multipollutant approach, to separate the possible effects of multiple pollutants, while accounting for confounding co-exposures.

METHODS

Study population

The study was part of the third cycle of the Flemish Environment and Health Study (FLEHS), which was set up for biomonitoring environmental exposure and health in Flanders, the Northern part of Belgium. General criteria for inclusion in the study were 1) to be 50 to 65 years old 2) living at least 10 years in Flanders 3) not having active cancer treatment or renal pathologies and 4) being able to fill out an extensive questionnaire in Dutch. In total, 1369 eligible subjects were invited through an information letter by the general practice of whom 18.1% consented to participate and 209 were included. Written informed consent was provided before start of the fieldwork. Proportional to the population number of each province, the number of subjects was geographically spread throughout Flanders [16]. The study enrolled 209 subjects from May 2014 to November 2014. The study was approved by the ethical committee of the University hospital of Antwerp.

Self-administered questionnaires obtained information on socio-economic status, lifestyle and medication use. Smoking habits were defined as never smoker, former smoker or current smoker. Socio-economic status was assessed based on the highest household educational level, and was coded as "none" (primary school, no diploma), "low" (early grades of secondary school), "middle" (secondary school) or "high" (college or university degree). Ethnicity was based on the native country of the subject and classified as Belgian (native country Belgium), European ancestry or non-European ancestry. Self-reported medication use was used to identify the use of anti-hypertension, hypercholesterolemia/dyslipidaemia or diabetes treatments. Blood pressure was measured during the study protocol

and HDL and triglyceride levels were quantified on collected blood samples. Hypertension was defined by the presence of elevated blood pressure (systolic blood pressure ≥ 140 mmHg or a diastolic blood pressure ≥90 mmHg) or on anti-hypertensive medication. Reduced HDL levels were defined as HDL <40 mg/dl for men and HDL <45 mg/dl for women [17]. Elevated triglyceride levels were defined as a triglyceride level >200 mg/dl [18]. Although lipid profiles were derived from non-fasting samples, the quantifications were valuable and provide information on the health status [19]. Information on the health status of the participants is provided as supplemental information (see Supplementary table 1).

After excluding 26 subjects without the outcome variables and 7 subjects with incomplete data on covariates, the main analysis was conducted on a total of 175 subjects (Figure 1).

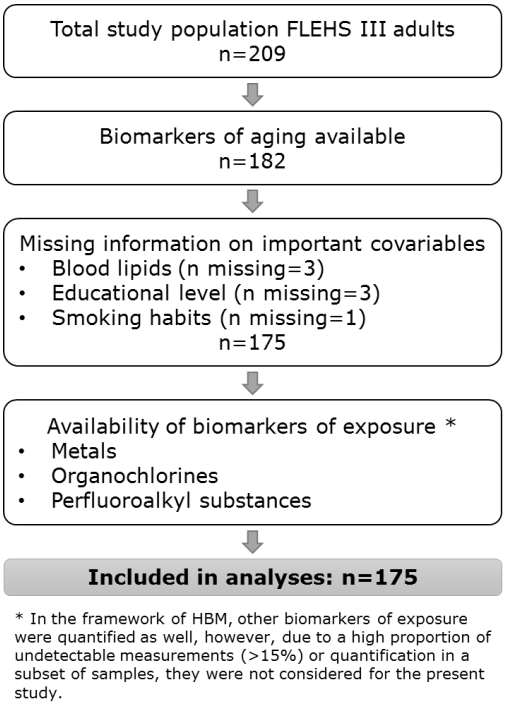

Figure 1: Flowchart illustrating the selection procedure of study participants from the FLEHS III cohort for the present study (n=175). HBM= human biomonitoring

Biomarkers of environmental exposure

Blood and urine samples needed for quantification of the biomarkers of exposure were collected during the study visit. Blood samples were immediately centrifuged after sampling for serum collection, and transported on dry ice and frozen within 24 hours. Urine was collected using a polyethylene container, which was free of metals and phthalates, and was frozen within 24 hours after collection.

Metals except Hg were quantified in urine by high resolution inductively coupled plasma-mass spectrometry (HR-ICP-MS), as described by Baeyens *et al*. [20] and included arsenic (As), cadmium (Cd), copper (Cu), chromium (Cr), nickel (Ni), thallium (Tl) and antimony (Sb). Mercury (Hg) was quantified by cold vapor atomic fluorescence spectroscopy (CV-AFS) as described by De Craemer *et al.* [21]. The limits of detection (LOD) for the metals were as follows: 0.173 µg/L for As, 0.018 µg/L for Cd, 0.655 µg/L for Cu, 0.199 µg/L for Cr, 0.022 µg/L for Hg, 0.262 µg/L for Ni, 0.014 µg/L for Tl and 0.012 µg/L for Sb. To correct for urinary concentration, metal concentrations normalized to the urinary gravity were calculated using the following formula: metal concentration[(1.024-1)/(specific gravity-1)]. Organohalogens were measured in blood serum samples by means of solid-phase extraction and gas chromatography- electron capture negative ionisation mass spectrometry, as described by Covaci *et al.* [22]. The quantified organohalogens included various polychlorinated biphenyls (PCBs; PCB-138, PCB-153 and PCB-180), oxychlordane (OXC), trans-nonachlor (TN), cis-nonachlor (CN), hexachlorobenzene (HCB), dichlorodiphenyldichloroethylene (p,p'-DDE), dichlorodiphenyltrochloroethane (p,p'-DDT), lindane (γ-HCH), β-hexachlorocyclohexane (β-HCH) and various polybrominated diphenyl ethers (BDEs; BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154 and BDE-183). The limits of quantification (LOQ) for the organochlorines were the following: 2 ng/L for PCBs, OXC, TN, CN and BDEs, 10 ng/L for HCB and p,p'-DDT, 20 ng/L for p,p'-DDE and 5 ng/L for β-HCH and γ-HCH. Organohalogen concentrations were normalized to the serum lipid concentrations [23]. Perfluorinated compounds were quantified in blood serum samples by HPLC with MS-MS detection in negative ionisation mode, as described by Midasch *et al.*, [24] and included perfluorobutanesulfonic acid (PFBS), perfluorohexane sulfonic acid (PFHxS), perfluorononanoic acid (PFNA), perfluorooctanoic acid (PFOA) and perfluorooctane

sulfonic acid (PFOS). The LOQs was 0.2 µg/L for PFOA, PFOS, PFHxS and PFBS and 0.1 µg/L for PFNA.

Handling of observations below detection limits.

Biomarkers of exposure with measurements below LOD or LOQ (depending on the cut-off employed by the measuring laboratory, as indicated above) were substituted with respectively LOD/2 or LOQ/2 and adjusted for urine density or lipids for subsequent statistical analyses. Possibly there might be some bias in this commonly used substitution technique and more accurate alternatives exist [25]. However, in statistical approaches for multipollutant studies [2-4] the use of more advanced techniques of handling undetectable levels is not supported. Therefore, we took into account the Environment Protection Agency (EPA) guideline, stating that a simple substitution method is only valid when not more than 15% of the measurements are undetectable [26]. As such, biomarkers of exposure with more than 15% below detection level were excluded from the analyses (see Supplemental table 2).

Biomarkers of biological aging

DNA was isolated from buffy coat, containing leukocytes, using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. The relative mitochondrial DNA (mtDNA) content and leukocyte telomere length were determined using quantitative PCR (qPCR) as previously described [27,28]. mtDNA content was measured by determining the ratio of two mitochondrial gene copy number (MTF3212/R3319 and MT-ND1) to one single copy nuclear control gene (RPLP0). Relative telomere length was determined by the ratio of the telomere sequence to the RPLP0 single copy nuclear control gene, proportional to the mean telomere length of the study population.

qPCR reactions were carried out in a 10 µl volume on a 384 well plate using the 7900 HT fast real-time PCR system (Applied Biosystems). All reactions were carried out in triplicate and every plate included three no-template controls. Each reaction contained 5 ng DNA input and 1x QuantiTect SYBR Green mastermix (Qiagen). Primer concentrations for the 36b4 assay were 300 nM of the forward and 500 nM of the reverse primers; for the mitochondrial assays 300 nM for both forward and reverse primers; and 900 nM for the telC and 300 nM for the telG

primers in the telomere assay. Primers for the mitochondrial genes [28] and telomeres [29] are reported elsewhere.

Thermal cycling profiles for the 36b4 gene started with an initial step of 10 minutes at 95 °C for activation of the enzyme, followed by 35 cycles of 15 s at 94 °C, 20 s at 62 °C and 100 s at 74 °C. Thermal cycling for the mitochondrial genes started with an initial step of 10 minutes at 95 °C, followed by 40 cycles of 15 s at 95 °C and 70 s at 58 °C. Thermal cycling for the telomere assay started with an initial step at 95 °C for 10 minutes, followed by two cycles of 15 s at 94 °C and 2 minutes at 49 °C, followed by 30 cycles of 15 s at 94 °C, 20 s at 62 °C and 100 s at 74 °C.

Raw qPCR data were evaluated using SDS software (version 2.3; Applied Biosystems). Only triplicates with a quantification cycle difference less than 0.5 were included in the study. qBASE software (Biogazelle) was employed to normalize the qPCR data. The measurements were carried out in two batches, for which we accounted in statistical analyses.

The coefficients of variation (CV) within triplicates of the single-copy gene runs, telomere runs, MTF and ND1 mitochondrial runs were respectively 0.37%, 1.78%, 0.54% and 0.50%. The CV within triplicates for the T/S ratio, MTF/S ratio and ND1/S ratio were as followed: 12.50%, 7.89% and 7.95%.

Statistical analysis

For analyses we used RStudio software (R version 3.3.1, packages: corrplot, sPLS).

Urinary metal concentrations were corrected for urinary density and organohalogens quantified in serum were normalized to the serum lipid content. To improve normality of the data, mtDNA content, relative telomere length and the biomarkers of exposure were $\log_{10}$ transformed. The distribution of the biomarkers of exposure is represented by the geometric means and 25^{th} and 75^{th} percentiles. Participant characteristics are represented as the mean (SD) for continuous variables or as the frequency (number) for categorical variables.

In a first exploratory analysis, we used single pollutants models to evaluate the relation between the selected biomarkers of environmental exposure and the

mtDNA content and relative telomere length separately for each pollutant, using multiple linear regression (ordinary least squares, OLS). We selected *a priori* covariates on the basis of a possible relation to outcomes and exposures. As such, linear regression models were adjusted for age, sex, BMI, smoking habits, household education, ethnicity, as well as thrombocyte count for mtDNA content. The association of biomarkers of aging with these chosen covariates is given in supplement (Table S3). In addition, we took the technical variables on storage time and qPCR plates into account. We corrected for multiple testing by setting the false discovery rate (FDR) below 5% using the Benjamini-Hochberg procedure. The results are presented by means of a Volcano plot, which depicts the effect size on the X axis and the significance (without FDR correction) on the Y axis. The effect estimates with 95% confidence intervals for a 50% increment in the biomarkers of exposure, p-values and FDR-adjusted p-values are provided as supplement (see Supplemental table 3 and 4). In the supplement, results for leukocyte telomere length in which we additionally accounted for lymphocyte percentage are provided by means of a Volcano plot (Figure S5).

In a second step, we used multipollutant models to evaluate the association between the markers of biological aging and all biomarkers of environmental exposures simultaneously. We used sparse partial least squares (sPLS) regression, a supervised dimension reduction technique, to select only relevant exposures out of all exposures. The dimension reduction in (s)PLS is obtained by projecting the predictor variables into a structure of a lower dimensionality, these are the latent factors or components. Latent variables are orthogonally constructed in a way that they reflect a linear combination of the original predictor variables that explains as much variation in the outcome variable as possible. On this account (s)PLS is able to accommodate the collinearity in the exposure dataset. sPLS is able to simultaneously perform dimension reduction and variable selection [30], by imposing L1 regularization during the process of decomposing the predictor variables into latent factors which are predictive for the response. Hereby, of all exposures that are included in the model, only a subset of exposure variables relevant to the outcome is retained. In the sPLS model, two different tuning parameters are defined: the number of latent components in the model (K) and the sparsity tuning parameter (η). For the sPLS modelling we prestandardized the exposures and outcomes by using the residuals of a regression model against

possible confounders and covariables [31,32]. For this, we accounted for the aforementioned variables in the explorative single pollutant analyses. To avoid the excessive contribution of biomarkers of exposure with a high variance, the exposure variables were centred and scaled prior to model fitting. The optimal tuning parameters for the sPLS model were determined using 10-fold cross validation, repeated 500 times. We tested models with K-values between 1 and 5 and η-values between 0.01 and 0.99, in steps of 0.01. For each K, the MSEP was averaged for the grid of η values (results provided in supplemental figure 1). We studied mtDNA content and telomere length separately, however, sPLS also supports a multivariate modelling (results shown in figure S3 and table S6). Multipollutant results for leukocyte telomere length in which we additionally accounted for lymphocyte percentage are provided in the supplement (results in figure S5 and table S7).

We constructed a multipollutant OLS model containing all the exposures selected by the sPLS model, to estimate the effects of the selected exposures on the outcomes. The reasoning behind this model is that it provides the opportunity to interpret the estimates as a percentage change, which is not feasible from the sPLS regression coefficients that lack interpretability since we inputted residuals in the model. Variance inflation factors (VIFs) of the multipollutant OLS models were checked to assess possible multicollinearity.

RESULTS

Study population

The study included 175 adults between 50 and 65 years old, Table 1 describes their demographic characteristics. Briefly, mean (SD) BMI of the participants was 25.8 (4.2) kg/m², with the majority being normal weight, BMI between 18.5 and 25 kg/m² (42.3%) and overweight, BMI between 25 and 30 kg/m² (41.7%). In our population, 12% of the participants were current smokers, while 40.6% were previous smokers. More than half of the participants scored high on household education and almost all participants were born in Belgium.

Table 1: Characteristics of the participants (n=175)

Characteristic	Mean ± SD or n (%)
Sex, male	90 (51.4%)
Age, years	58.3 ± 4.0
Body mass index, kg/m²	25.8 ± 4.2
< 18.5 kg/m²	4 (2.3%)
18.5 – 25 kg/m²	74 (42.3%)
25 – 30 kg/m²	73 (41.7%)
≥ 30 kg/m²	24 (13.7%)
Smoking habits	
Never smoked	83 (47.4%)
Stopped smoking	71 (40.6 %)
Current smoker	21 (12.0%)
Household education	
No diploma	8 (4.6%)
Low	29 (16.6%)
Middle	44 (25.1%)
High	94 (53.7%)
Ethnicity [a]	
Belgium	166 (94.9%)
European	6 (3.4%)
Non-European	3 (1.7%)

[a]: based on the country of birth of the participant

Biomarkers of aging

Figure 2 shows the correlation between mtDNA content and telomere length (Pearson's correlation coefficient=0.41; p<0.0001). The association of biomarkers of aging with important covariables is given in supplement (Table S3).

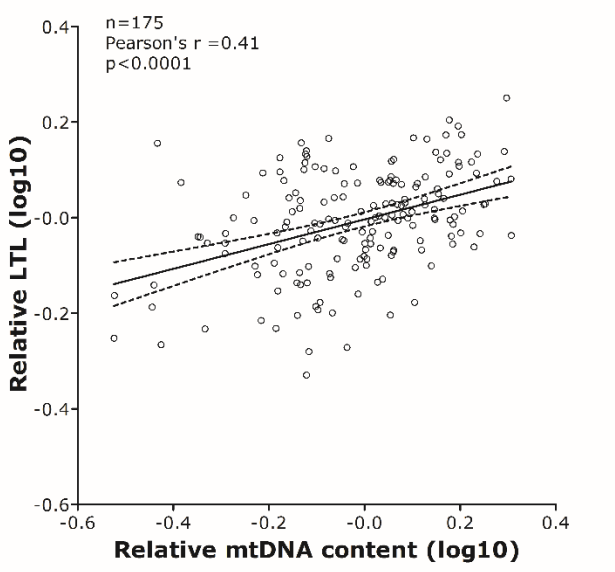

Figure 2: Pearson correlation plot of biomarkers of aging.

Biomarkers of exposure to environmental pollutants

The geometric means (with the 25th and 75th percentile) of the biomarkers of exposure are presented in Table 2 and their correlation structure is visualized in Figure 3. The correlation coefficients among the urinary metals ranged from -0.10 to 0.51. PCBs showed a strong pairwise correlation (correlation coefficients ranging from 0.67 to 0.92) and showed mainly strong correlations with other organohalogens, with correlation coefficients ranging from 0.01 (between PCB-180 and HCB, not significantly correlated) to 0.68 (between PCB-153 and DDE). PCBs showed an inverse correlation with Sb, Cd, Cu and Tl. Organohalogens were correlated with each other (correlation coefficients ranging from -0.17 to 0.92). Serum perfluoroalkyl compounds pairwise correlation coefficients varied between 0.42 and 0.77, yet they were only weakly correlated with other persistent organic pollutants.

Table 2: Description of the biomarkers of environmental exposure measured in the present study (n=175)

Pollutant	Unit	Percentage > LOD/LOQ	Geometric mean	P25	P75
Biomarkers of exposure to toxic metals [b]					
Antimony (Sb)	µg/L [a]	96	0.116	0.072	0.187
Arsenic (As)	µg/L [a]	100	24.1	9.57	50.4
Cadmium (Cd)	µg/L [a]	100	0.541	0.368	0.774
Copper (Cu)	µg/L [a]	100	12.3	9.50	15.3
Mercury (Hg)	µg/L [a]	95.4	0.385	0.212	0.840
Nickel (Ni)	µg/L [a]	89.7	1.29	0.758	2.02
Thallium (Tl)	µg/L [a]	100	0.320	0.256	0.404
Biomarkers of exposure to persistent organic pollutants [b]					
PCB-138	ng/g fat	100	41.0	29.3	61.6
PCB-153	ng/g fat	100	97.1	71.0	139
PCB-180	ng/g fat	100	87.0	61.9	119
Hexachlorobenzene (HCB)	ng/g fat	97.1	13.4	7.98	25.5
β-hexachlorocyclohexane (B-HCH)	ng/g fat	99.4	8.00	4.69	15.5
Oxychlordane (OXC)	ng/g fat	100	7.11	5.26	9.97
Cis-nonachlor (CN)	ng/g fat	96	1.05	0.730	1.64
Trans-nonachlor (TN)	ng/g fat	100	6.07	4.55	8.00
p,p'-dichlorodiphenyldichloroethylene (p,p'-DDE)	ng/g fat	100	216	126	379
p,p'-dichlorodiphenyltrichloroethane (p,p'-DDT)	ng/g fat	93.1	4.70	2.49	7.31
Brominated diphenyl ether-153 (BDE-153)	ng/g fat	100	1.08	0.730	1.55
Perfluorohexane sulfonate (PFHxS)	µg/L	99.4	1.60	1.20	2.48
Perfluorononanoic acid (PFNA)	µg/L	100	0.854	0.600	1.16
Perfluorooctanoic acid (PFOA)	µg/L	100	2.78	2.13	3.65
Perfluorooctanesulfonic acid (PFOS)	µg/L	100	7.52	5.31	10.9

[a]: corrected for urinary density; [b]: LODs were reported for biomarkers for metals and LOQs were reported for biomarkers of persistent organic pollutants.

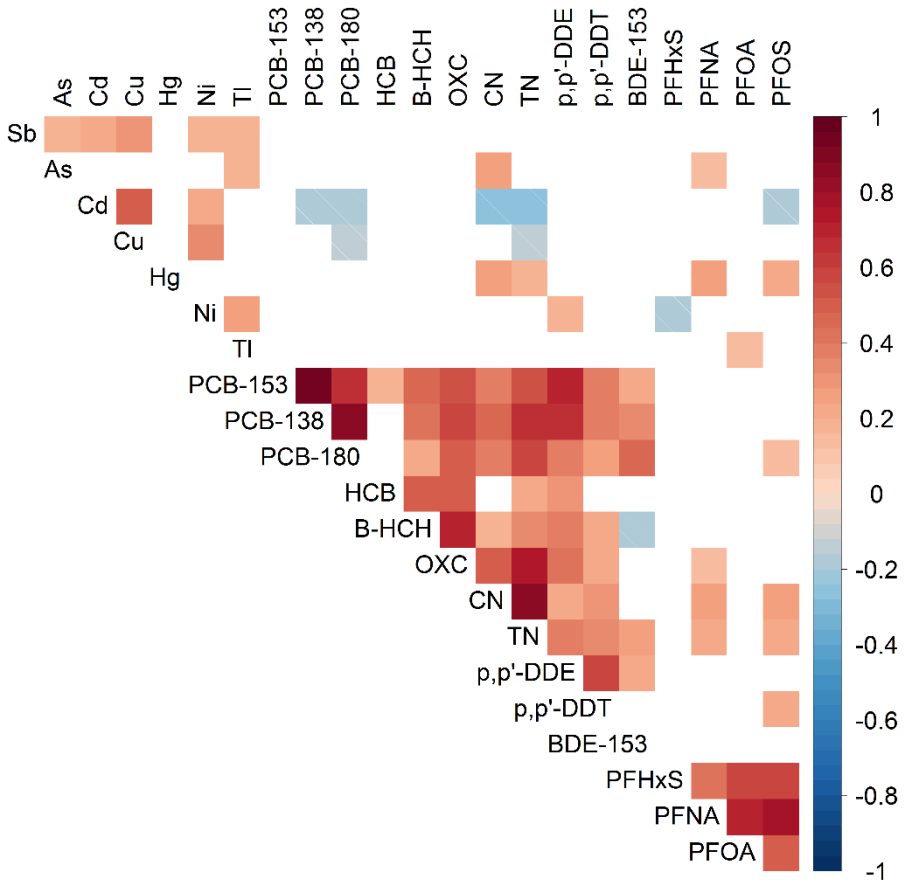

Figure 3: Pairwise Pearson correlation coefficients of the biomarkers of exposure (log_{10} transformed) presented as a heatmap. Only correlations with a p-value <0.05 were included.

