TESI DOCTORAL UPF / 2022

Paula de Prado Bert

Early-life environmental exposures and molecular markers in children

Universitat Pompeu Fabra Barcelona

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A l’entramat de vincles invisibles als ulls que han fet el camí més planer

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El doctorat és sens dubte, una experiència complexa, on es sap que la resiliència és la companya indispensable que et permet gaudir, superar-te i aprendre de tot el que passa i t'envolta. En el meu cas, el doctorat ha sigut una etapa de creixement personal i un repte, on he descobert que no poder amb tot no és un fracàs, i que posar límits de vegades és la millor solució per mantenir-se en peu. Com en tota experiència, el més bonic d'haver recorregut aquest camí ha sigut l'acompanyament que he tingut al llarg dels anys, el qual ha sigut un pilar invisible sense el que no hagués pogut viure de forma pausada aquesta etapa.

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També, m’agradaria agrair molt fortament a tota aquella gent que m’ha ajudat en el meu dia a dia, a totes aquelles persones que m’han fet tocar de peus a terra quan era necessari, i m’han fet aixecar el vol sempre que calia. Aquests quatre anys m’han ensenyat que la vida va

més enllà de la feina, i que trencar les bombolles és necessari per no perdre's.

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especial a en Berni, a qui descobrir m'ha fet diferent, a l'Agnès i les seves ganes de no parar, a l'Olmo i la seva ironia, a la Mariona i a la nostre manera d'estimar-nos i créixer, a la Pele i al seu inconformisme, a la Helen i a la seva manera d'apreciar-ho tot, i finalment, a la Martina i a l'Anna per la seva delicadesa, per ser abraçades quan calia, per ser orelles quan era necessari i per fer de *Puigmartí* una bombolla de calma i d'alliberacions.

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In nature nothing exists alone.

- Rachel Carson

Abstract

Exposure to environmental risk factors during *in utero* life or childhood has been linked to an increased risk of developing several diseases. However, the underlying biological mechanisms are poorly understood. The main aim of this doctoral Thesis is to investigate how early-life environmental exposures can affect molecular markers related to inflammation and biological aging in children.

On one hand, biological aging was assessed by measuring epigenetic age acceleration in two tissues: blood and placenta. In blood and in the context of the exposome, which included more than 100 exposures measured during the first years of life, it was observed that exposure to tobacco smoke during pregnancy and in childhood, and exposure at home to PM_{abs} in childhood were associated with increased epigenetic aging. In contrast, in the placenta, ambient air pollution and maternal smoking did not seem to affect epigenetic aging.

On the other hand, the inflammatory response to short- (1 day and 1 week) and medium- (1 year) term exposure to air pollution was examined by quantifying the levels of 36 proteins (adipokines, cytokines or apolipoproteins) in the children's plasma. Short-term exposure to different air pollutants during childhood was associated with increased levels of hepatocyte growth factor (HGF), which is involved in tissue reparation, and interleukin-8 (IL8), which activates inflammation. Furthermore, the results suggested that HGF might be

involved in the association between air pollution and systolic blood pressure, although further studies are needed to investigate its causality.

Finally, given the relevance of air pollution exposure in early life for child health, we aimed to identify the determinants of home-indoor and personal NO_2 levels in pregnant women. We found that non-European ancestry, the use of a gas cooker, outdoor levels of NO_2 and, exposure before Covid-19 pandemics increased home-indoor and personal NO_2 levels.

In conclusion, the results suggest accelerated biological aging and increased inflammation in children exposed to tobacco smoke and air pollution. These biological mechanisms may partly explain the effects on children's health, including increased systolic blood pressure. Future research on the molecular mechanisms of the early life exposome may help to give plausibility to the epidemiological associations and thus lead to further public interventions.

Resum

L'exposició a factors de risc ambientals durant la vida intrauterina o la infància ha estat relacionada amb un major risc a desenvolupar diverses malalties. Tot i això, els mecanismes biològics subjacents són poc coneguts. L'objectiu principal d'aquesta Tesi doctoral és investigar com les exposicions ambientals durant l'inici de la vida poden afectar diversos marcadors moleculars relacionats amb la inflamació i l'envelliment biològic en els infants.

D'una banda, es va avaluar l'envelliment biològic mesurant l'acceleració de l'edat epigenètica en dos teixits: sang i placenta. En sang i en el context de l'exposoma, el qual incloïa més de 100 exposicions mesurades durant els primers anys de vida, es va observar que l'exposició al fum del tabac durant l'embaràs i en la infància, i l'exposició dins la llar a PM_{abs} durant la infància s'associaven a un major envelliment epigenètic. En canvi, en la placenta, la contaminació atmosfèrica i el tabaquisme matern no semblaven afectar l'envelliment epigenètic.

D'altra banda, es va examinar la resposta inflamatòria a la contaminació atmosfèrica a curt (1 dia i 1 setmana) i mitjà termini (1 any) a través de la quantificació dels nivells de 36 proteïnes (adipoquines, citocines o apolipoproteïnes) en el plasma dels infants. Es va veure que l'exposició a curt termini a diferents contaminants atmosfèrics durant la infància s'associava a un augment dels nivells del factor de creixement dels hepatòcits (HGF), que participa en la

reparació dels teixits, i de la interleucina 8 (IL8), que activa la inflamació. A més, els resultats suggerien que l'HGF podria estar involucrat en l'associació entre la contaminació atmosfèrica i la pressió arterial sistòlica, encara que calen més estudis per investigar-ne la causalitat.

Finalment, atesa la importància de la contaminació atmosfèrica en els primers anys de vida per a la salut infantil, ens vam proposar identificar els determinants dels nivells personals i dins la llar de NO_2 en dones embarassades. Vam trobar que l'origen ètnic no europeu, l'ús d'una cuina de gas, els nivells de NO_2 a l'exterior de casa i, l'exposició abans de la pandèmia de la Covid-19 augmentaven els nivells personals i dins de la llar de NO_2.

En conclusió, els resultats suggereixen un envelliment biològic accelerat i major inflamació en els infants exposats al fum del tabac i a la contaminació atmosfèrica. Aquests mecanismes biològics podrien explicar, en part, els efectes sobre la salut infantil, inclòs l'augment de la pressió arterial sistòlica. Futures investigacions sobre els mecanismes moleculars de l'exposoma durant els primers anys de vida poden ajudar a donar versemblança a les associacions epidemiològiques i així incidir en millores de salut pública.

Preface

The research described in this Thesis has been carried out at the Barcelona Institute of Global Health (ISGlobal), Barcelona, Spain, between September 2018 and June 2022. It was conducted under the supervision of Dr. Mariona Bustamante and Prof. Dr. Jordi Sunyer. The present Thesis complies with the procedures and regulations of the Biomedicine PhD program of the Department of Medicine and Life Sciences of the University Pompeu Fabra, Barcelona, Spain.

The main aim of the thesis was to provide further knowledge on the underlying biological mechanisms of early life environmental exposures, with a special focus on maternal smoking and air pollution. In particular, the present thesis contributes to the understanding of 1) the influence of early life exposome on epigenetic age acceleration in children, 2) the influence of NO_2 and $PM_{2.5}$ exposure and active maternal tobacco smoking during pregnancy on placental epigenetic age acceleration, 3) the medium and short term effects of NO_2, PM_{10} and $PM_{2.5}$ exposure during childhood on blood plasmatic proteins and blood pressure, and 4) the determinants of indoor and personal NO_2 air pollution levels during pregnancy.

This Thesis contains four original research papers first authored by the PhD candidate (2 published, 2 in preparation). For all the

scientific papers, the PhD candidate formulated the research objective, conceptualized the study design, performed the data management and statistical analyses, interpreted the findings, and wrote the scientific articles. Besides, the four manuscripts enclosed in this Thesis, the PhD candidate have co-authored seven original research papers (see Appendix). Five of these co-authored articles also contributed on the evidence of the influence of environmental exposures in biological functions. Two of them evaluated the association between air pollution and greenspace exposure with telomere length in preschool children (Miri et al. 2020, Moslem et al. 2020). Another one was an epigenome-wide meta-analyses studying the levels of maternal iron status in early pregnancy and DNA methylation in offspring within the framework of the Pregnancy and Childhood Epigenetics (PACE) consortium (Taeubert et al. 2022). The last two are still under construction. In one of them we are studying maternal and paternal determinants in offspring epigenetic age acceleration, and the other one is a genome-wide meta-analyses on air pollution and lung function within the framework of the PACE consortium.

During this Thesis, the PhD candidate was closely involved in the development, fieldwork, and data management of two cohorts: *INfancia y Medio Ambiente – Environment and Childhood* (INMA) - Sabadell and Barcelona Life Study Cohort (BiSC). Throughout the Thesis period, she has collaborated with the redaction of different general standard operating procedures (SOPs) for the BiSC fieldwork, developed tasks as a laboratory technician within the BiSC

project, organized and updated codebooks for INMA cohort, participated in the Covid-19 BiSC study to evaluate the effect of the pandemics on mental health, and have been responsible of the news section of the BiSC website (https://www.projectebisc.org/en/news/). Additionally, the PhD candidate attended several national and international conferences, where she was able to present the results of the different manuscripts.

Besides the research projects, she was awarded from the 2nd call for Research Proposals of the Planetary Wellbeing Initiative 2020, through which she was able to attend to additional epidemiological and statistical courses. Moreover, she supervised one End of Degree's Thesis (Biomedical Science Degree, University of Barcelona), peer-reviewed three scientific articles and was involved in other dissemination activities including writing blogs and scientific talks for children.