Abbreviations: Sb= antimony, As=arsenic, Cd= cadmium, Cu= copper, Hg= mercury, Ni= nickel, Tl= thallium, PCB= polychlorinated biphenyl, HCB= hexachlorobenzene, β-HCH= β-hexachlorocyclohexane, OXC= oxychlordane, CN= cis-nonachlor, TN= trans-nonachlor, p,p'-DDE= p,p'-dichlorodiphenyldichloroethylene, p,p'-DDT= p,p'-dichlorodiphenyltrichloroethane, BDE= brominated diphenyl ether, PFHxS= perfluorohexane sulfonic acid, PFNA= perfluorononanoic acid, PFOA= perfluorooctanoic acid, PFOS= perfluorooctane sulfonic acid.

Association between environmental pollutants, mtDNA content and telomere length

The single pollutant associations showed that (i) serum PFOS, OXC and HCB were positively associated with mtDNA content; (ii) urinary Cu was inversely associated with the mtDNA content and (iii) urinary Hg and telomere length were positively associated (all at a nominal 0.05 significance level), after FDR correction, no association remained significant (Figure 4). The effect estimates, p-values and FDR-adjusted p-values are provided in supplementary tables S4 and S5.

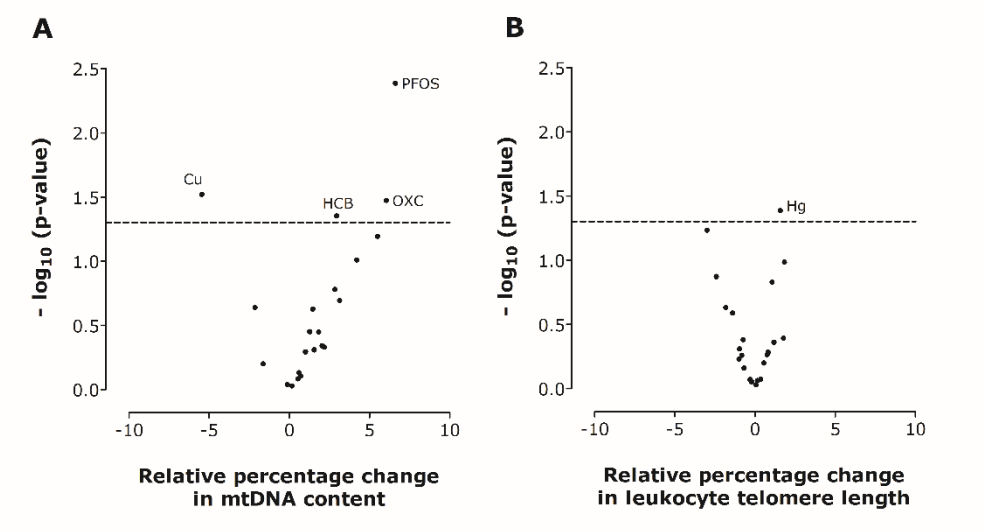

Figure 4: Volcano plot presenting the significance of the associations between biomarkers of environmental exposure and mtDNA content (A) and telomere length (B) in function of the magnitude of the estimated change. The models were adjusted for sex, age, BMI, household education, ethnicity, smoking habits, storage time and batch, and additionally for thrombocyte count in the models of mtDNA content. The dotted line in the Y-axis represents nominal p-value=0.05.

Using sPLS regression, we selected biomarkers of exposure predictive for either leukocyte mtDNA content or telomere length while considering other co-exposures. Cross-validation determined the number of components and sparsity for the optimal model according to the minimum MSPE (for mtDNA content: K=5; η=0.99; for RTL: K=4, η=0.99, supplemental figure 2).

The sPLS regression model for mtDNA content selected urinary Cd and Cu, serum HCB, BDE-153, PFHxS and PFOS as relevant predictors. However, serum BDE-153 was selected but non-significantly associated to mtDNA content (Table 3). Urinary Cd, serum HCB and PFOS exposure levels were positively associated with leukocyte mtDNA content, while urinary Cu and serum PFHxS were inversely associated. For telomere length, the sPLS regression model selected urinary Sb, Cu, Hg and serum PFOA as relevant predictors. Urinary Sb and Hg were positively associated with telomere length, whereas urinary Cu and serum PFOA were inversely associated with leukocyte telomere length. Contributions of the selected exposures in the different components is visualized in supplemental figure S2.

Effect sizes were estimated by OLS while accounting for possible confounding factors (i.e. age, sex, BMI, education, ethnicity, smoking habits, storage time and batch effects, and also thrombocyte count in case of mtDNA content), and other selected exposures (Table 3). The VIFs of the selected exposures ranged from 1.33 to 1.79 for the mtDNA content model, and from 1.20 to 1.43 for the telomere length model.

Table 3: Associations between biomarkers of environmental exposure and biomarkers of aging: the estimated effects from multipollutant modelling, using sPLS and OLS

Outcome	Selected exposure	sPLS model		OLS model	
		Estimate (95% CI) [a]	p-value	Estimate (95% CI) [b]	p-value
mtDNA content					
	Cd	0.033 (0.011, 0.058)	0.01	6.52% (1.06, 12.28)	0.02
	Cu	-0.051 (-0.073, -0.030)	<0.0001	-9.88% (-14.82, -4.66)	0.0004
	HCB	0.026 (0.001, 0.051)	0.04	2.89% (0.18, 5.68)	0.04
	BDE-153	0.017 (-0.010, 0.040)	0.19	3.66 (-0.89, 8.41)	0.12
	PFHxS	-0.033 (-0.063, -0.007)	0.02	-4.75% (-8.79, -0.54)	0.03
	PFOS	0.060 (0.035, 0.089)	<0.0001	11.38% (5.97, 17.08)	<0.0001
Leukocyte telomere length					
	Sb	0.018 (0.002, 0.037)	0.04	2.69% (0.45, 4.99)	0.02
	Cu	-0.018 (-0.033, -0.003)	0.02	-3.52% (-6.60, -0.34)	0.03
	Hg	0.019 (0.004, 0.033)	0.01	1.91% (0.42, 3.43)	0.01
	PFOA	-0.017 (-0.032, -0.002)	0.03	-3.64% (-6.60, -0.60)	0.02

[a]: Estimates are presented as the raw coefficients obtained from sPLS regression analyses. [b]: Estimates are presented as the relative change (%) in mtDNA content/leukocyte telomere length for a 50% increase in the mean concentrations of the biomarker of exposure. Models are adjusted for age, sex, BMI, smoking habits, household education, ethnicity, thrombocyte count, storage time and qPCR plates. Abbreviations: Cd= cadmium, Cu= copper, Hg= mercury, Sb= antimony, HCB= hexachlorobenzene, BDE-153= brominated diphenyl ether 153, PFOA=perfluorooctanoic acid, PFOS= perfluorooctane sulfonic acid, PFHxS=perfluorohexane sulfonate.

DISCUSSION

The key finding of our study is the identification of several environmental exposure indicators associated with molecular markers of aging. By means of a multipollutant analysis, we showed that urinary Cu and Cd, serum HCB, PFHxS and PFOS were associated with leukocyte mtDNA content, whereas urinary Sb, Cu and Hg and serum PFOA were associated to leukocyte telomere length. The usage of sPLS is deemed appropriate for this, as it employs the property of PLS to accommodate collinearity in the exposure data in a regression analysis, yet combined with a L1 penalty term, to retain only the most relevant exposures predictive of our outcomes [4].

The findings of the multipollutant approach and single pollutant models were partially consistent. Serum PFOS and HCB and urinary Cu were found significantly associated with mtDNA content in the single pollutant models (Figure 4). Contrary to the multipollutant approach, single pollutant models did not reveal associations between mtDNA content and urinary Cd or serum PFHxS. Serum OXC was associated with mtDNA content in a single pollutant model, but was not select by sPLS. Similarly, solely urinary Hg was selected as a relevant predictor from the single pollutant models whereas urinary Sb and Cu and serum PFOA were additionally selected by sPLS.

The use of multipollutant models has several advantages over the use of single pollutant models. First, in single pollutant models, co-exposures and joint patterns are not taken into account. For example, urinary Cd was correlated to both urinary Cu (Pearson's r=0.51) and serum PFOS (Pearson's r=-0.18), and serum PFHxS was correlated to serum PFOS (Pearson's r=0.57). When we do not account for correlated co-exposures, urinary Cd and serum PFHxS are not significantly associated to mtDNA content ($p>0.05$ in single pollutant models), but when accounting for co-exposures in multipollutant modelling, these are significantly associated to mtDNA content. Second, single pollutant models are less powerful to separate true associations from correlated exposures. This is illustrated by serum HCB and OXC, which are correlated (Pearson's r=0.50) and both are associated to mtDNA content in a single pollutant model ($p=0.04$ for HCB and $p=0.03$ for OXC). However, the single pollutant association of OXC may reflect

the (true) association of HCB, which is the only one selected by the sPLS model. Third, single pollutant models do not take confounding co-exposures into account which has an effect on the effect estimates of these associations. Comparing the effect estimates from single pollutant models (Supplemental table 3) to those of multipollutant models (Table 3) might show an underestimation for the effect estimates of Cu and PFOS in association with mtDNA content in single pollutant models.

We found a significant association between urinary Cu and mtDNA content, Cu is an essential element and necessary for mitochondrial function [33]. Cu is a co-factor for enzymes, predominantly those with oxygen-related functions, such as cytochrome c oxidase (COX) and superoxide dismutase (SOD). Hence, Cu is mainly accumulated in the mitochondria, with the majority mitochondrial Cu residing inside the matrix, where also the mitochondrial genome is located [33]. Although Cu is an essential element required for normal mitochondrial function, high concentrations of Cu can be toxic. Mitochondria are the primary target in Cu-toxicity [34], resulting in oxidative stress, impairment of the electron transport chain and ultimately apoptosis of the cells with a mitochondrial Cu overload [35-37]. Cu might also affect the mtDNA by influencing the mtDNA rearrangements [38,39], and it may play a role in the mtDNA instability by ROS induction. Since Cu is also an essential element this might reflect a physiological relationship, rather than a toxicological response. Up to now, no epidemiological study investigated mtDNA content in association with Cu nor with Cd, HCB, PFHxS or PFOS. However experimental evidence has linked exposure to these pollutants to mitochondrial toxicity. In general, mitochondria may be targets for metal toxicity, since they tend to accumulate in the mitochondria. Mitochondrial effects of Cd can include alterations of the mitochondrial gene expression, impairment of the respiratory activity, increased permeability of the inner mitochondrial membrane and excessive production of ROS [40]. Toxic manifestations of HCB exposure can include porphyria, for which the liver as well as the kidneys are the main targets. Although molecular mechanisms of toxicity responses remain to be elucidated, experimental evidence shows involvement of the mitochondria in which the induction of oxidative stress may play a role [41-45]. Some perfluorinated compounds are known to stimulate peroxisomes, which catabolise fatty acids that are then to be transported to the mitochondria for β-oxidation. Interference with

mitochondrial energetics and biogenesis is shown for some peroxisome proliferators. In rat models, there are indications of alterations of the mtDNA content through stimulation of mitochondrial biogenesis upon PFOA exposure [46,47], suggesting that perfluorinated compounds may also impact mitochondrial function. Furthermore, in experimental studies of exposure to perfluorinated compounds showed alterations in the mitochondrial bioenergetics [48] and induction of ROS [49,50].

Concerning mtDNA content, we observed both positive (urinary Cd, serum HCB and PFOS) and negative (urinary Cu and serum PFHxS) associations. During oxidative stress the mtDNA content can be increased in order to accommodate the reduced functionality of the mitochondrial genome [10]. However, with increasing mtDNA damage, this compensatory mechanisms may be insufficient and dysfunctional mitochondria may be removed, resulting in an overall reduced mtDNA content [10]. While PFOS and PFHxS are structurally related and may share exposure routes, their associations with mtDNA content are in opposite directions. Their correlation coefficient was 0.57, it is therefore likely that not all subjects with high PFOS levels display high PFHxS exposure levels. Moreover, the dose and timeframe (i.e. short or long-term) of the exposure may play a role in the dynamic responses of mtDNA content [51].

We observed a positive association between urinary Sb and Hg exposure and leukocyte telomere length, and an inverse association for urinary Cu and serum PFOA. Firstly, the NHANES study found an inverse association between urinary Sb and leukocyte telomere length. Compared to individuals with the lowest urinary Sb levels (1st quartile), individuals in the 3rd quartile and 4th quartile had respectively 4.78% and 6.11% shorter telomeres and the association was mainly driven by middle aged and older individuals [52]. The study sample of Scinicariello *et al.* is much larger (n=2307), and comprised a larger spread in the age of the participants (from 20 until 85 years old), but the geometric mean urinary Sb is comparable to that of our study population [52]. We observed a positive association between Sb and telomere length when accounting for confounding co-exposures, while the inverse association reported by Scinicariello was observed in a single pollutant model [52]. Concerning mercury, one study that investigated methylmercury exposure and telomere length in mother-newborn pairs did not

observe a significant association [53]. Furthermore, we found an inverse association for serum PFOA exposure, which is in line with a study in newborns where shorter telomere length was observed in association with exposure to perfluorinated compounds. These effects were shown to be partially mediated through increased ROS levels [54]. Finally, there are no other epidemiological studies investigating the relation between Cu and telomere length. In addition, Patel *et al.* investigated the correlation of 461 environmental factors with telomere length in the NHANES population, and observed a significant negative associations for urine and serum Cd and positive associations with serum PCBs [55]. Although the association between PCBs and telomere length is highly supported in literature by epidemiological [56-61] and experimental studies [62-64], we did not find an association with PCB exposure.

Telomeres consist of a high amount of guanosines which are susceptible for oxidative stress damage due to their lower reduction potential as compared to other nucleotides [65]. Therefore telomeres are highly vulnerable for ROS induced DNA damage. For example, 8-oxoguanosine formed by oxidation of guanosine is considered a biomarker for oxidative stress and may contribute to telomere attrition [66]. As a consequence, telomeres are sensitive to environmental factors that induce formation of ROS, hereby increasing oxidative stress [67]. This can be reflected by an increased telomere shortening in addition to cellular replication. Shorter telomere length is considered to contribute to genomic instability and biological aging. However, longer telomeres in association with environmental exposures are hypothesized to reflect a carcinogenic potential [68]. This is suggested since in spite of frequent proliferations, malignant cells often are able to maintain telomere length to circumvent degradation of the cell, and because some pollutants are known carcinogens [68]. We observed a positive association with leukocyte telomere length for urinary Sb and Hg. However, only some Sb compounds (Sb_2O_3) were classified as possible carcinogenic to humans (group 2B) and methylmercury and inorganic Hg compounds were not classifiable as carcinogenic (group 3) by IARC [69], suggesting their limited carcinogenicity. In explanation of our positive associations, it is also suggested that pollutants may induce acute inflammation, in which a shift in white blood cells is promoted towards an increase of immune cells [70]. T and B lymphocytes may have longer telomeres and a higher telomerase activity in support of their proliferative capacity during immune responses [71-73]. As such, an increase in leukocyte telomere length

may be attributed to shifts in cell distribution. Although accounting for lymphocyte percentage in single and multipollutant modelling did not affect our results (data in supplement), this does not fully correct for white blood cell distributions.

mtDNA content and telomere length are hypothesized to be interrelated [13]. A central role for p53 activation upon DNA damage is suggested to connect alterations in both telomere length and mitochondrial function and to maintain a cycle of genotoxic damage. Dysfunctional telomeres may activate p53, which by limiting the ROS defence mechanisms can in turn impair mitochondrial function [13]. Dysfunctional mitochondria produce more ROS, so a malicious cycle of DNA damage is introduced [74,75]. In our work, as well as in others', a positive correlation between mtDNA content and telomere length has been found [76-79]. Both mtDNA content and telomere length are dynamic features. Our findings markedly suggest individual signals for the environmental exposures associated to both markers. Furthermore, based on the number and strength of the associations we observed stronger effects of environmental exposure on mtDNA content compared to telomere length. Yet, as compared to the mtDNA, telomere length may be more protected for DNA damage by their association with shelterins and the presence of telomerase [80]. This suggests that the mitochondria may be more sensitive for environmental stressors. Additionally, telomere attrition in the early life occurs at a much faster pace than in adulthood [81]. It is suggested telomere length might be mainly affected by environmental factors in early life [14], and early life telomere lengths sets adult telomere length [82]. We studied environmental exposure in association with telomere length in adulthood, which may be a less critical period in life for telomere biology. The biomarkers of exposure all reflect exposure over a specific time span, and are as such not able to capture the totality of exposure throughout life, which may be particularly relevant for telomere length.

The findings of our study should be considered in light of its strengths and limitations. We had the opportunity to investigate our research questions in the FLEHS III adult cohort, which was set up as a part of the human biomonitoring (HBM) program in Flanders, Belgium. In the framework of multipollutant research, HBM studies are particularly valuable,[83] since they accurately quantify a larger set of biomarkers of environmental exposure relevant to human health in biological specimens. This is beneficial for exposure assessment, as the biological

quantification of the biomarkers reflects the totality of exposures and exposure misclassification is limited. Furthermore, we used a multipollutant approach, which contributes to better insights in the health effects of environmental factors, as we can identify the most influential exposures for our outcome while accounting for co-exposures. We used sPLS that combines PLS, which is flexible to deal with collinearity and searches for components that are predictive of the outcomes, with a L1 penalty that retains only the most influential exposures in the components. Employing this sensitive yet effective technique is compatible with the aims of our multipollutant approach to study all exposures as a whole and yielding information on which are most relevant for biological aging [3,4,84]. Our study has some limitations. Firstly, our study sample is rather small, limiting its power to identify some less sensitive effects. Second, we are unable to elucidate mechanisms underlying alterations in mtDNA content or leukocyte telomere length driven by environmental pollutants, for example increased oxidative stress or inflammation. Additionally, since biomarkers all reflect exposure over a specific time span, we are unable to conclude on life-time exposures although this might be important for telomere biology. We did not test for possible interaction effects between the exposures. We evaluated two biomarkers of aging, but more research on the integrated effects of combined exposure on molecular markers of aging is needed to fully understand the impact of various pollutants.

CONCLUSIONS

Using a multipollutant modelling approach to simultaneously assess the association between biomarkers of environmental exposure and molecular markers of aging, we identified environmental pollutants influential for mtDNA content or telomere length. Our study contributes to the hypothesis that environmental factors may impact putative markers of biological aging, which can be involved in the mechanism underlying exposure-related health outcomes.

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SUPPLEMENTAL INFORMATION

Table S1: Health aspects of the study population

Health parameters	Number (Frequency)
How would you consider your overall health [a]	
Very good	26 (15.03%)
Good	92 (53.18%)
Normal	51 (29.48%)
Bad	4 (2.31%)
Very bad	0
Hypertension	
Elevated blood pressure [b]	62 (35.43%)
Anti-hypertensive medication	50 (28.57%)
Elevated blood pressure or medication use	90 (51.43%)
Dyslipidaemia	
Reduced HDL levels [c]	27 (15.43%)
Elevated triglyceride levels [d]	52 (29.71%)
Medication for hypercholesterolemia or dyslipidaemia	36 (20.57%)
Hyperglycaemia / type 2 diabetes	
Elevated Hb1Ac [e]	2 (1.50%)
Metformin medication use	4 (2.29%)
Weight changes in the last 5 years [f]	
No	92 (61.74%)
Gained weight	42 (28.19%)
Lost weight	15 (10.07%)

a:n=173, self-reported; b: elevated blood pressure was defined as systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg; c: reduced HDL levels were defined as HDL < 40 mg/dl for men or HDL < 45 mg/dl for women; d: elevated triglycerides were defined as triglyceride > 200 mg/dl; e: n=133, elevated Hb1Ac levels was defined as Hb1Ac ≥ 6.5 %; f: n=149, self-reported.

Table S2: Overview of pollutants not included in the analyses, because of the high proportion undetectable measurements (n=175)

Biomarker of exposure	Method limit of detection (MLD)	Proportion below ML
Chromium	LOD = 0.199 µg/l	85.1%
Lindane (γ-HCH)	LOQ = 5 ng/L	96.0%
BDE-28	LOQ = 2 ng/L	98.9%
BDE-47	LOQ = 2 ng/L	34.9%
BDE-100	LOQ = 2 ng/L	68.6%
BDE-99	LOQ = 2 ng/L	72.0%
BDE-154	LOQ = 2 ng/L	98.3%
BDE-183	LOQ = 2 ng/L	100%
PFBS	LOQ = 0.2 µg/L	94.9%

Table S3: Association of biomarkers of aging and important covariables

	mtDNA content		**Leukocyte telomere length**	
	Estimate (95% CI)	**p-value**	**Estimate (95% CI)**	**p-value**
Sex, male	7.54% (-6.14, 23.21)	0.29	-1.62% (-9.19, 6.59)	0.69
Age, 4 years	1.21% (-5.01, 7.52)	0.70	-1.66% (-5.48, 2.21)	0.40
BMI, 4.2 kg/m²	4.39% (-1.78, 10.66)	0.16	1.39% (-2.51, 5.32)	0.48
Smoking habits	/	0.43	/	0.04
Never smoked	Ref	/	Ref	/
Stopped smoking	-0.29% (-12.81, 14.03)	0.97	-6.67% (-14.25, 1.58)	0.11
Current smoker	13.24% (-7.08, 38.01)	0.22	9.48% (-3.38, 24.05)	0.15
Household education	/	0.35	/	0.42
No diploma	26.47% (-6.31, 70.71)	0.12	10.54% (-8.51, 33.55)	0.30
Low	-4.30% (-19.31, 13.50)	0.61	2.72% (-7.81, 14.45)	0.62
Middle	-3.76% (-17.02, 11.62)	0.61	-4.34% (-12.94, 5.10)	0.35
High	Ref	/	Ref	/
Ethnicity [a]	/	0.28	/	0.77
Belgian	Ref	/	Ref	/
European	28.90% (-7.49, 79.60)	0.13	7.70% (-12.64, 32.77)	0.49
Non-European	-10.97% (-44.20, 42.03)	0.62	-2.51% (-27.40, 30.92)	0.87
Thrombocyte count, 61195	7.85% (1.35, 14.36)	0.02	Not included	/
Lymphocyte percentage, 7.7%	Not included	/	0.14% (-3.64, 3.94)	0.94

Effect estimates are presented as the relative change (%) with 95% CI in mtDNA content or telomere length for a SD increase for continuous variables or compared to a reference group for categorical variables. Models were adjusted for age, sex, BMI, smoking habits, household education, ethnicity, storage time and qPCR plates. In the mtDNA content model, thrombocyte count was additionally included, and in the telomere model the percentage of lymphocytes was additionally included. [a]: based on the country of birth of the participants.

Table S4: Single pollutant models evaluating the change in mtDNA content associated with the biomarkers of exposure

Biomarker	Estimate (95% CI)	p-value	FDR adj. p-value
Sb	0.60 (-2.90, 4.23)	0.74	0.90
As	-0.12 (-2.39, 2.20)	0.92	0.94
Cd	0.54 (-4.15, 5.46)	0.82	0.91
Cu	-5.46 (-10.14, -0.54)	0.03	0.24
Hg	1.47 (-0.96, 3.95)	0.24	0.52
Ni	-2.14 (-5.56, 1.40)	0.23	0.52
Tl	-1.63 (-8.02, 5.20)	0.63	0.82
PCB-138	1.55 (-2.81, 6.11)	0.49	0.70
PCB-153	2.04 (-3.26, 7.62)	0.46	0.70
PCB-180	2.19 (-3.65, 8.38)	0.47	0.70
HCB	2.95 (0.08, 5.91)	0.04	0.24
B-HCH	1.83 (-2.04, 5.86)	0.36	0.65
OXC	6.05 (0.46, 11.94)	0.03	0.24
CN	2.84 (-1.17, 7.01)	0.17	0.52
TN	5.50 (-0.32, 11.66)	0.06	0.28
p,p'-DDE	1.01 (-1.96, 4.07)	0.51	0.70
p,p'-DDT	1.27 (-1.41, 4.01)	0.35	0.65
BDE-153	3.14 (-1.67, 8.19)	0.20	0.52
PFHxS	0.16 (-3.71, 4.18)	0.94	0.94
PFNA	4.21 (-0.76, 9.43)	0.10	0.36
PFOA	0.71 (-4.26, 5.94)	0.78	0.91
PFOS	6.62 (2.08, 11.36)	0.004	0.09

Models were adjusted for age, sex, BMI, smoking habits, household education, ethnicity, thrombocyte count, storage time and batch effects. The estimated change is presented as the relative percentage (%) change in mtDNA content associated with a 50% increment in the mean concentrations in the biomarker of environmental exposure.

Abbreviations: Sb= antimony, As=arsenic, Cd= cadmium, Cu= copper, Hg= mercury, Ni= nickel, Tl= thallium, PCB= polychlorinated biphenyl, HCB= hexachlorobenzene, β-HCH= β-hexachlorocyclohexane, OXC= oxychlordane, CN= cis-nonachlor, TN= trans-nonachlor, p,p'-DDE= p,p'-dichlorodiphenyldichloroethylene, p,p'-DDT= p,p'-dichlorodiphenyltrichloroethane, BDE= brominated diphenyl ether, PFHxS= perfluorohexane sulfonic acid, PFNA= perfluorononanoic acid, PFOA= perfluorooctanoic acid, PFOS= perfluorooctane sulfonic acid.

Table S5: Single pollutant models evaluating the change in leukocyte telomere length associated with the biomarkers of exposure

Biomarker	Estimate (95% CI)	p-value	FDR adj. p-value
Sb	1.83 (-0.37, 4.08)	0.10	0.65
As	1.06 (-0.38, 2.52)	0.15	0.65
Cd	-0.21 (-3.17, 2.83)	0.89	0.93
Cu	-2.41 (-5.49, 0.76)	0.13	0.65
Hg	1.58 (0.06, 3.11)	0.04	0.64
Ni	0.55 (-1.68, 2.82)	0.63	0.87
Tl	1.77 (-2.37, 6.09)	0.41	0.86
PCB-138	-0.82 (-3.48, 1.91)	0.55	0.86
PCB-153	-0.31 (-3.53, 3.01)	0.85	0.93
PCB-180	-1.00 (-4.57, 2.70)	0.59	0.86
HCB	-0.74 (-2.50, 1.06)	0.42	0.86
B-HCH	0.75 (-1.68, 3.24)	0.55	0.86
OXC	-0.68 (-4.00, 2.76)	0.69	0.90
CN	0.82 (-1.67, 2.01)	0.52	0.86
TN	0.35 (-3.14, 3.98)	0.84	0.93
p,p'-DDE	0.16 (-1.67, 2.01)	0.87	0.93
p,p'-DDT	0.07 (-1.61, 1.77)	0.94	0.94
BDE-153	1.18 (-1.78, 4.24)	0.44	0.86
PFHxS	-1.40 (-3.79, 1.05)	0.26	0.81
PFNA	-1.84 (-4.79, 1.21)	0.23	0.81
PFOA	-3.00 (-6.01, 0.11)	0.06	0.64
PFOS	-0.97 (-3.67, 1.81)	0.49	0.86

Models were adjusted for age, sex, BMI, smoking habits, household education, ethnicity, storage time and batch effects. The estimated change is presented as the relative percentage (%) change in leukocyte telomere length associated with a 50% increment in the mean concentrations in the biomarker of environmental exposure.

Abbreviations: Sb= antimony, As=arsenic, Cd= cadmium, Cu= copper, Hg= mercury, Ni= nickel, Tl= thallium, PCB= polychlorinated biphenyl, HCB= hexachlorobenzene, β-HCH= β-hexachlorocyclohexane, OXC= oxychlordane, CN= cis-nonachlor, TN= trans-nonachlor, p,p'-DDE= dichlorodiphenyldichloroethylene, p,p'-DDT= dichlorodiphenyltrichloroethane, BDE= brominated diphenyl ether, PFHxS= perfluorohexane sulfonic acid, PFNA= perfluorononanoic acid, PFOA= perfluorooctanoic acid, PFOS= perfluorooctane sulfonic acid.

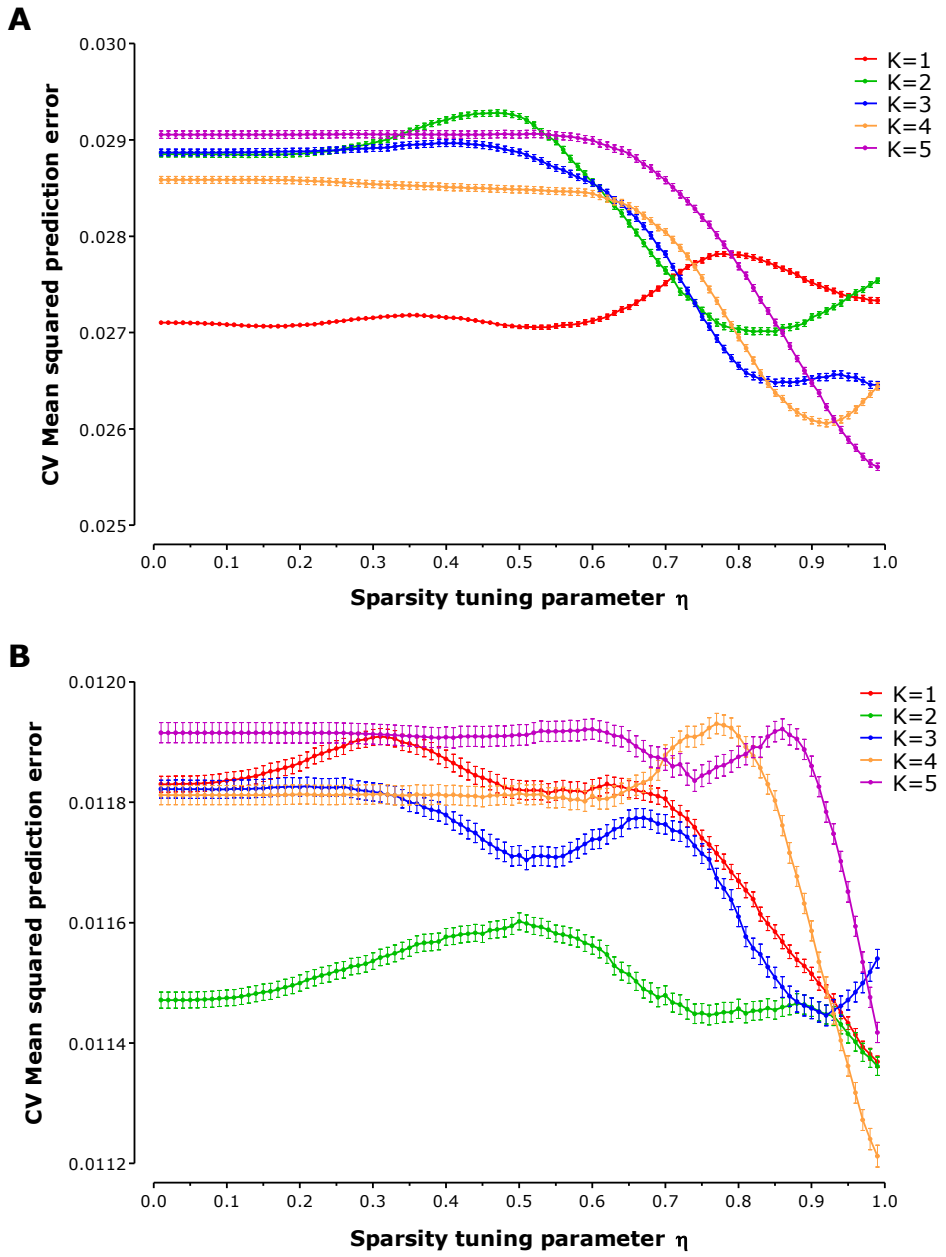

Figure S1: Error path for sPLS determined by 500x 10-fold cross-validation, depicting the cross-validated mean squared prediction error (MSPE) with the standard errors in function of the sparsity tuning parameter η, for different numbers of components ranging from 1 to 5. (A) error path for mtDNA content, with the optimal model K=5 components and very sparse with η=0.99; (B) error path for leukocyte telomere length with the optimal model K=4 components and very sparse with η=0.99.

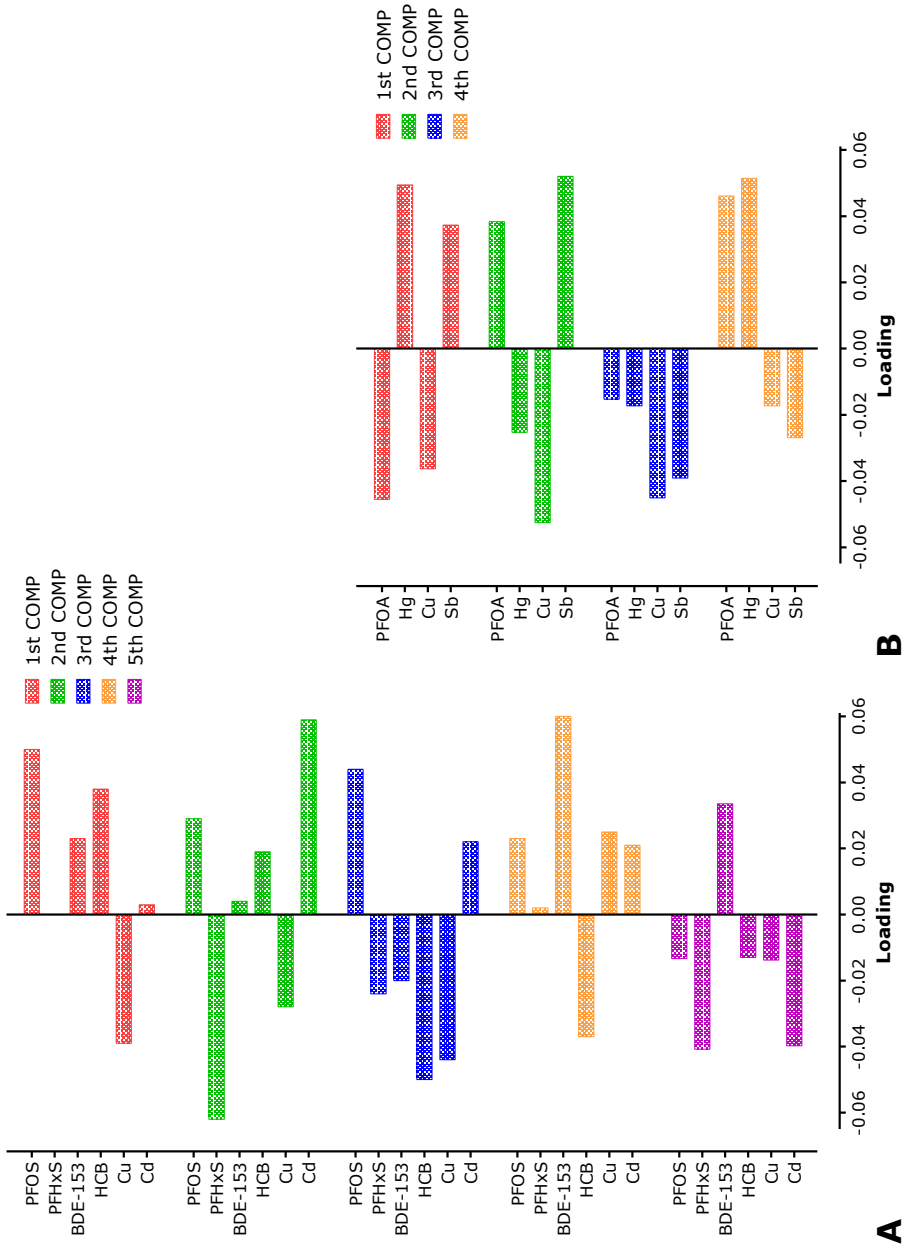

Figure S2: Loading of the selected biomarkers of exposure in the different components. (A) for sPLS regression model for mtDNA content, with six selected exposures and K=5 components; (B) for sPLS regression model for telomere lenght, with four selected exposures and K=4 components.

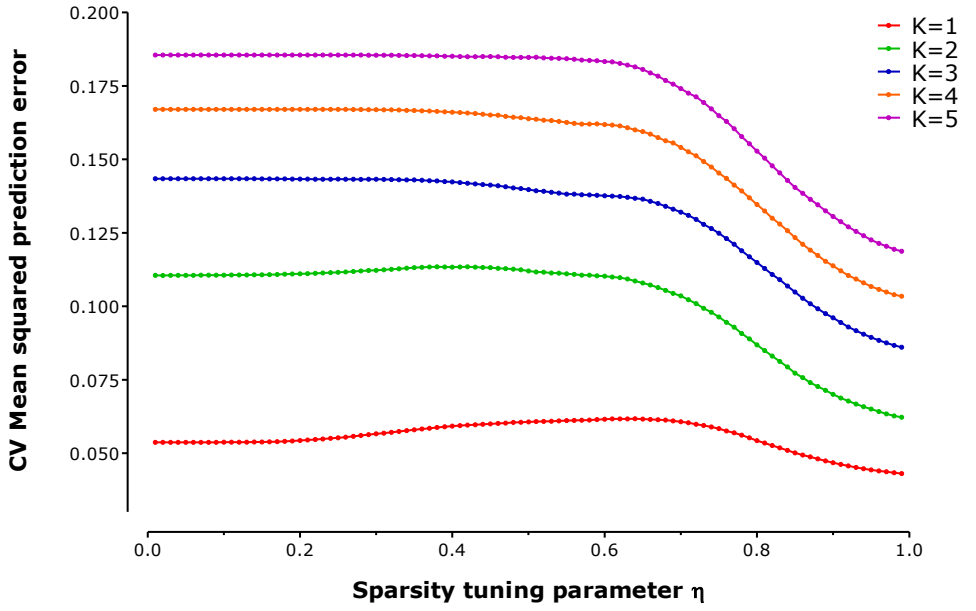

Figure S3: Error path for the multivariate sPLS model (including both mtDNA content and telomere length in the response matrix) determined by 500x 10-fold cross-validation, depicting the cross-validated mean squared prediction error (MSPE) with the standard errors in function of the sparsity tuning parameter η, for different numbers of components ranging from 1 to 5. The optimal model has K=1 components and very sparse with η=0.99.

Table S6: Beta coefficients* of selected pollutants by multivariate sPLS for biomarkers of aging

	mtDNA content	**Telomere length**
PFOS	0.036	-0.0056

* calculation of 95% CI is not supported for multivariate sPLS models in the sPLS package, however, from single pollutant models we learned that PFOS was only significantly associated to mtDNA content and not to telomere length.

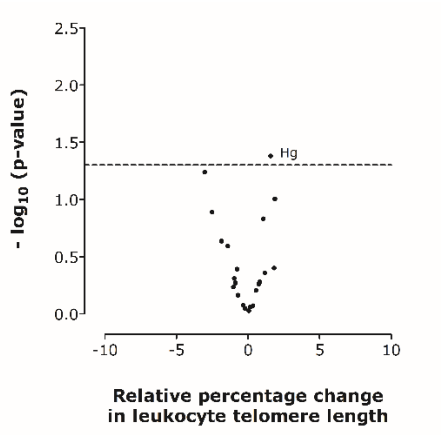

Figure S4: Volcano plot presenting the significance of the associations between biomarkers of environmental exposure and leukocyte telomere length (LTL) in function of the magnitude of the estimated change in LTL. Models were adjusted for age, sex, BMI, smoking habits, household education, ethnicity, lymphocyte percentage, storage time and batch effects.

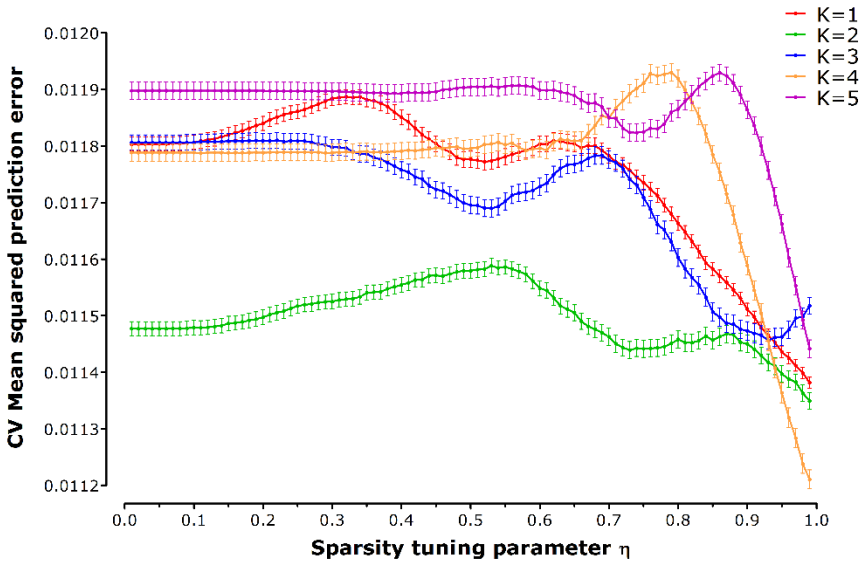

Figure S5: Error path for sPLS model for leukocyte telomere length, when additionally accounting for lymphocyte percentage, determined by 500x 10-fold cross-validation, depicting the cross-validated mean squared prediction error (MSPE) with the standard errors in function of the sparsity tuning parameter η, for different numbers of components ranging from 1 to 5. The optimal model K=4 components and very sparse with η=0.99.