Abbreviations

Bcell	B lymphocytes
BiB	Born in Bradford
BiSC	*Barcelona Life Study Cohort*
BMI	Body Mass Index
BP	Blood pressure
BPA	Bisphenol A
BUPA	N-Butyl paraben
CPC	Control placental clock
CRP	C-reactive protein
DBP	Diastolic blood pressure
DBPs	Water disinfection by-products
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DEP	Diethyl phosphate
DETP	Diethyl thiophosphate
DMDTP	Dimethyl dithiophosphate
DMP	Dimethyl phosphate
DMTP	Dimethyl thiophosphate
DNAm	Deoxyribonucleic acid methylation
DOHaD	Developmental Origins of Health and Diseases
EDEN	*Étudie des Déterminants Pré et Postnatals du Développement et de la Santé de l'Enfant*
Eos	Eosinophil
ESCAPE	European Study of Cohorts for Air Pollution Effects
ETPA	Ethyl paraben
ExWAS	Exposome-wide association study
FAS	Family Affluence Score
GIS	Geographical Information System
HCB	Hexachlorobenzene
HELIX	The Human Early-Life Exposome
HGF	Hepatocyte growth factor
IL	Interleukin
IL1	Interleukin 1
IL10	Interleukin 10
IL6	Interleukin 6

IL8	Interleukin 8
INMA	*INfancia y Medio Ambiente*
KANC	Lithuania – *Kaunus cohort*
LMM	Linear mixed model
LUR	Land Use Regression
MBzP	Mono benzyl phthalate
MECPP	Mono-2-ethyl 5-carboxypentyl phthalate
MEHHP	Mono-2-ethyl-5-hydroxyhexyl phthalate
MEHP	Mono-2-ethylhexyl phthalate
MEOHP	Mono-2-ethyl-5-oxohexyl phthalate
MEP	Monoethyl phthalate
MEPA	Methyl paraben
MiBP	Mono-iso-butyl phthalate
miRNA	microRNAs
MnBP	Mono-n-butyl phthalate
MoBA	Norwegian Mother, Father and Child Cohort Study
Mono	Monocytes
mRNA	messenger RNA
mtDNA	mitochondrial DNA
ncRNA	non-coding RNA
NDVI	Normalised Difference Vegetation Index
Neu	Neutrophil
NK	Natural killer
NO_2	Nitrogen Dioxide
nRBC	Nucleated red blood cells
OCs	Organochlorines
oh-MiNP	Mono-4-methyl-7-hydroxyoctyl phthalate
OP	Organophosphates
OXBE	Oxybenzone
oxo.MiNP	Mono-4-methyl-7-oxooctyl phthalate
PACE	Pregnancy and Childhood Epigenetics
PAI1	Plasminogen activator inhibitor-1
PBDE	Polybrominated diphenyl ethers
PBDE-153	Polybrominated diphenyl ether-153
PBDE-47	Polybrominated diphenyl ether-47
PBDEs	Polybrominated diphenyl ethers
PCB – 118	Polychlorinated biphenyls-118
PCB – 138	Polychlorinated biphenyls-138

PCB – 153	Polychlorinated biphenyls-153
PCB – 170	Polychlorinated biphenyls-170
PCB – 180	Polychlorinated biphenyls-180
PCBs	Polychlorinated biphenyls
PedBE	Paediatric-buccal-Epigenetic clock
PFHxS	Perfluorohexane sulfonate
PFNA	Perfluorononanoate
PFOA	Perfluorooctanoate
PFOS	Perfluorooctane sulfonate
PFUnDA	Perfluoroundecanoate
PM	Particulate matter
PM_{10}	Particulate matter with a diameter <10μm
$PM_{2.5}$	Particulate matter with a diameter <2.5μm
PM_{abs}	Particulate matter absorbance with a diameter <2.5μm
POPs	Persistent organic compounds
PRPA	Propyl paraben
RCP	Robust placental clock
refRCP	Refined robust placental clock
RHEA	*Mother Child cohort study - Greece*
ROS	Reactive oxygen species
rRNA	ribosomal RNA
SBP	Systolic blood pressure
SEI	Shannon's Evenness Index
SES	Socioeconomic status
SHS	Second-hand smoking
siRNA	small interfering RNAs
TCS	Triclosan
TEX	Toluene, ethylbenzene, and xylene
THMs	Trihalomethanes
TNF-α	Tumour necrosis factor-alpha
tRNA	transfer RNA
UFP	Ultrafine particles
WHO	World Health Organization

Table of contents

1. INTRODUCTION

1.1 Environmental exposures

1.1.1 Demographic challenges in the 21st century: aging and urbanization

The current world is experiencing two demographic challenges that can be considered as major forces to shape quality of life in 21st century: population aging and urbanization. On one hand, according to a recent report from the United Nations, between 2015 and 2050, the proportion of the world's population over 60 years will increase from 12% to 22% (Figure 1) (Nations Department of Economic et al., 2020). Therefore, healthy aging has become a public health priority worldwide, within which we need to consider not just elderly population, but also children as the aging processes starts early in life (Jagust, 2016).

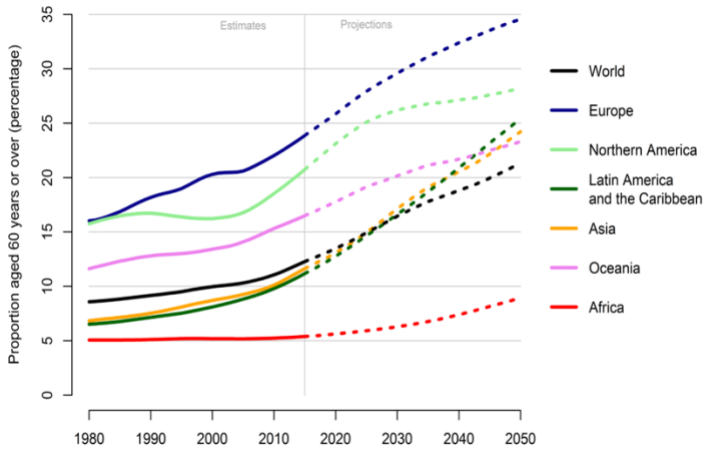

Figure 1. Percentage of population aged 60 years or over by region, from 1980 to 2050 (United Nations Organization, 2017).

On the other hand, the process of urbanization can be described as a massive movement of population from rural to urban areas, accompanied with all the physical changes in the urban settings (Kuddus et al., 2020). A recent study estimated that in 2019 more than half of the population was living in urban areas (4.2 billion people) and that this will increase to 6 billion people by 2041 (United Nations, 2018) (Figure 2).

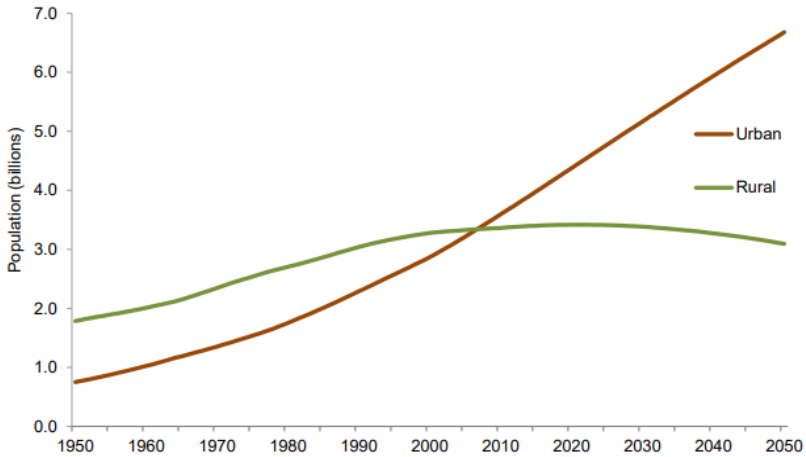

Figure 2. Urban and rural population of the world, 1950-2050 (United Nations Organization, 2018).

Throughout history, urbanization has been linked to human development and progress, as cities have been mainly associated with the evolution of ideas of public health and practice, and they have been also considered as sources of creativity and technology, and the engines for economic growth (McMichael, 2000). However, urbanization is also related with poverty, inequality and non-communicable diseases (Bettencourt et al., 2007; McMichael, 2000).

A rapid and unplanned urbanization within the developing world is known to be at the root of many of the environmental problems that cities currently face. Among these problems we must highlight an increase in urban transport, higher levels of air and noise pollution, direct loss in vegetation biomass and biodiversity, an increase in energy consumption, or impaired water quality (Moore et al., 2003; Yang et al., 2014). Consequently, most of the population is exposed to a few detrimental environmental risk factors. For example, in 2019, 99% of the world population was living in areas where the levels of air pollution were above the limits recommended by the World Health Organization (WHO) guidelines (World Health Organization, 2021a). Hence, in the last decades, the field of environmental health has expanded and updated the amount of evidence linking the environment to human health.

1.1.2 Environment and health

Environmental hazards, linked to urbanization, are responsible for a substantial fraction of human diseases. In 2012, a study estimated that a total of 12.6 million global deaths, which refers to a 23% of the worldwide deaths, were attributable to environmental stressors (Neira and Prüss-Ustün, 2016). Moreover, in children below age 5, if environmental risks were removed, up to 26% of the total deaths could be prevented (Neira and Prüss-Ustün, 2016). In line with this, it was also predicted that air pollution exposure and second-hand smoking (SHS) were responsible for a total of 52 million lower-

respiratory diseases each year, which represents 35% of the global cases. Furthermore, chemicals, SHS and air pollution exposure were responsible for 49 million cancers, 32 million chronic respiratory diseases, and 119 million cardiovascular diseases each year (Neira and Prüss-Ustün, 2016). In addition, a recent study published in 2020, provided estimates of the number of deaths per year attributed to three group of risk factors: metabolic factors such as high systolic blood pressure, behavioural factors as tobacco smoking or environmental factors as outdoor and indoor air pollution exposure (Abbafati et al., 2020). High blood pressure, smoking and air pollution are considered the main risk factors (Figure 3).