Table S7: Sensitivity analysis for leukocyte telomere length, additionally taking into account lymphocyte percentages

Selected exposure	sPLS		OLS	
	Estimate (95% CI) [a]	p	Estimate (95% CI) [b]	p
Sb	0.018 (0.001, 0.036)	0.04	2.70% (0.43, 5.01)	0.02
Cu	-0.018 (-0.033, -0.002)	0.02	-3.51% (-6.67, -0.25)	0.04
Hg	0.019 (0.003, 0.033)	0.01	1.91% (0.41, 3.44)	0.01
PFOA	-0.017 (-0.032, -0.001)	0.03	-3.65% (-6.63, -0.57)	0.02

[a]: Estimates are presented as the raw coefficients obtained from sPLS regression analyses. [b]: Estimates are presented as the relative change (%) in leukocyte telomere length for a 50% increase in the mean concentrations of the biomarker of exposure. Models are adjusted for age, sex, BMI, smoking habits, household education, ethnicity, lymphocyte percentage, storage time and qPCR plates.

Chapter 5

Cord blood leptin and insulin levels in association with mitochondrial DNA content

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ABSTRACT

Background: The developmental origins of health and disease theory states that a disturbance in the early life environment can contribute to disease risk in later life. Leptin and insulin are anorectic hormones involved in energy homeostasis and are crucial for foetal growth. Disturbances in the levels of these hormones contribute to obesity and diabetes. In adults, altered mitochondrial function is an important hallmark of metabolic disorders, including obesity and diabetes. However, the mitochondrial effects of early life metabolic variation are unexplored. We investigated whether there is an association between metabolic hormones and mitochondrial DNA (mtDNA) content in early life.

Methods: The study included 236 newborns from the FLEHS III birth cohort, Flanders (Belgium). Relative mtDNA content of cord blood leukocytes was determined using quantitative PCR. Cord blood levels of leptin and insulin were determined using immunoassays. We studied the association between these metabolic hormones and mtDNA content using multiple linear regression models, while accounting for covariates and potential confounders.

Results: Leptin and insulin levels were positively associated with cord blood mtDNA content. mtDNA content was respectively 4.49% (95% CI: 1.15, 7.93; p=0.008) and 1.60% (95% CI: 0.31, 2.91; p=0.02) higher for a interquartile range increase of respectively cord blood leptin and insulin levels. In a sensitivity analysis, we observed that insulin and leptin were independently associated to mtDNA content and that insulin was stronger associated to mtDNA content in boys than in girls.

Conclusion: Neonatal metabolic hormones were associated with cord blood mtDNA content, which suggests that in early life the variation of mtDNA content might accommodate or reflect changes in the metabolic status.

BACKGROUND

According to the developmental origins of health and disease theory, a suboptimal perinatal environment can lead to metabolic programming, which may affect one's susceptibility for disease in adult life [1]. Perinatal metabolic determinants, such as birth weight and maternal BMI, impact the risk of the development of cardiovascular and metabolic disorders in later life which is characterised by a U-shaped association [2-4].

Leptin and insulin are hormones involved in energy homeostasis. Leptin is an anorectic hormone, which is mainly produced by adipocytes and functions in a negative feedback mechanism to regulate adipocyte size, energy intake/expenditure and the metabolism. Leptin concentrations are correlated with the adipose tissue mass and leptin insensitivity is often observed in obesity [5]. During gestation, the placenta produces leptin and leptin plays an important role in foetal development [6]. Insulin is produced by pancreatic β-cells to regulate glucose homeostasis. Peripheral insulin resistance and dysfunction of pancreatic β-cells cause type 2 diabetes, which is among the most prevalent metabolic disorders in humans [7]. During gestation, maternal insulin levels may rise to facilitate maternal fat storage for maternal energy, while maintaining carbohydrates for placental-foetal transport and foetal growth [8,9]. In the foetus, β-cells are functional from the 10th week, but only become responsive to glucose in the last trimester [10]. Neonatal concentrations of leptin [11] and insulin [12,13] are correlated with growth measures at birth. As such, they can be reflective of foetal growth and the metabolic status of the newborn. Furthermore, neonatal levels of leptin [14,15] and insulin [16] may be predictive of the metabolic status in childhood.

Oxidative stress is the imbalance between production of reactive oxygen species (ROS) and antioxidant defence mechanisms resulting in excessive ROS. It is an important feature in the aetiology of disorders such as type 2 diabetes, obesity and cardiovascular disease. Oxidative stress causes damage to mitochondrial macromolecules, hereby affecting mitochondrial function. As such, alterations in mitochondria and mitochondrial function have been observed in relation to metabolic disorders, in which oxidative stress appears to be a key player [17]. Mitochondrial DNA (mtDNA) content can be indicative of mitochondrial

(dys)function [18] and was recently suggested as a biomarker for type 2 diabetes in adults [19]. It is hypothesized that early life metabolic variation or challenges may result in foetal programming, which contributes to the predisposition for disease in adulthood. The relation between metabolic variation and mitochondrial function might already be established in early life. Here, we studied cord blood leptin and insulin levels, which reflect metabolic challenges during gestation, in association with cord mtDNA content, an indicator of mitochondrial function, in healthy neonates.

METHODS

Study population

This study was part of the third cycle of Flemish Environment and Health study (FLEHS), which recruited mother-newborn pairs in six hospitals in Flanders, Belgium to obtain a representative sample of the population [20]. All women that lived at least five years in Flanders, who were able to fill out an extensive Dutch questionnaire, who gave birth in one of the participating hospitals, were eligible to participate in the study.

In total, 281 mother-newborn pairs were recruited from November 2013 to November 2014. Of these 281 pairs, we collected sufficient cord blood from 277 participants to obtain DNA for cord blood mtDNA content measurements. Participants with missing quantification of the hormones insulin (n=9) or leptin (n=10), or missing information for important covariables (n=23) were not included in the analyses. Furthermore, we excluded mothers with gestational diabetes and/or insulin medication use, as well as mothers that delivered through caesarean section. In this study, 236 mother-newborn pairs were included (detailed information in flow chart in supplemental figure 1). The medical ethical committee of University of Antwerp and University Hospital of Antwerp as well as the local ethical committee of each participating hospital approved the study. All subjects in the study gave informed consent to participate.

Data collection

The mothers filled out a questionnaire addressing their general health status (e.g. gestational weight gain, gestational diabetes, medication use during pregnancy), lifestyle (e.g. smoking during pregnancy), socio-economic status (e.g. occupation, education), household composition & housing conditions and dietary patterns. Information on birth weight, birth length, head circumference and gestational age were obtained from medical records at the maternity ward. Small for gestational age (SGA) was defined as a birth weight below the 10th percentile for gestational age and sex, according to the sex-specific references curves. Similarly large for gestational age (LGA) was defined as a birth weight above the 90th percentile for gestational age and gender. The references were based on data of singleton births in Flanders from the Study Centre for Perinatal Epidemiology in the period 2001-2010 [21].

Sample collection

Umbilical cord blood was collected immediately after delivery using polypropylene Na-EDTA tubes, which were tested for metal contamination. Blood cell counts were determined on a fresh sample using an automated haematology analyser (HST-N302; Sysmex XE-2100 and SP-1000i). Samples were centrifuged at 3200 rpm for 15 min to retrieve buffy coats for DNA isolation and plasma for the hormone quantification. Samples were stored at –80 °C until further analyses.

Determination of the mitochondrial DNA content

DNA was isolated from buffy coats containing cord blood leukocytes using the QIAamp DNA mini kit (Qiagen). Mitochondrial DNA content (mtDNA) in cord leukocytes was measured by determining the ratio of two mitochondrial gene copy numbers (*MTF3212/R3319* and *MT-ND1*) to a single-copy nuclear control gene (*RPLP0*) using a real-time quantitative polymerase chain reaction (qPCR). qPCR reactions were carried out in triplicate on a 384-well plate on the 7900HT Fast Real-Time PCR System (Applied Biosystems) in a 10 µl volume containing: 5 µl QuantiTect SYBR Green (Qiagen) mastermix, 0.3 µl of forward and reverse primers (300 nM) and 1.9 µl RNAse-free water and 6 ng DNA diluted in 2.5 µl RNAse-free water. Primer sequences for mitochondrial genes are reported elsewhere [22]. Six interrun calibrators and no-template controls were included in

each qPCR run. The thermal cycling profile for the three transcripts was 10 minutes at 95 °C for activation of the polymerase enzyme and initial denaturation, followed by 40 cycles of 15 seconds at 94 °C for denaturation and 70 seconds at 58 °C for annealing and extension. After thermal cycling, the raw data were collected and processed using SDS 2.3 software (Applied Biosystems). The cycle quantification (Cq) values were normalized relatively to the *RPLP0* gene using qBase+ software (Biogazelle) taking into account the run-to-run differences [23].

Quantification of metabolic hormones in cord blood

Leptin and insulin levels were determined in cord blood plasma. Leptin was quantified using a radio-immuno assay (Human-Leptin-RIA-CT (KIPMR44); DIAsource ImmunoAssays). Insulin was measured using a chemiluminescence microparticle immuno assay (Architect Insulin Reagent Kit (8K41); Abbott Laboratories) carried out on a Abbott Architect i2000sr analyser. For successful quantification of insulin, plasma samples were frozen within 6 hours after collection.

Statistical analysis

Data management and statistical analysis was done using SAS software (version 9.4; SAS Institute Inc.). mtDNA content, insulin and leptin were $\log_{10}$-transformed to normalize their distribution. Continuous variables are presented as arithmetic means (standard deviation (SD)) or in case of insulin and leptin represented by geometric means (25^{th} – 75^{th} percentile). Categorical variables are presented as numbers (frequency in percentage).

We performed univariate linear regression models to assess the associations between potential important covariates and the hormone levels or mtDNA content. The effect estimates of the univariate associations are represented as the mean percentage change (standard error) in respectively mtDNA content (see Table 1) and hormone levels (see Table 2), compared to a reference group for the categorical variables or for a standard deviation increase in the continuous variables.

We then first evaluated the association between mtDNA content and the metabolic hormones with Pearson and Spearman correlation coefficients and we built multiple linear regression models to account for covariates and potential

confounders, including cord blood thrombocyte counts, gestational age, newborns' sex, growth rate (normal, LGA, SGA), maternal pre-pregnancy BMI, maternal age, smoking during pregnancy (yes, no), parity (0, 1, ≥2), ethnicity (Belgian, European ancestry, non-European ancestry) and the highest educational level of the household (low, middle, high). The *a priori* chosen covariates were included in the model regardless of the p-value. Q-Q plots of the residuals were checked to test the assumptions of all linear models. The effect-estimates were calculated as the relative percentage change in mtDNA content associated with an interquartile range (from the 25th to the 75th percentile) increase in cord blood leptin and insulin levels (log10), which corresponds to a 28.1% difference in insulin and a 84.5% difference in leptin.

In a sensitivity analysis, we examined the associations between cord blood mtDNA content and the metabolic hormones in the same model, to indicate if the effects were independent of each other. Furthermore, we evaluated effect modification of the growth rate (SGA, normal, LGA), newborns' sex and maternal BMI. Lastly, we performed sensitivity analyses in which we excluded mothers with a BMI above 30 kg/m³. The results for the associations of mtDNA content and other metabolic factors, including maternal pre-pregnancy BMI, birth weight and growth rate, were provided as supplementary information (Table S1).

RESULTS

Population characteristics

An overview of the population characteristics is presented in Table 1. On average (standard deviation, SD), the mothers were 30.2 (4.1) years old and had a normal pre-pregnancy BMI of 23.7 (4.1) kg/m². 19.5% of the mothers were overweight and 8.9% was obese. 9.8% of the mothers reported smoking during the pregnancy. The majority of the households had a high education level (65.3%) and for 44.5% of the mothers, this newborn was their first child. 51.3% of the newborns were boys, and the birth weight was on average 3,449 (429) grams. 11% of the newborns was LGA and 8% of the newborns was SGA. The geometric mean cord blood insulin and leptin levels were respectively 27.3 (25th -75th percentile: 18 – 40.5) pmol/L and 5.6 (25th – 75th percentile: 3.4 – 9.3) µg/L.

Table 1: Characteristics of the study population (n=236) and their association with cord blood mtDNA content

Characteristic	Mean ± SD or n (%)	Mean effect (SE)	p-value
Maternal characteristics			
Maternal age, years	30.2 ± 4.1	1.23% (1.63)	0.45
Maternal pre-pregnancy BMI, kg/m²	23.7 ± 4.1	-1.71% (1.65)	0.30
Maternal pre-pregnancy BMI			
< 18.5 kg/m²	7 (2.5 %)	10.21% (11.08)	0.36
18.5 – 25 kg/m²	163 (69.1 %)	Ref	-
25 – 30 kg/m³	46 (19.5 %)	0.82% (4.31)	0.85
> 30 kg/m²	21 (8.9 %)	-1.21% (6.03)	0.84
Smoking during pregnancy	23 (9.8 %)	7.79% (5.67)	0.17
Highest educational level in household			
Lower high school	17 (7.2 %)	4.82% (6.66)	0.47
Higher high school	65 (27.5 %)	-0.15% (3.80)	0.97
College/ University	154 (65.3 %)	Ref	-
Parity [a]			
0 children	105 (44.5 %)	Ref	-
1 child	88 (37.3 %)	7.96% (3.68)	0.04
≥ 2 children	43 (18.2 %)	7.04% (4.63)	0.13
Newborn characteristics			
Boys	121 (51.3 %)	5.96% (3.32)	0.08
Ethnicity [b]			
Belgian	172 (72.9 %)	Ref	-
European	20 (8.5 %)	3.41% (6.05)	0.57
Non-European	44 (18.6 %)	12.42% (4.29)	0.01
Gestational age, weeks	39.3 ± 1.3	-5.45% (1.67)	0.001
Birth weight, grams	3449 ± 429	-1.11% (1.64)	0.50
Large for gestational age	26 (11 %)	0.98% (5.42)	0.85
Small for gestational age	19 (8 %)	-3.58% (6.26)	0.55
Leptin, µg/l [c,d]	5.6 (3.4 – 9.3)	1.38% (1.50)	0.36
Insulin, pmol/l [c, d]	27.3 (18 – 40.5)	1.94% (0.62)	0.002

The mean (SE) effects are represented as a % change in the cord blood mtDNA content for a SD change in the continuous variable or compared to a reference category for the class variables. a: Parity is indicated based on the number of children before the child in our study; b: ethnicity was defined by country of birth of the grandparents of the child; c: for leptin and insulin the geometric mean (25^{th} – 75^{th} percentile) is given; d: effect sizes for an interquartile range increase in the mean hormone levels.

Correlates of cord blood mtDNA content

Of the aforementioned variables, we assessed univariate associations to determine if they are relevant predictors of cord blood mtDNA content (Table 1). Compared with mtDNA levels of first-born neonates, mtDNA levels were respectively 7.96% (± 3.68%) higher in neonates who were the second (p=0.04) child in the family. Neonates from non-European ancestry had 12.42% (± 4.29%) higher cord blood mtDNA copy numbers (p=0.01). A 1.3 week longer gestational age was associated with 5.45% lower (± 1.67%) mtDNA content in cord blood (p=0.001).

Correlates of metabolic hormones

In unadjusted analyses, birth weight and growth rate were determinants of both cord blood leptin and insulin levels (Table 2). An SD increase in birth weight (429 grams) was associated with 22% (± 4.2%) higher leptin levels (p=0.03) and 9.3% (± 4.2%) higher insulin levels (p=0.03). Compared with newborns with a normal growth rate, LGA babies had 68.2% (± 14.6%) higher leptin levels (p=0.0002) and 39.7% (± 13.9%) higher insulin levels (p=0.01). Similarly, SGA babies had on average 23.5% (± 17%) lower leptin levels (p=0.09) and 34.6% (± 16.2%) lower insulin levels (p=0.01). Furthermore, parity was associated with insulin levels (overall p=0.14, difference between 0 and ≥2 children p=0.05). Maternal pre-pregnancy BMI and newborn's sex were associated with cord leptin levels (p=0.002). Each SD increase in pre-pregnancy BMI (4.1 kg/m²) was associated with 13.6% (± 4.4%) higher leptin (p=0.004) in cord blood. Boys had on average 38.38 % (± 8.48 %) lower levels of cord blood leptin (p=0.002). Compared with newborns of Belgium ethnicity, newborns with non-European ancestry had 34.7% (± 11.3%) higher cord blood insulin concentrations (p=0.01).

Table 2: Correlates of cord blood leptin and insulin levels

	Leptin		Insulin	
	% change (SE)	**p-value**	**% change (SE)**	**p-value**
Maternal characteristics				
Maternal age, years	8.4% (4.4)	0.06	3.8% (4.2)	0.36
Maternal pre-pregnancy BMI, kg/m²	13.6% (4.4)	0.002	5.6% (4.2)	0.19
Maternal pre-pregnancy BMI				
< 18.5 kg/m²	-38.5% (31.9)	0.08	14.9% (30.6)	0.60
18.5 – 25 kg/m²	Ref	/	Ref	/
25-30 kg/m³	24.3% (11.7)	0.05	17.7% (11.3)	0.13
> 30 kg/m²	38.9% (16.7)	0.03	14.7% (16.1)	0.36
Smoking during pregnancy	-9.5% (16)	0.50	-3.8% (15.2)	0.78
Highest educational level in the family				
Lower high school	-6.4% (18.9)	0.70	25% (17.8)	0.18
Higher high school	-8.1% (10.6)	0.40	7.7% (10)	0.43
College/ University	Ref	/	Ref	/
Parity				
0 children	Ref	/	Ref	/
1 child	-2.2% (10.3)	0.82	6.1% (9.7)	0.52
≥ 2 children	-2.1% (13.1)	0.86	25.8% (12.3)	0.05
Newborn characteristics				
Boys	-37% (8.7)	<0.0001	-11.8% (8.7)	0.13
Ethnicity				
Belgian	Ref	/	Ref	/
European	0.3% (17.4)	0.98	10.1% (16.2)	0.52
Non-European	2.3% (12.2)	0.84	34.7% (11.3)	0.01
Gestational age, weeks				
Birth weight, grams	22% (4.2)	0.03	9.3% (4.2)	0.03
Large for gestational age	68.2% (14.6)	0.0002	39.7% (13.9)	0.01
Small for gestational age	-23.5% (17)	0.09	-34.6% (16.2)	0.01

The mean (SE) effects are represented as a relative % change in the cord blood hormone levels for a SD change in the continuous variable or compared to a reference category for the class variables.

Metabolic hormones in association with cord blood mtDNA content

In an unadjusted analysis (Figure 1), we observed a positive correlation between cord blood insulin and mtDNA content (Pearson r = 0.23, p=0.0004; Spearman r=0.20, p=0.002), contrary cord blood leptin and mtDNA content were not correlated (Pearson r =0.09, p=0.15; Spearman r=0.05, p=0.43). For an interquartile range increase in insulin levels, we observed a 1.94% (± 0.62%; p=0.002) higher mtDNA content in cord blood (Table 1).

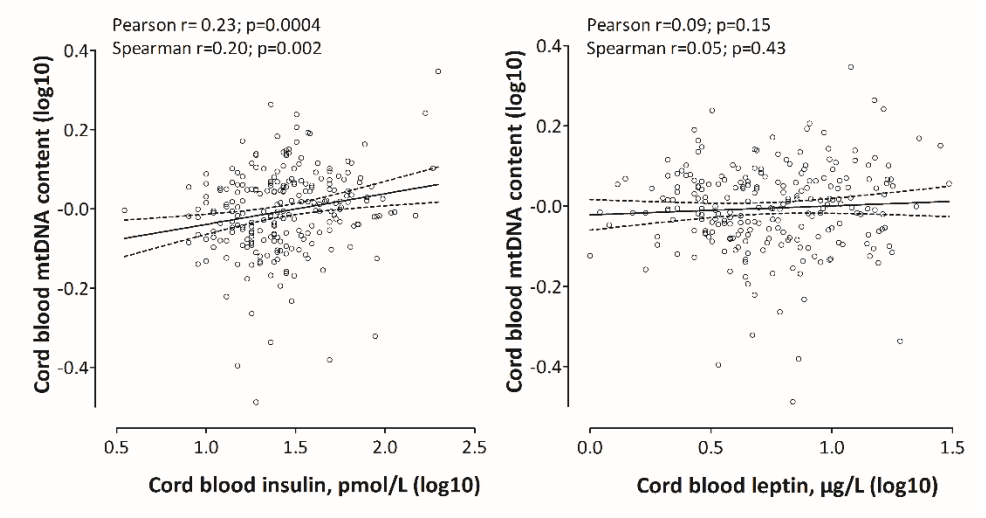

Figure 1: Correlation plots between mtDNA content in cord blood and metabolic hormones (n=236).