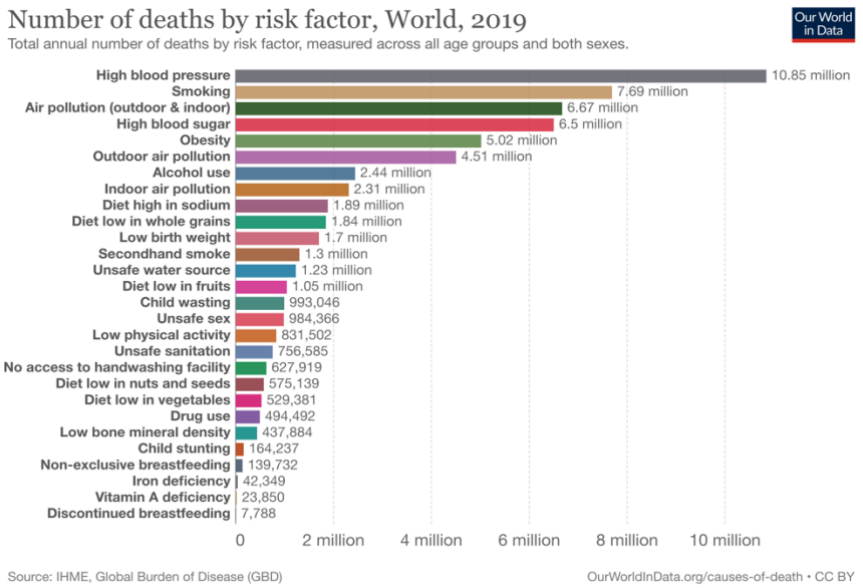

Figure 3. Number of worldwide deaths by risk factor (Our World in Data, 2022).

1.1.3 The relevance of the early-life period

The impact of environmental factors on human health may differ based on the vulnerability of the population subgroup exposed (Poore et al., 2017). Current evidence shows that early-life, including prenatal and early postnatal periods, are important windows of susceptibility to environmental exposures (Wright, 2017). Throughout history we find several examples of how exposure to certain environmental factors during pregnancy or early life is a risk to human health:

- The "Hunger Winter" in the Netherlands is also a well-known historical event. The Dutch population experienced a period of widespread starvation during the winter of 1944-1945, which "naturally" created a cohort of babies who were conceived during this period and who were exposed to an energy-poor environment during gestation. These children were found to have an increased risk of obesity, glucose intolerance and cardiovascular disease compared to those born before the famine period. Moreover, infants who had been exposed to famine during the prenatal period were found to have an increased risk of schizophrenia in adulthood.

- In the late 1950s in a fishing village in Japan, Minamata, there was an epidemic of persistent mental retardation and spastic

palsy[1] in children. It was noted that in all cases the children's mothers had eaten seafood contaminated with methylmercury discharged from a factory located in Minamata Bay.

- In France during the 1960s it was observed that it was very common for babies born to mothers who drank alcohol continuously during pregnancy to be mentally retarded. These observations helped to identify the well-known foetal alcohol syndrome, which is characterized by chronic mental health and developmental disorders in the offspring.

Pregnancy and first years of life are characterized by a rapid development and body growth, in which a great number of physiological changes occur (Davis and Narayan, 2020). First, it is a period known for its unique developmental plasticity as cells are differentiating and tissues are developing. This plasticity is part of the organism's adaptability to the environment which promotes an optimal functioning when the individual is exposed. However, this adaptation process can induce modifications and changes in the human body that may persist over time resulting in maladaptation to the later environment (Hochberg, 2011). Second, the organism might not be able to counteract the effect of the exposures, which can interfere directly with the proper development. Moreover, the

[1]Spastic palsy or spastic cerebral palsy is a condition in which affected people find it difficult to control some or all the muscles in their body, which tend to stretch or weaken.

susceptibility of children can differ from that of adults as they have immature detoxification processes and are still developing.

Consequently, being exposed to different environmental risk factors during *in utero* life or childhood might permanently change the body's structure, metabolism, and physiology, and hence promote health or diseases in later stages of life (Barouki et al., 2012) (Figure 4). This idea is enclosed on the Developmental Origins of Health and Diseases paradigm (DOHaD), which was proposed in 1990 by a British epidemiologist called David Barker (Barker, 1990).

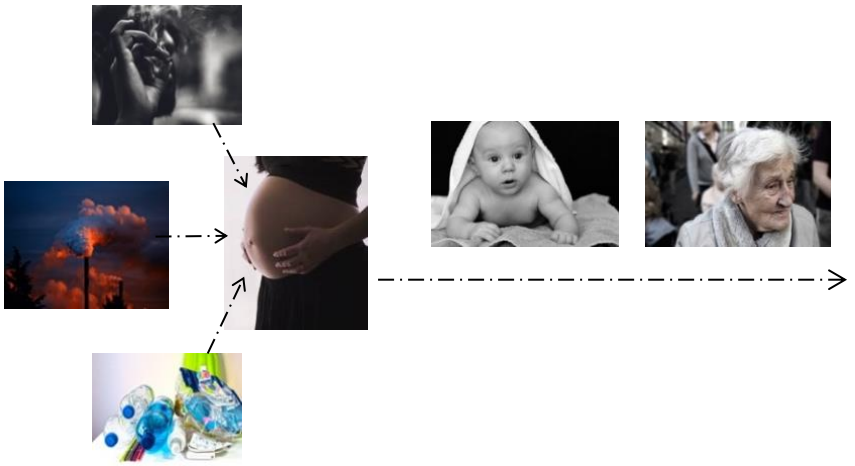

Figure 4. Environmental exposures during pregnancy and early-life could influence health later in life (DOHaD paradigm).