After adjustment for sex, gestational age, growth rate, ethnicity, maternal pre-pregnancy BMI, maternal age, parity, smoking status during pregnancy, education and thrombocyte count, leptin and insulin levels were significantly associated with cord blood mtDNA content (Table 3). An interquartile range increase in the cord blood leptin levels was associated with a 4.49% (95% CI: 1.15, 7.93%) higher mtDNA content in cord blood (p=0.008). Similarly, a interquartile range increase in the mean insulin levels was associated with a 1.60% (95% CI: 0.31, 2.91%) increase in mtDNA content (p=0.02). Other metabolic factors were not associated with cord blood mtDNA content (Table S1).

In sensitivity analyses, we tested the hormones in the same model, indicating that the effects of insulin and leptin on mtDNA were independent (Table 3). Exclusion of obese (BMI > 30 kg/m³) mothers did not affect our results. Furthermore, effect modification of newborn's sex, growth rate or maternal pre-pregnancy BMI was evaluated. The interaction terms were not significant for growth rate, pre-pregnancy BMI and for newborn's sex and leptin. Only, the effect of insulin on mtDNA content was different for boys and girls, as shown by a trend of the interaction between insulin and newborn's sex (p=0.10). The association between mtDNA content and insulin was more pronounced in boys, while it was not significant in girls (Table 3, supplemental figure 2).

Table 3: The association between metabolic hormones and mitochondrial DNA content

	Effect size (95% CI)	**p-value**
Main results: Separate models for metabolic hormones (n=236)		
Insulin	1.60% (0.31, 2.91)	0.02
Leptin	4.49% (1.15, 7.93)	0.008
Sensitivity: Combined model including both metabolic hormones (n=236)		
Insulin	1.29% (-0.02, 2.61)	0.05
Leptin	3.77% (0.40, 7.25)	0.03
Sensitivity: Exclusion of obese mothers (n=215)		
Insulin	1.72% (0.37, 3.09)	0.01
Leptin	4.75% (1.25, 8.37)	0.008
Sensitivity: Effect modification of newborns' sex for the relation between mtDNA content and insulin*		
Girls	0.57% (-1.19, 2.36)	0.53
Boys	1.61% (0.32, 2.91)	0.01

The estimated effects (95% CI) are represented as a relative % change in the mtDNA content for a interquartile range increase in hormone levels. Models were adjusted for newborns' sex, gestational age, growth rate, ethnicity, maternal pre-pregnancy BMI, maternal age, household education, parity, smoking during pregnancy and cord blood thrombocyte count. * p for interaction term =0.10.

DISCUSSION

Early life molecular markers implicated in metabolic programming are widely unexplored. In adults, altered mitochondrial function is a hallmark in the aetiology of cardio-metabolic disorders [17]. In this study, we showed that metabolic hormones and mtDNA content are associated in newborns, which suggests that mtDNA content might reflect or accommodate metabolic variation from early life onwards.

Early life metabolic disturbances may affect mitochondrial function and these effects can persist into adulthood. Experimental evidence showed that long-term adaptations in mitochondrial function arise as a consequence of malnutrition during early life [24-27]. The following epidemiological studies further support a putative role of the mitochondria in programming as a consequence of metabolic challenge. First, placental oxidative stress and mitochondrial dysfunction was observed in maternal obesity coincided with gestational diabetes and correlated with neonatal leptin levels [28]. Also, Clemente *et al.* reported a positive association between placental mtDNA content and birth weight [29]. In newborns, associations were shown between the mtDNA content and foetal growth rate, showing lower umbilical cord mtDNA content in small for gestational age and large for gestational age neonates [30]. However, in the latter study, the authors did not find an association between mtDNA content and leptin levels [30]. In addition, in a population of obese children between 2 and 18 years old, mtDNA content was positively correlated with BMI [31]. Skeletal muscle mitochondrial oxidative phosphorylation function was measured by phosphocreatine recovery time after exercise measured using ^{31}P-MRS, as an indicator of ATP synthesis in healthy children of 8 to 18 years old [32]. Phosphocreatine recovery time was associated with insulin sensitivity, HDL and triglyceride levels and with the resting energy expenditure [32]. Furthermore, a recent study in 12 to 19 years old healthy children studied muscle mitochondrial function by means of indicators of the ATP synthesis quantified using ^{31}P-MRS. They reported an association between the mitochondrial function and triglyceride to HDL ratio, but not with insulin sensitivity [33]. Taken together, these studies demonstrate that alterations in mtDNA content and the mitochondrial energy production might be indicative of the metabolic phenotype, already in early life without the presence of morbidities. A recent study by

Clemente *et al.* provided evidence that placental mtDNA is associated with infant height at 6 months of age, suggesting that neonatal mtDNA content can be predictive of infant growth [34]. This finding further supports our hypothesis that the relation between mitochondrial function and metabolic balance might already be established from birth onwards.

Perinatal metabolic challenges are linked to oxidative stress, which is a key factor affecting the mitochondria. Luo *et al.* [35] studied the relationship between markers for oxidative stress (lipid peroxidation) and foetal metabolic factors (insulin, IGF1, IGF2, leptin, adiponectin and ghrelin as markers for foetal growth, insulin sensitivity and energy regulation). The authors reported a negative association between maternal and foetal oxidative stress indicators and the foetal concentrations of ghrelin. These results suggest that metabolic programming may occur in healthy neonates [35]. Furthermore, oxidative stress increases with foetal growth retardation and/or malnutrition, which was exemplified by (i) a study on small for gestational age neonates from malnourished mothers [36] and [16] differences in the oxidative balance were observed in small for gestational age neonates from mothers without nutritional problems [37]. Our study adds to this evidence that even in a normal physiological range of neonatal concentrations of the metabolic hormones leptin and insulin, variation is associated with the mtDNA content, which may imply foetal metabolic programming. However, we observed no significant association between the maternal BMI or birth weight, other metabolic parameters and mtDNA content (Table 1 and Table S1). A discrepancy with other studies showing an association between placental mtDNA content and birth weight [29] or between cord blood mtDNA and growth rate [30], might be due to differences in the populations, to difference in the biological samples used, to difference in statistical power or due to differences in residual confounding. Clemente *et al.* [29] had a much larger sample (926 subjects) and studied placental mtDNA content while we focused on cord blood mtDNA content. While the study of Gemma *et al.* [30] had a smaller sample, they had a higher portion of newborns with an abnormal weight for gestational age (12.5% LGA and 19.3% SGA) compared with our study.

Since prenatal stressors have an impact on the future health of the newborns, physiological metabolic variation, as exemplified by metabolic hormone levels, can

be linked with predisposition to risk factors for disease. Neonatal levels of these hormones are sensitive to metabolic challenges. Cord blood levels of leptin are positively associated with birth weight and may explain up to 22% of the variation in birth weight [11]. Lower leptin levels are linked to more rapid growth (or "catch-up growth") in early life [15]. Serum leptin levels in early childhood (at 12 months) are associated with a slower foetal growth [38] and a higher increase in BMI in the first four months of life [38,39]. Leptin levels in adulthood are associated inversely to birth weight [39] and are related to obesity [40]. Neonatal leptin levels are higher when gestational diabetes mellitus occurred during pregnancy [41-43] and when mothers had a higher BMI [44]. Leptin levels are also associated with neonatal weight and fat mass [11,45]. Similar, neonatal insulin levels are correlated with maternal BMI [46]. birth weight as well as growth rate were associated with cord blood leptin and/or insulin levels. Also in our population of healthy neonates, the insulin and leptin levels reflect metabolic variation.

We observed more marked effects of insulin on mtDNA content in boys than in girls. Sex differences in the mitochondrial function and oxidative capacity have been previously described (reviewed in [47]). Animal studies indicate that in female rodents mitochondrial function is more specific and efficient, and less oxidative damage occurs [47]. Accordingly, mitochondria might be better protected in females, which may explain why we observe more marked effects in boys.

The study has some limitations. We only studied mtDNA content, leptin and insulin, however to study the metabolic variation and associated mitochondrial function considering more markers would be advantageous. Furthermore, we cannot exclude reverse causality or discuss the temporal relationships in this study. In other words, we do not know if higher levels of the metabolic hormones affect the mtDNA, or if it is the other way around that altered mtDNA content, as a reflection of mitochondrial function, influences the levels of the metabolic hormones.

CONCLUSION

Our study is the first to evaluate mtDNA content in relation to metabolic hormones in healthy neonates. mtDNA content reflects the metabolic variation demonstrated by foetal leptin and insulin concentrations. These findings indicate that the association between mitochondria and the metabolic status might be established at birth. Furthermore, our results contribute to the hypothesis that mitochondria may play an important role in foetal metabolic programming.

Nevertheless, it remains unclear if variation in the mtDNA content is a consequence or the cause of the metabolic status. Longitudinal studies are needed to provide insight in the role of mitochondria in relation to early life metabolic changes and the potential role in programming of later life disease.

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SUPPLEMENTAL INFORMATION

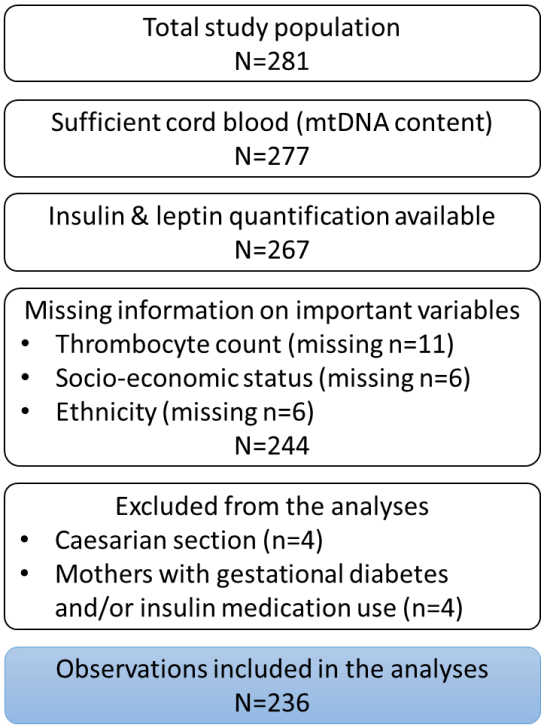

Figure S2: Flow chart of data availability and observations in the analyses.

Table S1: The association between metabolic factors and mitochondrial DNA content

	Effect size (95% CI)	**p-value**
Maternal pre-pregnancy BMI, + 4.1 kg/m²	-1.30% (-4.59 – 2.02)	0.44
Birth weight, + 429 grams	0.68% (-2.92 – 4.27)	0.71
Growth rate		
Small for gestational age	-1.94% (-12.85 – 10.35)	0.74
Appropriate for gestational age	Ref	-
Large for gestational age	1.01% (-8.78 – 11.84)	0.85

The estimated effects (95% CI) are presented as a relative % change in the mtDNA content for a SD increase in maternal pre-pregnancy BMI or birth weight / compared to appropriate for gestational age newborns. Models were adjusted for newborns' sex, gestational age, ethnicity, maternal age, household education, parity, smoking during pregnancy and cord blood thrombocyte count. n=236

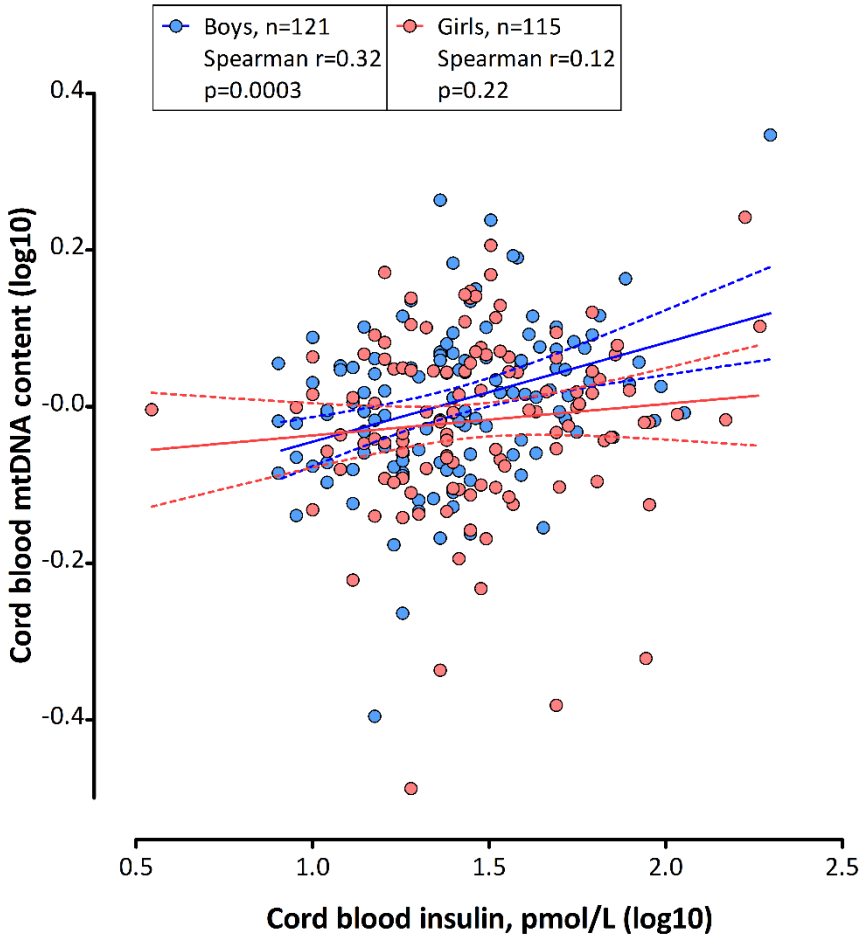

Figure S3: Correlation plot between cord blood insulin levels and cord blood mtDNA content, for boys (blue) and girls (orange).

Chapter 6

Children's screen time alters the expression of saliva extracellular miR-222 and miR-146a

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Vriens A. *et al.* Scientific Reports (2018)

ABSTRACT

Background: An imbalance between energy uptake and energy expenditure is the most important reason for increasing trends in obesity starting from early in life. Extracellular miRNAs are expressed in all bodily fluids and their expression is influenced by a broad range of stimuli. We examined whether screen time, physical activity and BMI are associated with children's salivary extracellular miR-222 and miR-146a expression.

Methods: In 80 children the extracellular fraction of saliva was obtained by means of differential centrifugation and ultracentrifugation. Expression levels of miR-222 and miR-146a were profiled by qPCR. We studied the association between children's salivary extracellular miRNA expression and screen time, physical activity and BMI using mixed models, while accounting for potential confounders.

Results: We found that higher screen time was positively associated with salivary extracellular miR-222 and miR-146a levels. On average, one hour more screen time use per week was associated with a 3.44% higher miR-222 (95% CI: 1.34, 5.58; p=0.002) and 1.84% higher miR-146a (95% CI: -0.04, 3.75; p=0.055) level in saliva. BMI and physical activity of the child were not significantly associated with either miR-222 or miR-146a.

Conclusions: A sedentary behaviour, represented by screen time use in children, is associated with discernible changes in salivary expression of miR-146a and or miR-222. These miRNA targets may emerge attractive candidates to explore the role of these exposures in developmental processes of children's health.

BACKGROUND

A rising prevalence in obesity, both in children and adults has emerged over the last decade[1]. Obesity fosters the development of a variety of morbidities, among which type 2 diabetes and cardiovascular disease [2]. The onset of these diseases could occur already early in life, with obesity being an important risk factor [3].

An unhealthy lifestyle, with an imbalance between energy intake and expenditure is the predominant cause of obesity. Insufficient energy expenditure can directly be linked to a lack of physical activity [4]. Although it is well-accepted physical activity is an effective measure to reduce multiple health risk factors, a sedentary lifestyle is frequent in all age groups [5]. Children spend a substantial amount of time in sedentary pursuits, such as watching television, using the computer and playing videogames. This behaviour not only increases the risk to develop obesity, but also increases the risk to develop metabolic syndrome and cardiovascular disease [6].

miRNAs are short (~22 nt long) single-stranded RNA molecules which control gene networks and play a central role in a various (patho)physiological processes [7]. miRNAs are present both intracellular and in the extracellular environment. Extracellular miRNAs, which are protected from degradation and carried by vesicles and protein aggregates, are stably present in all bodily fluids. Recently, they are recognized as important signal molecules and/or biomarkers in the pathology of various morbidities [8]. As such, circulating miRNAs are implemented in cardiometabolic disease mechanisms [9]. Studies focusing on differential miRNA expression patterns in patients with various cardiometabolic disorders compared with healthy subjects showed the importance of miR-222 and miR-146a. In this regard, the inflammatory miR-146a is linked to metabolic and cardiovascular disease [9]. miR-222 is a cell cycle regulator, which is involved in both physiological and pathological cardiac processes [10]. The (extracellular) expression profiles of miRNAs in body fluids are suggested as potential biomarkers for cardiometabolic processes. However, these studies either focus on subjects already having a specific metabolic or cardiovascular disorder or on specific interventions in adults. Studies linking miR-146a or miR-222 with life-style factors related to cardiometabolic outcomes in childhood are currently lacking. We investigated

whether miR-146a and miR-222 in the extracellular fraction of saliva are associated with screen time use, physical activity and BMI in children.

METHODS

Study population

This study was based on the COGNAC (COGNition and Air pollution in Children) study [11-13], which is a panel study with three repeated measures. Between 2011 and 2013, we invited children (grades three to six) from three primary schools in Flanders (Belgium) to participate. 43.4% of all invited children participated in the study. The examinations took place between December 2011 and February 2014 on Monday, Tuesday, Thursday, and Friday between 9:00 a.m. and 2:00 p.m. Of the 334 children within the COGNAC cohort, we randomly selected 80 children, from two participating schools. Saliva samples of the first two study visits, which were approximately 3 months apart, were selected. For each child, all visits were scheduled at the same day of the week and same time point, to rule out diurnal variation. The study was approved by the medical ethical committee of Hasselt University and the Eastern-Limburg Hospital (Belgium) in accordance with the Helsinki Declaration [14]. Parental informed consent was obtained prior to participation in the study. Information on the socio-economic status (by parental education and occupation), exposure to second-hand smoke through parental smoking and the use of TV and PC screens was accessible by a questionnaire filled out by the parents. Based on the reported weekly TV and PC screen use, we calculated the total screen time as the sum of TV and PC screen use. Based on information of the reported sport activities, out-of-school sport activities were identified and categorized as "none" (i.e. no out-of-school sport activities), "low" (i.e. ≤ 3 hours per week), "middle" (> 3 to < 6 hours per week) and high (≥ 6 hours per week). Passive smoking was defined as exposure to indoor tobacco smoke, when one or more family member(s) smoked inside the house. Height and weight were recorded and body mass index (BMI) was calculated. Overweight and obesity were defined according to international childhood BMI thresholds [15].

Saliva extracellular miRNAs

In order to avoid contamination of the samples, children refrained at least 30 minutes from eating, drinking or hygienic procedures prior to saliva donation. Additionally, they rinsed three times with tap water to eliminate possible food residues. Saliva (2 ml) was collected using the Oragene® RNA self-collecting kits (DNA Genotek Inc.) and immediately stabilized by mixing with RNA stabilizer. Within 6 hours, the samples were stored at -20°C until further analyses.

The extracellular fraction of the saliva was obtained by differential centrifugation and ultracentrifugation, protocol was adapted from Théry et al. [16] to integrate the processing of saliva specific to the Oragene collection kits in the procedure. After thawing, the saliva was incubated at 50°C for one hour. Subsequently, a 1 ml aliquot was incubated at 90°C for 15 minutes. Next, the debris present in saliva was pelleted by adding 40 µl of neutralizer solution (DNA Genotek Inc.) and centrifuging samples at 1500 x *g* for 10 minutes. The supernatant was collected and centrifuged at 16000 x *g* for 20 minutes. Then, the supernatant was ultracentrifuged at 160000 x *g* for one hour (Optima LE-80K ultracentrifuge equipped with a ti70 fixed angle rotor; Beckman). The polyallomer tubes for ultracentrifugation were pre-treated with RNA*Zap* (Life Technologies) to remove RNAse activity. After the first ultracentrifugation step, the pellet was resuspended in 1x PBS (pH 7.4) and ultracentrifuged at 160000 x *g* for one hour. Afterwards, the pellet, containing vesicles and protein aggregates [17], was resuspended in RNAse-free water and stored at -80°C.

Total RNA, including small miRNAs was isolated from the extracellular fraction of saliva using the miRNeasy mini kit (Qiagen), following the manufacturer's instructions. Samples were spiked with 250 fmol *C. elegans miR-39* for normalization of the expression data. 125 ng total RNA was reverse transcribed using looped primers for specific miRNA cDNA synthesis (Megaplex RT primers human pool A & Taqman mircoRNA RT kit; Life Technologies) on a PCR gradient thermal cycler (TC-5000; Techne). For all samples, 1:75 dilutions were made and an equal volume of products was mixed with reagents of the Taqman miRNA assay and the Taqman Fast Advanced mastermix (Life Technologies) for quantification of the miRNAs. qPCR was carried out on an ABI 7900HT sequence detection system (Life Technologies) and thermal cycling was for 10 minutes at 95 °C,

followed by 40 cycles of 15 seconds at 95 °C and one minute at 60 °C. All runs were carried out in triplicate and with a no-template control (NTC) on 384-well plates with three inter run calibrators (IRC). Raw qPCR data were analysed using the SDS Relative Quantification Software (version 2.3; Applied Biosystems). Cq values were transformed to a relative quantity to the external spike-in miRNA *cel-miR-39* in qbase+ software (Biogazelle).

Statistical analysis

We used SAS (version 9.3, SAS institute Inc., Cary, NC, USA) software for data management and statistical analyses. Demographic characteristics are represented as mean (standard deviation) for continuous variables or number (frequency) for categorical variables. miRNA expression data were $\log_{10}$-transformed to improve normality of the data.

We correlated the total screen time use with BMI (Figure 1A) and we evaluated the associations of screen time, physical activity and the BMI of the child using linear regression models, taking into account children's age and gender. Using the LSMEANS option, we studied mean BMI values (with 95% confidence intervals (CI), Figure 1B) while accounting for gender and age and mean screen time values (with 95% CI, Figure 1C) for each physical activity category.

We evaluated the possible associations between screen time, BMI and physical activity and salivary extracellular miR-222 and miR-146a expression using the MIXED procedure to account for the hierarchical structure of the data (i.e. repeated measures for the miRNAs). This implies correlation between measurements within the same child, but no correlation between different children. The child identifier was included as a random effect nested within the schools in the mixed model. Restricted maximum likelihood estimation (REML) with unstructured autocorrelation was employed to estimate the coefficients and standard errors. A priori selected covariates were added to the model as fixed effects to correct for possible confounding. In models evaluating the associations between screen use / BMI / physical activity and miRNA expression as a dependent variable, the following variables were chosen: age of the child (continuous), gender, maternal education (two categories: up to high school diploma/college or university diploma), passive smoking (yes/no) and the extracellular RNA

concentration. Effect estimates of significant covariates were presented based on the model that evaluated the effect of screen time. The effect estimates are represented as a percentage change in extracellular miRNA expression. Q-Q plots of the residuals were checked to test the assumptions of the models. Due to variability and heterogeneity in gene expression profiles, it is difficult to distinguish true gene expression alterations associated with the outcome from passenger signals [18]. To study the robustness, we applied Monte Carlo simulation (10,000 times) for adjustment of the parameter estimates of the significant predictors of miRNA expression using the PLM procedure. Finally, we evaluated the prediction of the models using receiver-operating characteristics (ROC) plots. The children were stratified according to their reported screen time use with the 75th percentile as a cut-off point (19.5 hours per week). The ROC plots of the models are provided (Supplementary information).

RESULTS

The characteristics of the study population are reported in Table 1. The number of boys and girls included in the study were approximately equal. The average (standard deviation) age of the children was 10 (1) years and BMI averaged 17 (2.4) kg/m². Of the 80 children, 14 (17.5%) were underweight and 10 (12.5%) were overweight. 9 children were exposed to tobacco smoke by parental indoor smoking. The average TV screen use was 9.3 (5.5) hours per week, and average PC screen use was 4.8 (3.7) hours per week. The TV screen use was not correlated with the PC screen use (Pearson correlation coefficient r= 0.11; p=0.34). Total screen use, defined by the sum of TV and PC use, is on average 14.11 (6.93) hours per week.

Both before (Figure 1a) and after adjustment of BMI for child's age and gender, total screen use was positively correlated with children's BMI. Each one hour increment of screen use per week was associated with a 0.08 kg/m² higher BMI (95% CI: 0.004, 0.163; p=0.04), independent of children's age and gender. Compared with children without out-of-school physical activities, children with a high activity level have a 2.37 kg/m² (95% CI: -4.56, 0.17; p=0.0.4, overall p=0.16) lower BMI (Figure 1b). Physical activity levels were not associated with the total screen time use (overall p=0.36; Figure 1c).

Table 1: Demographic characteristics of the study population (n=80)

Characteristic	**Mean ± SD or n (%)**
Boys	37 (46.3%)
Age, yrs	10.44 ± 0.97
BMI, kg/m² [a]	17.01 ± 2.42
Underweight	14 (17.5%)
Normal weight	56 (70%)
Overweight	10 (12.5%)
Passive smoking	9 (11.3%)
Maternal level of education	
Up to high school diploma	26 (32.5%)
College or university diploma	54 (67.5%)
Caucasian	73 (91.3%)
TV screen use, hours per week	9.32 ± 5.46
Computer screen use, hours per week [b]	4.77 ± 3.65
Total screen use, hours per week [b]	14.11 ± 6.93
Physical activity [c]	
None	7 (9%)
Low	33 (42.3%)
Middle	21 (26.9%)
High	17 (21.8%)

[a] BMI categorization based on age and gender specific children's growth curves for Flanders 2004; [b] n=77 (included in the analysis); [c] low: ≤ 3 hours, middle: >3 and <6 hours per week, high: ≥6 hours per week, n=78.

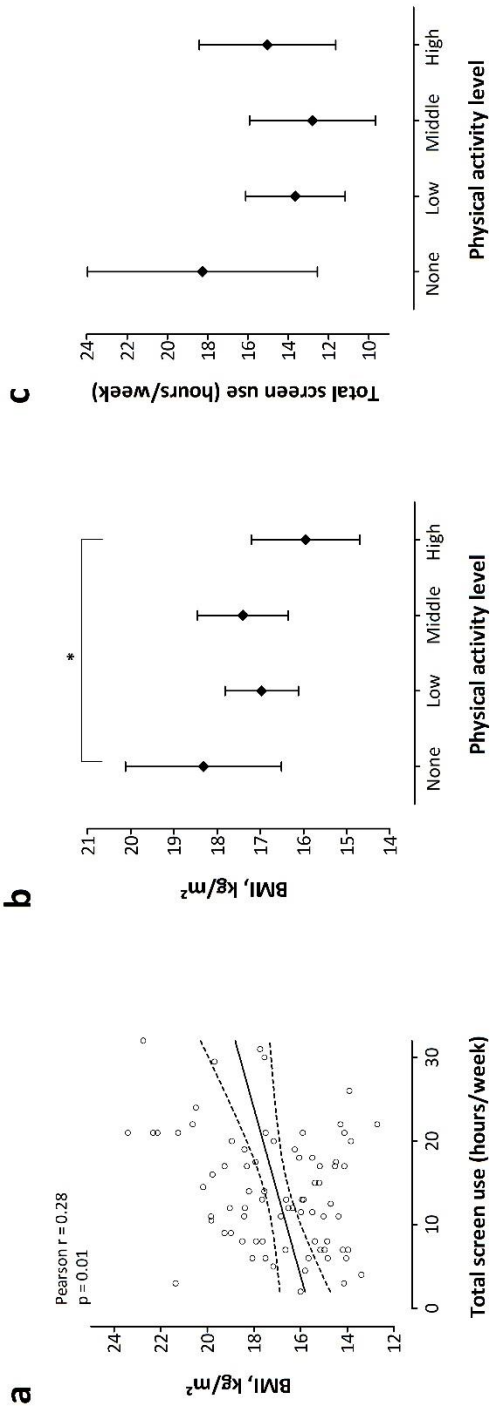

Figure 1: Correlation between total screen use, physical activity and body mass index (BMI). a: correlation between total screen use and BMI (line represents the linear fit of the correlation with the 95% confidence intervals), b: Mean (95% CI) BMI in function of the physical activity level category, adjusted for age and gender of the child, c: Mean (95%CI) total screen time in function of the physical activity level. * p<0.05

The association between total screen time use, BMI and physical activity on salivary extracellular miR-146a and miR-222 expression was evaluated using a mixed model, which took into account the repeated measurements of the miRNAs for each child, while accounting for gender, age, passive smoking, maternal education and the RNA content of saliva (Table 2).

Total weekly screen time use was significantly associated with salivary extracellular miR-146a and miR-222 expression. One hour increase in weekly screen time was associated with 3.44% higher (95% CI: 1.34, 5.58; p=0.002) extracellular miR-222 levels in saliva. One hour increase in weekly screen time was associated with 1.84% higher (95% CI: -0.04, 3.75; p=0.055) levels of salivary extracellular miR-146a. Monte Carlo adjusted confidence intervals and p-values for the associations of the miRNAs with screen time are confirmative and given in Table 3. No significant association between either BMI or physical activity and miR-222 and miR-146a was observed.

In addition to screen time use, salivary extracellular miR-222 was significantly higher in children exposed to passive smoking. On average, passive smoking was associated with 55% higher (95% CI: 1, 139; p=0.044) miR-222 levels in the extracellular fraction of the saliva. Age of the child was inversely associated with both miR-222 and miR-146a. A 1 year increase in age was associated with a 16.11% lower (95% CI: -27.15, -3.41; p=0.01) saliva extracellular level of miR-222 and a 15.86 % lower (95% CI: -25.95, -4.37; p=0.01) saliva extracellular level of miR-146a.

Table 2: The estimated changes in miR-222 and miR-46a associated with screen time, BMI and physical activity as evaluated in separate models

	Effect size (95% CI)	**p-value**
miR-222		
Total screen time	3.44 (1.34, 5.58)	0.0016
BMI, continuous	-2.20 (-8.07, 4.04)	0.48
BMI, categorical		0.59
Underweight	18.51 (-18.57, 72.46)	
Normal weight	Ref	
Overweight	13.26 (-25.97, 73.30)	
Physical activity		0.74
None	Ref	
Low	10.19 (-35.15, 87.24)	
Middle	-2.74 (-44.31, 69.82)	
High	-12.75 (-51.85, 58.09)	
miR-146a		
Total screen time	1.84 (-0.04, 3.75)	0.055
BMI, continuous	-4.08 (-8.75, 0.84)	0.10
BMI, categorical		0.43
Underweight	12.74 (-16.88, 52.90)	
Normal weight	Ref	
Overweight	-15.35 (-40.23, 19.90)	
Physical activity		0.71
None	Ref	
Low	27.67 (-17.99, 98.79)	
Middle	15.97 (-28.12, 87.07)	
High	19.81 (-27.22, 97.24)	

Effect sizes represent the mean % change with the 95% confidence intervals in miRNA for a 1-hour increment in weekly screen time/ 1kg/m² increase in BMI or compared to a reference group . Models were adjusted for gender, age, passive smoking exposure, maternal education and RNA content of the extracellular fraction.

Table 3: The estimated changes in miR-222 and miR-146a associated with screen time, adjusted using Monte Carlo simulation

	Effect size (adjusted 95% CI)	**Adjusted p-value**
miR-222	3.44 (1.34, 5.62)	0.0022
miR-146a	1.84 (-0.07, 3.78)	0.060

Effect sizes represent the mean % change with the 95% confidence intervals in miRNA for a 1-hour increment in weekly screen time. Models were adjusted for gender, age, passive smoking exposure, maternal education and RNA content of the extracellular fraction. Monte Carlo simulation was repeated 10,000 times.

DISCUSSION

Already from childhood onwards, sedentary behaviour is associated with adverse health outcomes [19]. From both intervention and longitudinal studies it is apparent that watching TV is related to overweight and obesity in children [19]. A meta-analysis including 170 studies in children, indicated a reduction in BMI with a reduction in the sedentary behaviour [19]. Due to their established role in developmental processes miRNAs have emerged as attractive candidates to explore the impact of exposures during critical windows of susceptibility [7].

In this context the key finding of our current paper is that children's weekly screen time use is positively associated with BMI and this parallels differential profiles of salivary extracellular miR-146a and miR-222 expression.

The relevance of these miRNAs in the framework of physical (in)activity becomes clear from studies in adults on acute exercise [20-22] and fitness [23], obesity [24], atherosclerosis [25], and diabetes [26-30]. For example, after acute exercise a transient up-regulation in circulating levels of miR-146a and miR-222 is observed [20,22]. Furthermore, this upregulation is also observed short-term after a training session in athletes on a sustained training schedule [20]. miR-222 can be indicative of a subject's fitness level based on its positive association with the maximal oxygen uptake (VO_{2max}) in healthy adults [23]. Compared to normal weight subjects, circulating miR-146a [31] and miR-222 levels [24] can be alternatively expressed in obese subjects. Many studies report altered expression levels of miR-146a in type 2 diabetes patients [26-30,32]. The functional significance of changes in the expression of these miRNAs in the extracellular fraction of saliva remains to be elucidated. Extracellular miRNAs could be involved in cell communication, but the process is currently poorly understood. Extracellular miRNAs are promoted as promising biomarkers [8], also for aspects related to lifestyle and diet [33]. Alterations in the (extracellular) miRNA abundance can reflect a dynamic response to metabolic stress in an attempt to re-establish homeostasis or a continuing response involved in disease development.

The findings of this study should be interpreted in the context of its limitations and strengths. As with other studies investigating the effect of screen time use or physical activity, the use of self-reported quantities of screen time and sport

activities might give some exposure misclassification. In our study, physical activity was based on the reported out-of-school sport activities. It is important to note, that apart from the out-of-school sport activities other leisure activities contribute to the total physical activity level of the child. Future studies could benefit from the implementation of activity trackers and diaries, to measure physical inactivity in a more objective manner. Second, we only quantified two miRNAs, which is insufficient to draw conclusions on biological mechanisms contributing to the early onset of adult disease. Finally, observational studies cannot prove the utility of a biomarker. Nevertheless, our proof-of-concept findings indicate changes in extracellular miRNAs in relation to childhood life-style factors that are particularly relevant in the development of obesity. miR-222 and miR-146a have been implicated in cardiovascular health outcomes in adults. Our findings add to this by highlighting a correlation between these miRNAs and life-style in healthy children, suggesting that their expression profiles might be linked to cardiometabolic processes, already from early-life onwards. Therefore there is a possible role of these miRNAs in translating the impact of sedentary behaviour on our genome, which should be further validated in intervention studies. Gene expression can be highly heterogeneous and this hampers capturing true alterations in gene expression associated with health outcomes or life style factors instead of passenger signals reflecting the heterogeneity [18]. We confirmed the robustness of our findings by employing a statistical resampling technique. A strength of our study is that we explored a non-invasive matrix by use of saliva which is preferable in population based studies including children.

CONCLUSION

To conclude, we showed expression of two extracellular miRNAs in saliva is associated with screen time use in children. The alterations in the salivary miRNA signature reflects epigenetic alterations related to an increased sedentary behaviour in school-aged children, which is particularly relevant since these miRNAs are associated with fitness and metabolic health in adults. Further research on this topic is warranted to understand the mechanism underlying the effects early-life inactivity on epigenetic alterations.

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SUPPLEMENTAL INFORMATION

Table S1: Logistic regression between high and low screen time use in association with saliva miR-146a and miR-222

	Odds ratio (95% CI) [c]	**p-value**
miR-222 [a]	6.39 (1.58, 25.82)	0.0092
miR-146a [b]	2.70 (0.37, 3.61)	0.33

Screen time use was dichotomized into two groups: high (>P75 reported screen time use) and low (<P75 reported screen time use). The logistic regression model was adjusted for age and gender, maternal education, exposures to passive smoking and the extracellular RNA content. [a]: the results correspond to panel C of the supplemental figure 1; [b]: the results correspond to panel B of the supplemental figure 1; [c]: the odds ratios for having a high screen time use are expressed for a 10-fold increment in microRNA expression.

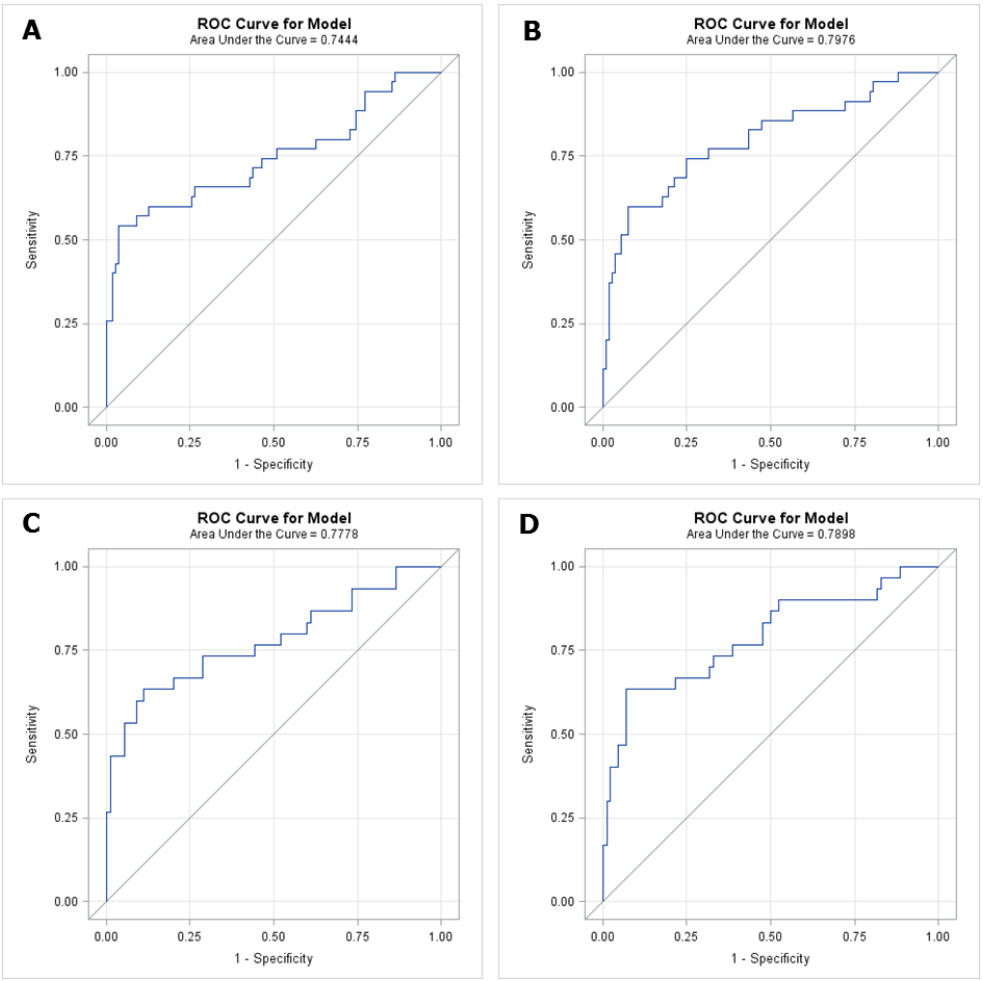

Figure S1: Receiver Operating Characteristic (ROC) curves to indicate the predictive power of our model to differentiate between individuals with high (>75th percentile) and low (<75th percentile) reported screen time use. (A) Performance of the model including all covariates (age, gender, maternal education, passive smoking exposure and extracellular RNA content). (B) Performance of the model including miR-222 and covariates. (C) Performance of the model including miR-146a and covariates. (D) Performance of the model including both miR-146a and miR-222 and the covariates.

Chapter 7

General discussion

In this doctoral dissertation the biological impact of environmental exposures and indicators of metabolic health was investigated on molecular markers using observational studies. We assessed the effects of fine and ultrafine particulate matter exposure, as well as indicators of physical (in)activity on two extracellular miRNAs, which were specifically targeted based on their relation with air pollution exposures and cardiometabolic outcomes, as shown by previous studies. Furthermore, we evaluated mitochondrial DNA (mtDNA) content and telomere length in association with a large set of environmental exposure biomarkers by means of a multipollutant approach. Both markers are considered sensitive molecular sensors for environmental effects. Additionally, we studied mtDNA content in association with metabolic hormones in newborns, to evaluate if the relation between metabolic balance and mitochondrial function may already be established from early life onwards. For this purpose, we employed studies on subjects of various age groups, including newborns, children and adults, hereby representing a large part of the population. The main findings of this work are summarized in Table 1.

The novelties of this dissertation include:

- The development of a technique for investigation of salivary extracellular miRNAs which embodies a non-invasive alternative in children studies to evaluate the molecular effects of environmental factors.
- The proof-of-concept of a relation between extracellular miRNAs and air pollution exposure and physical (in)activity indicators in childhood that may be exemplified by studying targeted miRNAs.
- The use of multipollutant models to capture the totality of environmental exposures for the evaluation of mtDNA content and telomere length as sensitive molecular sensors for a wide range of environmental pollutants.
- The evaluation of the relationship between metabolic status and mitochondrial function, assessed by mtDNA content, in early life.