Based on this paradigm, an increasing number of studies evaluating the association between environmental exposures early in life, even before birth, and diverse health outcomes have been published during the last years. Their aim is to define whether and how being exposed

to environmental risk factors during early life can induce permanent changes and increase risk of morbidity later in life.

The placenta is a crucial organ during pregnancy as it acts as the interface between maternal and foetal circulations, it serves as a barrier, and it helps to create the *in utero* environment, in which a complex sequence of interactions between maternal and foetal cells happen to regulate the exchange of nutrients and gas, and to produce and secrete hormones to control foetal growth and development (Fowden et al., 2008; Vlahos et al., 2019). It has been observed that an impaired placental structure or function leads to the majority of adverse pregnancy outcomes such as preeclampsia, placental abruption, foetal growth restriction, or an increased risk of stillbirths (Heazell, 2015; Lean et al., 2017). Moreover, a recent experimental study with 103 mouse knockout lines found that placental defects correlated with abnormal brain, heart, and vascular development. This highlighted the importance of studying placental functioning in its relation with adverse health outcomes (Perez-Garcia et al., 2018).

1.1.4 The *exposome* concept and adverse health outcomes

The exposome is a holistic concept that involves all non-genetic risk factors that can be experienced during an individual's life and their link with the biological responses that occur to maintain homeostasis (Santos et al., 2020). The exposome was firstly described as "the totality of human environmental exposures from conception

onwards, complementing the genome" (Wild, 2012). For instance, it provides to the environmental field the chance to move to a more comprehensive analyses of the exposures and their effects over the life course.

Measuring the exposome can be challenging as individual's exposome is dynamic. First, as it has been commented in the previous section, there are several critical life stages, such as the *in utero* life, in which some exposures can have a greater impact to future diseases. Second, throughout life the levels and the group of exposures to which the individuals are exposed may vary. For example, during life *in utero* exposures are mainly due to diet, pharmaceutical use or environmental exposures, however occupational exposures occur mainly during working years and exposure to pharmaceuticals might be related with age as the use of them tends to increase with it. Third, some chemical compounds have the property to bioaccumulate, which might lead to a higher body burden with age (DeBord et al., 2016). Fourth, human health status is the product of a complex system of interactions, in which the genetic factors are also involved and can influence. Hence, the study of the interaction between the genome and the exposome needs to be considered and studied to protect and promote health. Fifth, the length of exposure is also important, distinguishing between acute and chronic exposures. The magnitude of the estimated effects depending on the length of exposure is still unclear (Pope, 2007). Finally, determining the time scale over which the adverse health outcome is more probable to appear is essential for policy makers. Thus, disentangling between

short and long-term effects is essential to interpret and estimate the influence of risk factors and also understand the benefits of reducing their exposure (Beverland et al., 2012; Künzli, 2005).

The exposome concept has evolved during the last decades integrating new dimensions. Nowadays, it can be divided in the external and the personal exposome depending on whether the exposure is related to the environment in which the individual live (external), or if the exposure is directly related to the diet, lifestyle, toxic chemicals, or social factors (personal). The exposome concept also includes the different biological functions that take place in the body in response to exposure to maintain homeostasis (Figure 5). These biological responses are the link with later health outcomes.

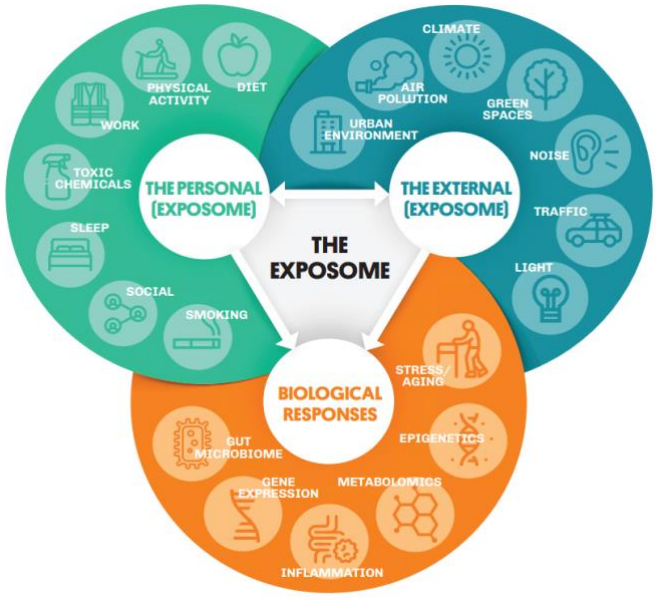

Figure 5. The exposome: Understanding the Effect of the Environment on Our Health (ISGlobal, 2020).

The external and personal exposures can be divided in families, which are briefly described below.

A. Outdoor and urban exposures

This family of exposures includes air pollution, natural and blue spaces, built environment, traffic and road traffic noise.

Ambient air pollution corresponds to the outdoor air pollution, which is principally originated from different natural and anthropogenic sources and it collects information on the levels of exposure to which the individual can be exposed when they are not at home, work, school or other indoor buildings (Fromme, 2019). In contrast indoor air pollution includes gases or particles that contaminate the air inside buildings such as home or workplace, and their main determinants are outdoor air pollution, building conditions such as the windows frame, the season of the year or socioeconomic status (SES) (Vardoulakis et al., 2020).

Air pollution comprises different groups of pollutants as particulate matter (PM) or gaseous pollutants. PMs resulting from different chemical reactions, can be classified according to their size: (a) particulate matter with an aerodynamic diameter of fewer than 10 μm (PM_{10}); (b) particulate matter with an aerodynamic diameter of fewer than 2.5 μm ($PM_{2.5}$) and (c) ultrafine particles with an aerodynamic diameter of 0.1 μm (UFPs) (Figure 6).

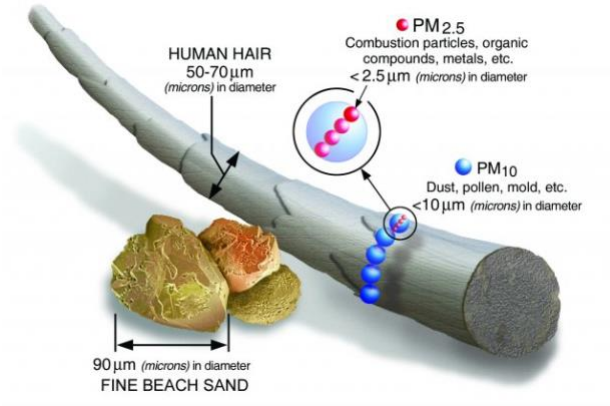

Figure 6. Size of particulate matter (US EPA, 2021)

The penetration of PMs through the respiratory tract to the human body depends on the particle size (Manisalidis et al., 2020): PM_{10} affects mainly the upper respiratory tract, $PM_{2.5}$ can reach the lungs altering the lower respiratory tract and the alveoli and UFP which have the potential to cross bodily barriers, even the blood-brain barrier (Danel, 2022). Moreover, it has been seen that the toxic effects of PMs may differ according to their physical and chemical properties (Cheung et al., 2011). During pregnancy, it is important to consider that PM can be transferred and cross the placenta throughout maternal blood (Ghazi et al., 2021) and therefore, influence its functioning. Previous studies have already proved the presence of pollutant particles in placental tissues (Bové et al., 2019; Liu et al., 2021).

Besides particulate matter, other air pollutants are considered harmful for human health, such as nitrogen dioxide (NO_2), which is a gaseous air pollutant mainly used as a marker for traffic-related air

pollution (WHO, 2003). Based on a recent review, there is consistent evidence on the relationship between ambient air pollution exposure and children's health, mainly with the respiratory system, and birth or neurodevelopment adverse health outcomes (Leung, 2015). Indoor air pollution exposure have also been related with adverse health outcomes during childhood in a recent report, mainly with respiratory, skin and neurological effects (Royal College of Paediatrics and Child Health (RCPCH), 2020).

Apart from air pollution, the urban exposome includes natural or blue spaces, noise or factors related with the physical parts of where we live and work, including access to infrastructures or open spaces, which is known as built environment. Previous studies established that green space exposure is associated with reduced birth weight (Nieuwenhuijsen et al., 2019); that noise, air pollution, ambient temperature and different features of the built environment are related with increased blood pressure (BP) (Warembourg et al., 2021); and that greenness exposure, ambient air pollution and connectivity density are linked to impaired cognitive and motor function (Binter et al., 2022).

B. Contaminant exposure biomarkers

This family includes a wide range of toxic chemicals or their metabolites. The most relevant due to their public health concern are metals, persistent organic compounds, organophosphates, phthalates, and phenols.

- Metals are natural chemical compounds which can be found at different levels of the environment such as water or soil. Due to human activities such as industry or farming, metals can also be found in food (Masindi and Muedi, 2018). Therefore, humans can be exposed to these compounds through the environment or by the ingestion of contaminated food or water. Some of them are essential for the functioning of the organism, however, when their concentrations in the human body are high, they become toxic and dangerous. Mercury, lead, cadmium, and arsenic are considered as one of the top health menacing metals, and it has been shown that its exposure during pregnancy might lead to neurodevelopmental deficits and related disorders during childhood (Tran and Miyake, 2017).