Table 1: Summary of this doctoral dissertation

Chapter	What is known	What this study adds	Conclusions and perspectives
Chapter 2	▪ Ultrafine particles (UFP) are a part of particulate matter (PM) that due to their small size can penetrate the body more deeply ▪ Children are particularly vulnerable for PM-associated health effects ▪ Epigenetic mechanisms including microRNAs (miRNAs) may be involved in the development of PM-induced health effects	▪ Saliva can be used as a non-invasive biofluid for the characterization of extracellular miRNAs in children ▪ UFP concentrations at the school site are associated with children's saliva extracellular miR-222 expression ▪ miRNAs present in the extracellular environment are biomarker candidates to evaluate the biological effects of air pollutants in children	▪ Alterations in miRNAs levels may present a rapid response to air pollutants ▪ The effect was only observed in relation to UFP and not to $PM_{2.5}$, suggesting a potential health risk of UFP ▪ The biological effects of UFP exposure remain to be further explored in parallel to health effects of PM_{10} and $PM_{2.5}$
Chapter 3	▪ The placenta enables passage of substances, including exogenous compounds, to the foetus ▪ *In utero* chemical exposure may contribute to adverse health outcomes at birth and in later life ▪ mtDNA content can reflect environmental perturbations	▪ Neonatal exposure to environmental pollutants may affect placental mtDNA content ▪ Neonatal exposure to β-hexachlorocyclohexane (β-HCH), arsenic and thallium is associated with placental mtDNA content	▪ Placental mtDNA content is sensitive to low levels of exposure, which can be detected using a multi-pollutant approach ▪ The placental mitochondria may be important targets for numerous pollutants, including metals and organochlorines even at low concentrations ▪ The impact of environmental exposure on placental function should be further investigated

Table 1 (continued): Summary of this doctoral dissertation

Chapter	What is known	What this study adds	Conclusions and perspectives
Chapter 4	▪ Telomere length and mtDNA content are associated with the aging phenotype ▪ Exposure to environmental chemicals contributes to (premature) onset of age-related diseases ▪ Environmental chemical exposures can affect biomolecular markers of aging	▪ Exposure to Cd, Cu, HCB, PFHxS and PFOS was associated to mtDNA content in adults ▪ Exposure to Cu, Sb, Hg and PFOA was associated to telomere length ▪ Multipollutant modelling captures the complexity of co-exposures in relation to biological responses	▪ Multipollutant approaches are powerful techniques to limit the influence of confounding co-exposures ▪ Our results support the hypothesis that environmental pollution may impact biological aging, which can be on the path of exposure-related health effects
Chapter 5	▪ Neonatal insulin and leptin levels reflect variation in the metabolic status of the newborn ▪ Metabolic disturbance is linked to mitochondrial dysfunction in adults ▪ mtDNA content is a marker of mitochondrial function	▪ Cord blood insulin and leptin levels showed a positive association with mtDNA content ▪ Mitochondria can reflect metabolic variation in early life	▪ Variation in mtDNA content associated with the metabolic status may suggest metabolic programming already at birth ▪ The relation between metabolic balance and mitochondrial function in early life should be further explored
Chapter 6	▪ Physical inactivity and sedentary behaviour contribute to the onset of diseases and premature deaths ▪ Extracellular miRNAs are linked to a wide range of health and disease outcomes, such as cardiovascular and metabolic disorders	▪ miR-222 and miR-146a are positively associated with the screen time, but not with physical activity or body mass index	▪ Proof-of-concept that candidate extracellular miRNAs, which are associated with metabolic health in adults, are linked to childhood sedentary behaviour indicators ▪ Mechanisms underlying the molecular effects of early life physical inactivity and sedentary behaviour need further investigation

FINDINGS IN CONTEXT TO THE LITERATURE

Extracellular miRNAs

miRNA expression levels are dynamically regulated and are responsive to various environmental stimuli [1]. Furthermore, alterations in miRNA expression profiles can highly impact cellular gene expression and hereby distort biological functions, which is involved in various disease mechanisms. Extracellular miRNAs may embody a unique communicative purpose of the cell that is shown to be involved in diverse physiological and pathological mechanisms [2]. We and others provided evidence linking the extracellular miRNA expression profiles to environmental factors. In this thesis, we showed that ultrafine particulate matter exposure and screen time were associated to extracellular miRNAs in saliva of children by using a targeted approach. As a proof-of-concept, we specifically chose to evaluate miRNAs that were relevant for air pollution and cardiometabolic health status based on previous literature.

Extracellular miRNAs in relation to air pollution

Currently there is evidence supporting a role for extracellular miRNAs in the biological effects of environmental pollutants in humans, including particulate air pollution [3-5], health outcomes related to asbestos exposure [6], phthalates [7] and cigarette smoke [8]. Here below, the evidence on particulate matter (PM) exposure will be summarized.

PM exposure can induce a rapid increase in the generation and release of microparticles that may have prothrombotic features, shown by an *in vitro* model [9] as well as findings in adults [10,11]. Additionally, an overall increase in expression of extracellular vesicle-associated miRNAs was observed after PM exposure [5]. The effect of PM exposure on extracellular miRNAs was studied in steel plant workers, who are exposed to high concentrations of metal-rich PM at their workplace. Shortly after PM exposure, an upregulation of plasma vesicles expression of miR-128, which is related to cardiovascular disease development, was observed [3]. Furthermore, long-term workplace PM exposure was associated to differential expression levels of extracellular vesicle miRNAs related to inflammation and coagulation [4]. In overweight and obese subjects, short-term PM_{10} exposure was associated with increased extracellular vesicle numbers, mainly from

macrophages and thrombocytes. PM_{10} exposure was associated to extracellular vesicle levels of nine miRNAs, of which some mediated the procoagulant effects of PM_{10} [12]. In an elderly population, extracellular vesicle miRNA levels were mainly associated with long-term $PM_{2.5}$ exposure, but not with short-term exposures. A set of extracellular miRNAs, that are involved in cardiovascular-related pathways, was differentially expressed in association with long-term (6 months or one year) $PM_{2.5}$ exposure levels [5]. All together, these results provide a framework that supports the hypothesis that extracellular vesicles, which may contain specific miRNA cargo for intercellular communication, are involved in the mechanisms underlying exposure-related health effects, among which cardiovascular disorders [13]. As such, air pollution exposure may result in an increased shedding of vesicles that may have procoagulant properties and may result in an adapted transport of extracellular miRNAs. The extracellular miRNAs, that can be complexed in vesicles or with proteins, may be captured by recipient cells and favour post-transcriptional regulation of the gene expression in these cells.

Extracellular miRNAs in relation to metabolic health status

A wealth of evidence relates extracellular miRNA expression to metabolic health disorders, including obesity, type 2 diabetes and non-alcoholic fatty liver disease [14]. While the evidence of adapted extracellular miRNA expression profiles in relation to metabolic health indicators in adults is abundant, studies in children are far more limited. However, life style factors, including insufficient physical activity and increased sedentary behaviour may predispose to such metabolic disorders already from the early life onwards.

Recently, some studies investigated extracellular miRNAs in relation to obesity and obesity-related health outcomes in paediatric populations (Table 2). In children, extracellular miRNAs were associated to BMI [15,16] and sets of extracellular miRNAs were suggested as biomarkers for insulin sensitivity [17], endothelial dysfunction [18] and non-alcoholic fatty liver disease (NAFLD) [16]. Prats-Puig *et al.* were the first to study extracellular miRNAs in relation to childhood obesity [15]. The expression of 15 extracellular miRNAs differed between lean and obese children, and was associated with BMI and other obesity indicators. Additionally, they showed in a longitudinal cohort that alterations in extracellular miRNA levels can be associated to changes in the obesity status over time [15].

Second, in obese 2 to 6 years old children, extracellular miRNAs were differentially expressed between subjects with and without insulin resistance, and a set of three miRNAs (miR-200c-3p, miR-190a and miR-95) was suggested as biomarkers hereof [17]. Next, in 5 to 10 years old overweight or obese children, a set of extracellular miRNAs implemented in cardiovascular disease was studied in relation to endothelial function [18]. Three extracellular miRNAs were differentially expressed in endothelial dysfunction, independently of the obesity status [18]. Finally, twenty extracellular miRNAs related to NAFLD were studied in obese children and normal children from 8 to 18 years old [16]. For almost all miRNAs, the expression levels were elevated in the obese children and could discriminate between obese and healthy children. Furthermore, of the studied miRNAs, five correlated with the BMI of the children and the liver-specific miR-122 correlated with serum transaminases, that are indicative of liver damage [16].

Numerous studies in adults indicate that extracellular miRNAs are dynamically regulated in response to acute physical activity and they may reflect adaptive physiological responses to both acute and chronic exercise [19,20]. Yet, there is only one study investigating extracellular miRNAs in response to physical activity in children. The study included healthy competitive young male cyclists with a mean age of 14 years old [21]. Extracellular miRNAs related to angiogenesis and vascular integrity miR-16, miR-21 and miR-126 were studied in response to high-intensity interval trainings and low-intensity high volume training. The angiogenic miRNAs were upregulated during and shortly after a low-intensity high volume training session, but were not affected by high-intensity interval trainings [21]. The epidemiological evidence of extracellular miRNAs in association to sedentary behaviour and/or physical inactivity remains scarce. To the best of our knowledge, no studies reported on extracellular miRNAs in relation to sedentary behaviour or inactivity either in adults or in children. To conclude, extracellular miRNA profiles may be associated with clinical manifestations of metabolic adversity in childhood. In addition, already in childhood extracellular miRNAs may be sensitive to stimuli such as exercise to accommodate adaptive physiological responses. In the aforementioned studies, the extracellular miRNAs are mainly studied to discriminate between affected individuals and healthy children in light of their application as biomarkers. Yet, this limits the insights of miRNA expression and metabolic health in a general population of children.

Table 2: Overview of studies on extracellular miRNAs in association with obesity-related health outcomes in children

Population	Outcomes	Identified miRNAs	Functions related to the miRNAs	Ref
Discovery: 10 healthy children, aged 9 years Validation study 1 (cross-sectional): 40 obese children and 85 lean children, aged 9 years	Obesity Obese children compared to lean children	Characterization of miRNA expression levels using an untargeted approach ↑ expression in obese children: miR-486-5p, miR-486-3p, miR-142-3p, miR-130b, miR-423-5p, miR-532-5p, miR-140-5p, miR-16-1, miR-222, miR-363, and miR-122 ↓ expression in obese children: miR-221, miR-28-3p, miR-125b and miR-328	miR-486, miR-142-3p, miR-221 and miR-28-3p were put forward as novel biomarkers for obesity risk and classification. miR-142-3p: inflammatory events & obesity miR-486: NF-κB signalling & insulin and triglyceride metabolism miR-221: cell cycle	15
Validation study 2 (longitudinal): 45 normal weight children at age 7 and 10	Change in BMI over time	Increase in BMI over time: ↓: miR-486-5p ↑: miR-532-5p, miR-222 and miR-122 Decrease in BMI over time: ↑: miR-28-3p and miR-142-3p	miR-28-3p: insulin sensitivity & stress responses	
10 normal and 20 obese children, aged 8 to 18 years	Obese children compared to normal children BMI	↑ expression in obese children: miR-15b, miR-199a, miR-222, miR-223, miR-181, miR-122, miR-23a, miR-27b, miR-21, miR-34a, miR-192, miR-29a, miR-214, miR-155, miR-191 and miR-103a miR-223, miR-21, miR-29a, miR-150a and miR-103a were positively correlated to BMI	All miRNAs studied were selected on the basis of having a known role in non-alcoholic fatty liver disease (NAFLD) pathogenesis and/or being previously evaluated in adult NAFLD patients	16

Population	Outcomes	Identified miRNAs	Functions related to the miRNAs	Ref
	Serum transaminases	miR-122 was positively correlated to both alanine transaminase and aspartate transaminase		
12 obese children of which 6 were insulin resistant (IR), aged 2 to 6 years	IR children compared to matched controls	miR-200c-3p, miR-190a and miR-95 were differently expressed in IR obese children, both at fasting and after an oral glucose tolerance test	miR-200c: obesity & putative role in regulation of food intake and body mass accretion miR-190a: IGF and insulin signalling miR-95: adipocyte differentiation & adipogenesis	17
60 overweight or obese children, aged 5 to 10 years	Endothelial dysfunction compared to normal endothelial function	↑ expression in endothelial dysfunction: miR-365b-3p ↓ expression in endothelial dysfunction: miR-125a-5p and miR-342-3p	miR-125a, miR-342 and miR-365b were previously reported in association with cardiovascular disorders	18

Contribution of our findings to the literature

Considering their communicative capacity, extracellular miRNAs are put forward as a putative mechanism involved in numerous health outcomes. They are attractive candidates to study as early molecular effect markers and interesting biomarker candidates, because they are stably present in bodily fluids.

From the aforementioned studies, it is apparent that there is increasing evidence on extracellular miRNAs in association with PM exposure and the metabolic health status. The early life period embodies an important time window in life for the influence of environmental stressors and their impact on health outcomes in later life [22]. Nevertheless, studies investigating possible mechanisms underlying health effects associated with environmental factors in children remain scarce. Our findings add to this evidence as we specifically contribute to deepen the knowledge on early life environmental factors. We were the first to show that extracellular miRNAs are associated with PM exposure in children. While studies on extracellular miRNAs in relation to metabolic health indicators already were available in children, they mainly employed a case-control design: comparing levels of obese children to normal children. This limits their translation to a general child population. We studied metabolic health indicators in a set of healthy children representing the general population of children. While there is little or no overlap in the findings of differential extracellular miRNAs in relation to childhood obesity (Table 2), our findings add to the current evidence that differential expression of extracellular miRNAs implemented in inflammatory processes and cardiometabolic disorders may already occur in childhood, even before clinical manifestations hereof.

Furthermore, adult studies as well as the available studies in children on extracellular miRNAs predominantly focused on expression levels in blood. Our findings contribute herein, by proving the possibility of using extracellular miRNAs in saliva when investigating the effects of environmental factors. Furthermore, saliva miRNAs signatures of healthy individuals are shown to exhibit variability comparable to other biofluids [23]. Together with the ease to collect saliva in comparison to blood samples, especially in children studies, this highlights the applicability of saliva to study extracellular miRNA signatures. Although the use of saliva to study molecular effects has already shown potential and is attractive

because of many advantages over the use of blood [24], more research in support hereof is needed to validate and promote its use over blood in future research. Yet, the major challenge hindering the application of saliva in clinical and epidemiological studies is the lack of standardized procedures. As compared to other biological specimens such as blood or urine, no general strategies for the collection (e.g. stimulated vs. unstimulated, fasted vs. non-fasting, whole saliva vs. addition of stabilization liquids), the storage (e.g. time before freezing, ideal storage temperature) and the analysis (e.g. exclusion of bacterial components) of saliva exist.

mtDNA content and telomere length

mtDNA is particularly vulnerable to ROS-induced DNA damage, since they lack protective histones, their repair mechanisms are limited and the mitochondria are primary sources of ROS in the cells. Similarly, telomeres, that cap the end of the chromosomes, contribute genomic stability and are prone to ROS-induced damage due to their high guanosine content. While maintenance of mitochondrial function and telomere length is pivotal for normal cellular functioning, both can be affected by environmental stressors that may hereby distort cellular homeostasis. As such, mtDNA content and telomere length may be considered cellular sensors for environmental disturbances. We and others demonstrate the link between mtDNA content and telomere length with diverse environmental pollutants in different subgroups of the population. However, we were among the first to study these cellular sensors for early biological effects in a multipollutant approach, hereby investigating the associations of multiple pollutants simultaneously.

mtDNA content and telomere length in relation to environmental pollutants

Although the mitochondria are contemplated as important cellular targets of environmental pollutants and are often involved in toxicological mechanisms, the evidence on mtDNA content and environmental pollution is still limited. There is cumulative epidemiological evidence linking mtDNA content to air pollutants in different age groups. PM exposure was associated to mtDNA content in newborns [25-27], in adults [28-31] and in elderly [32]. Furthermore, associations were shown between mtDNA content and nitrogen dioxide [33], polyaromatic hydrocarbons [34-36] and benzo(a)pyrene [28], black carbon [37], benzene [38,39] and tobacco smoke [40-44]. We

studied mtDNA content in association with toxic metals, organochlorines and perfluorinated compounds, for which there is substantial less evidence available. Metal-components of PM were not associated with mtDNA content [31]. Studies linking mtDNA content to metal exposure in humans are limited. In a population with high arsenic exposure through drinking water, urinary arsenic levels were positively associated with mtDNA content [45]. Skin lesions of arsenic-induced carcinoma have higher mtDNA contents than skin lesions of non-arsenic induced carcinomas or normal skin [46]. *In vitro* evidence shows alterations in the mtDNA content of cells upon hexavalent chromium exposure [47,48]. Contrary, in a chromium-exposed population, mtDNA content was not significantly different compared to controls, although a trend for higher mtDNA content was observed [49]. In nematodes, methylmercury exposure resulted in a decrease in mtDNA content [50]. To our knowledge, no evidence of associations between mtDNA content and organochlorine exposure in humans is available. Additionally, there are no studies in humans but experimental evidence in rats linked perfluorinated compound exposure to higher mtDNA content [51,52]. From the evidence on mtDNA content in relation to environmental pollutants, a dual response is apparent. The amount of oxidative stress may play a role in this duality [31,53]. In response to mild oxidative stress or DNA damage, mitochondrial biogenesis can be stimulated and the number of mitochondria might be increased in order to compensate functional losses. In this way, an increase in the mtDNA content of the cell can be observed. In contrast, excessive oxidative stress may increasingly affect the mitochondria as such that apoptosis becomes inevitable, resulting in an overall decrease of the mtDNA content.

The association between environmental pollutants and telomere length is much more explored, and the evidence linking telomere length to air pollution is evident (as reviewed by Martens in [54]). Hence, here the existing literature on their association with toxic metal, organochlorine and perfluorinated compound exposure is summarized (Table 3), on which this dissertation focussed as well. Firstly, there is evidence suggesting a relation between telomere length and exposure to toxic metals, including antimony, arsenic, cadmium, chromium, lead, manganese and mercury. In adults of NHANES, antimony exposure was associated with shorter telomere length [55]. Exposure to arsenic was shown to be associated to both shorter [56] and longer [45,57,58] telomere lengths. In subjects with

arsenic-induced carcinoma due to high arsenic exposure (i.e. in Bangladesh), leukocyte telomere length was longer compared to non-exposed controls and exposed subjects without skin lesions [59]. However, a recent study showed no association between arsenic exposure and telomere length, but having a shorter baseline telomere length was associated with an increased risk for developing skin lesions [60]. Cadmium exposure was associated with shorter telomere length in placental tissue [61], in adolescents [62] and in adults [63-65]. In subjects occupationally exposed to lead, shorter telomere length was observed with higher exposure levels [66,67]. The inverse association between lead exposure and telomere length was also observed in children [68]. Contrary, in inhabitants of a highly polluted area, no association between placenta lead concentrations and placental telomere length was found [61]. In the US biomonitoring NHANES, lead exposure was not associated to telomere length [65]. One study investigated the methylmercury and telomere length in mothers and their newborns and did not found an association [69]. Second, there are studies pointing towards a link between telomere length and persistent organic pollutants. Findings on organochlorines indicate both increases and decreases in telomere length in association with exposure. In NHANES, PCB exposure was associated with longer telomere length [63,70,71]. Also in other studies, a positive association between PCB exposure and telomere length was observed, in both high [72] and low [73] exposed populations. However, another study reported shorter telomeres in association with occupational PCB exposure [74]. In a longitudinal study which investigated the telomere length 10 years apart, exposure to oxychlordane, trans-nonachlor and PCB-153 was associated to telomere attrition [75]. Furthermore, p,p'-DDE was associated to longer telomere length [73]. Exposure to perfluorinated compounds was associated with shorter telomere length in newborns, which could be mediated by increased oxidative stress [76]. Yet, another study investigated telomere length of pregnant women in association with various endocrine disruptors, among which PCBs and perfluorinated compounds and did not observe any significant associations [77]. A duality is also observed in telomere length's responses to environmental pollutants. While pollutants associated with shorter telomeres are thought to contribute to biological aging [54], pollutants associated with longer telomere length are hypothesized to show some carcinogenic potential [78] or to contribute to acute inflammation upon exposure [79-82].

Table 3: Overview of studies on telomere length and exposure to metals, organochlorine and perfluorinated compounds

Exposure	Population	Effect on TL	Ref
Antimony	Adults	↓ LTL	55
Arsenic	Adults	↓ LTL	56
	Adults	↑ TL in whole blood	45, 57-59
	Adults	TL in whole blood not associated with As	60
Cadmium	Newborns	↓ placental TL	61
	Adolescents, 12-16 yrs	↓ salivary TL	62
	Adults	↓ LTL	63-65
Lead	Adults	↓ TL in whole blood	66
	Adults	↓ LTL	67
	Children, 8 year old	↓ TL in whole blood	68
	Newborns	Placental TL not associated with Pb	61
	Adults	LTL not associated with Pb	65
Methylmercury	Mother newborn pairs	TL not associated with methyl-Hg	69
PCBs	Adults	↑ LTL	63, 70-73
	Adults	↓ lymphocyte TL	74
	Adults	↓ LTL	75
	Pregnant women	TL not associated with PCB exposure	77
Oxychlordane	Adults	↓ LTL	75
Transnonachlor	Adults	↓ LTL	75
p,p'-DDE	Adults	↑ LTL	73
Perfluoroalkyl substances	Newborns	↓ LTL	76
	Pregnant women	TL not associated with PFAS exposure	77

TL= telomere length, As= arsenic, Pb= lead, Hg= mercury, yrs= years, LTL= leukocyte telomere length, PCBs= polychlorinated biphenyls.

mtDNA content in relation to metabolic health status

Maintenance of mitochondrial function is pivotal for normal physiological functions, even beyond the cellular level. Mitochondrial dysfunction is a hallmark that is observed in a myriad of disorders [83]. Since mitochondria provide cellular energy and are able to modulate global energy, it may not be surprising that they are linked to metabolic disorders in which energy homeostasis is disrupted. Indeed, mitochondrial dysfunction is observed in metabolic disorders, including obesity or type 2 diabetes [84]. It is hypothesized that ROS and oxidative stress are key players implicated in the link between metabolic disorders and mitochondrial dysfunction. The inflammatory state associated with metabolic disorders may contribute to the increased production of ROS. Furthermore, the energy imbalance in metabolic disorders confronts the mitochondria with an overload of energy substrates without increased energy needs. As a consequence mitochondrial oxidative capacity may be overwhelmed, resulting in an overall increase of oxidative substrates and a shift in the oxidative balance [85,86]. Moreover, ROS associated with metabolic disorders such as obesity or diabetes may also contribute to associated (cardiovascular) health complications, since they add to inflammatory signalling and alterations in the vascular tone [87]. Nevertheless, the exact mechanisms underlying the relation between the mitochondria and metabolic disturbances remain poorly understood.

It is still under debate whether mitochondrial dysfunction plays a causal role, or increased ROS may deteriorate mitochondrial function. While the relation between metabolic disorders and mitochondrial dysfunction is acknowledged from studies in adults, little is known about a possible link in early life. Recently, there is evidence supporting a link between mtDNA content and metabolic determinants in early life. Intra-uterine growth restriction was already associated with higher placental [88] and cord blood mtDNA contents [89]. Placenta mtDNA content was lower in small for gestational age neonates [90] and cord blood mtDNA content was found to be lower in neonates that were small for gestational age and large for gestational age [91]. Additionally, placental mtDNA content was associated to birth weight [33] and infant growth [92]. BMI was associated with mtDNA content of obese children [93].

Contribution of our findings to the literature

In spite of a wealth of experimental studies that suggest involvement of the mitochondria in the toxicity mechanism of many environmental pollutants, the studies linking their exposure to mtDNA content in humans are scarce, especially in newborns. Until now, mtDNA content is mainly studied in relation to airborne pollutants. However, for telomere length there is more scientific evidence available for the effects of toxic metals and persistent organic compounds. Yet, the majority reported on single pollutant associations, without taking into account confounding co-exposures. We studied exposure to environmental pollutants in relation with mtDNA content in newborns and in adults, as well as their relation with telomere length in adults. Hereby we provide evidence that exposure to toxic metals, organochlorines and perfluorinated compounds may be linked to perturbations in the mtDNA content and telomere length. This further supports their application as cellular sensors to study early biological effects of environmental pollutants. Furthermore, studying these associations in a multipollutant approach has several advantages (i) taking into account co-exposures contributes to a better understanding of the influential exposures, of which effects can be different in presence of a correlated co-exposure, and to a better understanding of the health consequences of exposure to multiple pollutants (ii) investigating and reporting associations on all available pollutants diminishes scattered information by ignoring null-findings.

Mitochondrial dysfunction is considered a key hallmark of various diseases, among which metabolic disorders. In spite of their manifestation in later life, these disorders may already find their origin in early life. Since the exact mechanism underlying the relation between mitochondrial dysfunction and metabolic disturbances remains to be elucidated, the early life period may be pivotal in understanding this link. However, the evidence of mitochondrial effects of metabolic disturbances is limited. We showed in healthy neonates an association between mtDNA content and metabolic hormones, insulin and leptin. This suggests that mitochondria might reflect metabolic variation already in early life as exemplified by alterations in the mtDNA content that may accommodate a difference in the energy requirements or may reflect variation in the ROS levels.

FINDINGS IN CONTEXT TO THE STUDY DESIGN

General strengths and limitations of the study design

We had the opportunity to work with cross-sectional studies. In chapter 3, 4 and 5 we worked with the third cycle of the Flemish Environment and Health Study (FLEHS III, 2012 to 2015), which is a cross-sectional study that was designed and set up for human biomonitoring purpose in Flanders, Belgium [94]. In chapter 3 and 5 we used data from FLEHS III in newborns, and in chapter 4 we made use of data in adults. Cross-sectional studies are defined by their observational nature, indicating that there is no interference in the exposures or the outcomes. Yet, the observational study design comes with some strengths and weaknesses. Firstly, observational studies are ethically reasonable to perform since there is no interference in the exposure of participants. In this way no participants were deliberately exposed to environmental factors, instead we employed measurements of their actual exposure. Secondly, the cross-sectional design implies that all measurements of the exposures as well as the outcomes are quantified at the same time point. This constraints the temporal identification of events necessary to discriminate cause and effect. As such, no conclusion on causality can be made and reverse causation cannot be excluded. With reverse causation, the direction of cause leading to effects was wrongfully presumed, and the exposure might be affected by the outcome rather than vice versa. Even though for some associations reasonable logic may allude to which variable would be rather the cause than the effect, for other associations the temporality is less intuitive. Next, although observational studies are merely descriptive they contribute to the identification of novel hypotheses by means of the identification of influential predictors of the outcome. Furthermore, associations might be affected by confounding, which cannot be fully eliminated. Confounding variables may simply explain the findings, because of their independent relation to both the outcome and the exposure variable. While adjusting for potential confounders is possible in statistical analysis, residual confounding may still occur when a confounding factor remains unobserved or unidentified. Lastly, in spite of the lower level of evidence attributed to cross-sectional studies (e.g. compared to randomized experiments with matched individuals), they are pivotal for deriving scientific evidence of the biological effects of environmental factors.

Additionally, we made use of a panel study (the COGNAC study in chapter 2 and chapter 6) in which repeated measures of the participants were collected. Since this panel study is observational by nature, the aforementioned strengths and limitations remain relevant. Nevertheless, some additional perspectives should be addressed. Firstly, the collection of repeated measures enables the investigation of dynamics in the alterations in the outcomes. Furthermore, the repeated measures provide a greater statistical power than an equal number of observations derived from a cross-sectional study. Next, panel studies might provide stronger evidence of causality compared to cross-sectional observational studies. However, in the COGNAC study the repeated measures were collected on a short-term, which may be less ideal to derive evidence for a causal relationship.

Quality of the exposure and outcome variables

In observational studies, an accurate characterization of the exposures and outcomes is pivotal for valid analyses. Exposure misclassification presents a bias in the attribution of one's exposure, that contains a deviation of the true exposure. Exposure misclassification is important to acknowledge, since it may impact on associations. We explain this for the different study chapters below.

In chapter 2 the association between extracellular miRNAs and fine and ultrafine PM was studied. Here, we employed on-site measurements of $PM_{2.5}$ and UFP, as well as interpolated estimates of $PM_{2.5}$. While the on-site measurements represent the accurate, exact concentrations the children were exposed to, the interpolated concentrations present an estimation of the average $PM_{2.5}$ concentration during the last 24 hours at the home address and may have some exposure misclassification. First, they represent an estimation that approximates the true concentration at the home. However, the model used for this incorporates (i) information from CORINE land-cover data, obtained through satellite images, that provide information on the environment and land use with (ii) information from fixed monitoring sites, where the exact concentrations of PM in the air are measured and has a good predictive power [95]. Second, they reflect the $PM_{2.5}$ concentration at the residence, yet, children also spend a substantial amount of time at other places, such as school.

In chapter 3 and 4, we used internal biomarkers of exposure to environmental pollutants which quantify the pollutant or a metabolite in a biological matrix, such as urine or blood. Due to differences in uptake, metabolism, elimination and differences in behaviours and exposure routes, exposures can differ between individuals. Internal biomarkers of exposure are very accurate representations of an integrated exposure at the individual level. However, one of the most important problems when employing such data is the way of dealing with non-detectable values. They can be considered as left-censored missing values, for which we know the value is in the range between zero and the detection limit. By excluding observations with non-detectable values, some information on the exposure in the study population will be lost and may as such influence the inferences of the results. We included these observations with the use of a single-substitution method, in which all the non-detectable values were assigned the value of LOD/2. However, since the substitution technique lacks variation in the undetectable values and does not account for the uncertainty of the imputation, some exposure misclassification is introduced that might affect the estimates. Although multiple imputation is nowadays recommended [96], the use of this technique is not always reconcilable with statistical techniques used for dealing with multiple correlated pollutants. We limited the possibility of undetectable exposure levels to distort our associations by refraining exposures with a high undetectable portion based on EPA guidelines, which advices to solely use the LOD/2 substitution technique when no more than 15% of the observations are undetectable, or by including them as a binary variable.

In chapter 5, we investigated the association between mtDNA content and metabolic hormones insulin and leptin. Both hormones were quantified in cord blood and represent the actual concentrations of the hormones. Insulin concentration was undetectable for solely one observation, which was replaced by LOD/2, and leptin concentrations were detectable in all samples. As such, it is unlikely that exposure misclassification was present and distorted our associations.

In chapter 6, we employed self-reported data on screen time and physical activity. Self-reported data may be conflicted by response bias, in which subjects deliberately answer parts of the questionnaire wrongfully, for example with

answers that they think are socially acceptable or if they do not understand the question. In this case, we cannot exclude the presence of response bias in our exposures, which may impact our associations.

On the other hand, errors in the measurements of the outcome may also introduce information bias in observational studies. In this PhD dissertation, we used validated and reliable laboratory techniques for measurements of mtDNA content [27] and telomere length [97]. For quantification of the extracellular miRNAs in saliva, no golden standard or validated technique is available yet. We adopted techniques that are commonly used by other studies in the field. Further, we acknowledged the drawback of this method, which is unable to discriminate the origin of the miRNAs that may be from vesicles or protein complexes.

Environmental factor – molecular marker associations in observational studies

Observational studies are deemed appropriate to evaluate the association between environmental factors and molecular markers of early biological effects. Both are characterized at the same point in time, which may precede adverse health outcomes and disease onset. However, for interpretation of the associations it is important that the time window of exposure and effect are in accordance with each other. The exposures studied in this dissertation mainly reflect recent exposures (chapter 2: recent UFP and $PM_{2.5}$ exposure; chapter 3 and 4: some biomarkers of toxic metals reflect short-term metal exposure) or recent life style patterns (chapter 5: neonatal metabolic hormones; chapter 6: screen time and physical activity). Contrary, in chapter 3 and 4 some biomarkers of exposure (e.g. persistent pollutants) reflect a longer time window or chronic exposure. The molecular markers evaluated in this doctoral work are known to respond to both recent and chronic environmental factors [1,54,98]. As such, they can be associated with short-term or recent exposures to reflect a dynamic response. Additionally, their association with chronic exposure may rather reflect a steady-state adaptation. Nevertheless, the molecular markers can be indicative of (early) biological effects, which may be transient, as they may represent an intermediate molecular response on the continuum between exposure and disease. As such, they provide insight in (i) the potential of the environmental factor to induce

biological effects and (ii) the mechanism of the exposure-related disease development.

CONCLUSIONS

In this dissertation we aimed to identify associations between environmental factors and molecular markers of early biological effects, which may precede or link to adverse health outcomes. In newborns, we have investigated the association between mtDNA content in placenta and environmental pollutants and the association between cord blood mtDNA content and metabolic hormones. In primary school children, we focused on extracellular miRNAs and their association with exposure to fine and ultrafine PM as well as indicators of physical (in)activity. In adults, we studied mtDNA content and telomere length as biomarkers of aging in association with environmental pollutants.

We observed associations between placental mtDNA content and neonatal exposure to arsenic, thallium and β-hexachlorocyclohexane. In adults, mtDNA content was associated to cadmium, copper, hexachlorobenzene, perfluorohexane sulfonic acid and perfluorooctanesulfonic acid. Telomere length was associated to antimony, copper, mercury and perfluorooctanoic acid exposure. These findings contribute to evidence suggesting mtDNA content and telomere length as sensitive cellular sensors for environmental toxicants. Furthermore, they highlight the importance of studying associations to multiple pollutants, for which a multipollutant approach should be adopted.

We showed a positive association between saliva extracellular miR-222 and UFP exposure in primary school children. This contributes to the evidence linking particulate air pollution to extracellular miRNAs. Furthermore, extracellular miR-222 and miR-146a in saliva were associated with self-reported amounts of screen time use. We provided evidence in support of the use of saliva, and saliva extracellular miRNAs to study molecular effects of environmental factors in children. Additionally, our proof-of-concept findings favour the applicability of extracellular miRNAs to study environmental influences in healthy children.

Lastly, we showed a positive association between mtDNA content in cord blood and neonatal levels of insulin and leptin. This adds to the wealth of evidence that

relates disturbances in the metabolic balance to alterations in mitochondrial function, by suggesting a connection from birth onwards.

In general, the presented results contribute to the understanding that a wide range of environmental factors may impact on biological systems throughout the life course. The characterization of early molecular effects plays a crucial role in the identification of potential detrimental environmental factors and in the understanding toxicological responses.

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Scientific output

INTERNATIONAL PEER-REVIEWED PUBLICATIONS

1. **Vriens A**, Plusquin M, Baeyens W, Bruckers L, Den Hond E, Loots I, Nelen V, Schoeters G, Janssen BG, Nawrot TS. *Cord blood leptin and insulin levels in association with mitochondrial DNA content.* J Transl Med. 2018 August 13;16(1):224.

2. **Vriens A**, Provost EB, Saenen ND, De Boever P, Vrijens K, De Wever O, Plusquin M, Nawrot TS. *Children's screen time alters the expression of saliva extracellular miR-222 and miR-146a.* Sci Rep. 2018 May 29;8(1):8209.

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Vafeiadi M, Van Ijzendoorn MH, Vonk JM, **Vriens A**, Vrijheid M, Wang P, Wiemels JL, Wilcox AJ, Wright RJ, Xu CJ, Xu Z, Yang IV, Yousefi P, Zhang H, Zhang W, Zhao S, Agha G, Relton CL, Jaddoe VWV, London SJ. *Cohort Profile: Pregnancy And Childhood Epigenetics (PACE) Consortium.* Int J Epidemiol. 2018 Feb 1;47(1):22-23u.

5. **Vriens A**, Nawrot TS, Baeyens W, Den Hond E, Bruckers L, Covaci A, Croes K, De Craemer S, Govarts E, Lambrechts N, Loots I, Nelen V, Peusens M, De Henauw S, Schoeters G, Plusquin M. *Neonatal exposure to environmental pollutants and placental mitochondrial DNA content: A multi-pollutant approach.* Environ Int. 2017 Sep;106:60-68.

6. Schoeters G, Govarts E, Bruckers L, Den Hond E, Nelen V, De Henauw S, Sioen I, Nawrot TS, Plusquin M, **Vriens A**, Covaci A, Loots I, Morrens B, Coertjens D, Van Larebeke N, De Craemer S, Croes K, Lambrechts N, Colles A, Baeyens W. *Three cycles of human biomonitoring in Flanders - Time trends observed in the Flemish Environment and Health Study.* Int J Hyg Environ Health. 2017 Mar;220(2 Pt A):36-45.

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CONFERENCE PRESENTATIONS

1. **Vriens A**, Plusquin M, Baeyens W, Bruckers L, Den Hond E, Loots I, Nelen V, Schoeters G, Nawrot TS. *Cord blood leptin and insulin in association with mitochondrial DNA content.* DOHaD World Congress 2017 "Life course health and disease: Observations, experiments and interventions", Rotterdam, The Netherlands, 15-18 October 2017 **(poster presentation)**

2. **Vriens A**, Nawrot TS, Baeyens W, Bruckers L, Covaci A, De Craemer S, Den Hond E, Govarts E, Nelen V, Schoeters G, Plusquin M. *Placental mtDNA content and environmental exposure: a multipollutant approach.* Society of Toxiclogy 56th Annual Meeting and ToxExpo, Baltimore, Maryland, USA, 12-16 March 2017 **(poster presentation by Plusquin M)**

3. **Vriens A**, Nawrot TS, Baeyens W, Bruckers L, Covaci A, De Craemer S, Den Hond E, Govarts E, Nelen V, Schoeters G, Plusquin M. *Placental mtDNA content and environmental exposure: a multipollutant approach.* ISEE "Old and new risks: challenges for environmental epidemiology", Rome, Italy, 1-4 September 2016 **(poster presentation)**

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Curriculum Vitae

Annette Vriens was born on the 14th of February 1991 in Genk, Belgium. In 2009, she graduated from secondary school at Sint-Jozefsinstituut Bokrijk in Genk. In October 2009, she started her higher education in Biomedical Sciences at Hasselt University. She had a specific interest for the molecular functioning of the human body and the interaction between the environment and human health and did her master thesis in this field. In 2014, she obtained her master's degree in Biomedical Sciences cum magna laude. Her curiosity, eagerness to learn and to explore new things founded her interest in conducting a PhD research project. She acquired a personal PhD scholarship of the Bijzonder Onderzoeksfonds of Hasselt University. In October 2014, she started a PhD in the research unit of prof. dr. Tim Nawrot at the Centre for Environmental Sciences of Hasselt University. During her PhD, she studied early molecular markers of effect in relation with different environmental exposures as well as life-style factors. Furthermore, she was involved in human biomonitoring studies in Flanders and the hotspot region Genk-Zuid, which were organized and steered by the Flemish Government.

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Bureau C107b, wat initieel een ongunstige locatie leek wegens te ver van alle andere bureaus (en het steeds dreigende risico op administratieve taakjes van de promotor), bleek eigenlijk een gezellig bureau waar iedereen (ja, heel de onderzoeksgroep) graag vertoeft. We gaven vaak de aanzet voor feestjes en koffie met dat ietsje meer, waar iedereen meer dan welkom was, want de frigo, de mandjes op de frigo of de 'magical closet for researchers only' waren vaak gevuld… Koffie (met of zonder lekkers) bleef dan ook vaak niet alleen bij een korte pauze, maar mondde uit in toffe babbels waar we ons hart konden luchten. Deze momenten ga ik blijven koesteren!! Dries, een bureau delen met vijf vrouwen, met al ons gekakel moet je het als enige haan in het kippenhok toch vaak benauwd hebben gekregen (of in ieder geval omdat we de verwarming graag op het allerhoogst hadden in de winter)… Toch wist je altijd de rust (ten minste uiterlijk) en concentratie te bewaren. Ik denk dat dat goed was om ons af en toe te doen inzien dat we beter je voorbeeld volgden. Diana, een bureau delen met jou was altijd super. In het begin van jouw doctoraat waren we beiden vaak de 'uitverkorenen' om administratieve taakjes uit te voeren, maar het is niet alleen daarom dat ik je gemist heb tijdens je periode in Barcelona. We konden ook over alles babbelen en hebben een voorliefde voor huisdiertjes, schoenen en handtassen gemeen. Hoe leuk was dat?! Narjes, het organiseren van feestjes, lekker eten maken en denken aan alles, niets was je te veel en je deed het allemaal met zo veel enthousiasme dat het aanstekelijk was… Ik denk niet dat ik kan uitdrukken hoezeer ik dat in jou bewonder, Superwoman bestaat dus echt! Nog heel veel succes in de eindfase van je doctoraat! Maria, you're always kind, positive and warm and you just breathe tranquillity. I admire how you stayed true to this while working combined with raising Dimitris and little sleep. I guess you must have superpowers as well! While I'm writing this, you are finishing up your PhD work, close to your family in Greece. I wish you all the best!! Ellen, op de dagen dat je niet in Heist-op-den-Berg werkte, waren de dagen in Diepenbeek steevast leuker! We zijn samen door de laatste loodjes gesparteld, dat maakte het zeker aangenamer. We zagen ons bureau langzaam leeglopen en konden het naast de thesis-stress toch ook over ontspanning, toekomstplannen buiten de C107b en je prachtige dochter Estelle hebben.

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Working hard is important, but there is something that matters even more: believing in yourself. - Harry Potter and the Order of the Phoenix

Annette Vriens
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