- Persistent organic compounds, also known as POPs, are a group of chemical substances characterized by its ability to remain in the environment for long periods of time as they are resistant to degradation. They also have the capacity to accumulate in the adipose tissue and be incorporated into trophic chains, a phenomenon known as bioaccumulation. These POPs are generated in the process of manufacturing, use and disposal of organic chemicals such as pesticides or agrochemicals, and by the emission of smoke from cars and tobacco. POPs can be divided in different subfamilies: (1) organochlorine compounds, such as polychlorinated biphenyls (PCBs) mainly found in electronic products, or dichlorodiphenyltrichloroethane (DDT) and dichlorodiphenyldichloroethylene (DDE), which are used as

pesticides, (2) perfluoroalkylated substances (PFASs or PFOAs) found in food packaging, drinking water or commercial household products, and (3) polybrominated diphenyl ethers (PBDEs) found in various products such as old televisions or computers, in furniture or textile clothing. Previous evidence has shown that POPs influence in different aspects of the pre- and postnatal development such as birthweight or gain weight (Krönke et al., 2022), and they are associated with adverse effects on growth, metabolism, neurodevelopment and sexual development and reproduction (World Health Organization, 2010).

- Organophosphates (OPs) are organic compounds used as pesticides. Individuals can be exposed to them in their workplace, from the environment if living in communities where there is an intensive agricultural production, and through diet in the general population. Published studies have found a relationship between exposure to OPs and impaired neurodevelopment (Hertz-Picciotto et al., 2018; Sapbamrer and Hongsibsong, 2019).

- Phthalates or phenols are found in cosmetic products, plastics, carpets, or toys and medical or cleaning products such as bisphenol A (BPA) (Wang and Qian, 2021). These chemicals are difficult to assess as they have a short-half-life, are quickly excreted from the body, and show a high intra-individual variability. However, recent studies have shown that the exposure to these chemicals can be associated with respiratory health

(Abellan et al., 2022; Vindenes et al., 2021), cardiovascular diseases (Montazeri et al., 2022) or neurodevelopment (Braun et al., 2017).

C. Water disinfection by-products (DBPs)

One of the main public health practices to protect population from water-borne infections is the disinfection of public drinking water supplies. This process, however, leads to the formation of a group of chemical substances that can be toxic for humans (Villanueva et al., 2015). General population is daily exposed to them through ingestion (main route), dermal and respiratory intake. It has been seen that exposure to DBPs is related to adverse reproductive outcomes (Lewis et al., 2006; Wright et al., 2003) and bladder cancer (Villanueva et al., 2004).

D. Lifestyle

Finally, the exposome concept also includes variables related with lifestyle and SES. This group of variables is challenging as information on them is mainly collected via questionnaires. Life-style variables include a wide range of exposures, that go from dietary patterns to physical activity, social relations, stress and alcohol and tobacco consumption. This last one is one of the most threatening stressors known for many years. Tobacco smoke is a reactive mixture that contains an estimated amount of 5,000 chemical, of which 90 of them have already been identified as

harmful (Hwang et al., 2012). Indeed, several compounds contained in tobacco smoke have been found in placental samples (Mohammadi et al., 2017). Besides own smoking that increases the risk of several diseases especially lung cancer, maternal smoking and SHS have many implications for the foetus and children. On one hand, maternal smoking has been related to lower birth weight and higher risk for cardiovascular diseases later in life, furthermore smoking during pregnancy have resulted in more than 1,000 infants deaths annually (Everson et al., 2021; Taylor et al., 2021). On the other hand, SHS have been linked to numerous health problems in children such as asthma attacks, respiratory infections, or ear infections (United States Department of Health and Human Services, 2014). Moreover, passive smoking was estimated to be responsible for 50.000 deaths and 4.500.000 disability-adjusted life years in children under the age of 14 (Alla, 2021).

1.2 Biological functions

Published evidence has revealed the contribution of the external and the personal exposome to more than 100 adverse health outcomes (Prüss-Üstün and Corvalán, 2006). Before clinical manifestation there are several biological responses that take place in the body. Understanding these biological responses provides knowledge to improve and develop new biomarkers of exposure or disease risk. In addition, the identification of the environmental factors responsible and the comprehension of how adverse health effects are triggered

provides biological plausibility. This is essential to influence the decision-making process of authorities with the main aim of mitigating adverse health effects and protecting public health.

1.2.1 From classical biomarkers to omics profiles

1.2.1.1 Classical biomarkers

Biomarkers have been traditionally defined in the epidemiology field as those measurable events or measures of a physiological state that occurs in a biological system such as the human body (Grandjean, 1995). They can be divided into three groups: (1) biomarkers of exposure, (2) biomarkers of effect and, (3) biomarkers of susceptibility (Owen et al., 2008). First one refers to the exposure itself, and it corresponds to a substance found in the human body after exposure to an environmental factor, which can be measured in different biological samples such as blood, saliva, urine, or hair. A biomarker of effect refers to a measurable biological alteration observed in the human body that is consequence of an exposure, which can also be assessed in biological samples. Finally, biomarkers of susceptibility, such as genetic polymorphisms or metabolic phenotypes, are used to identify individuals with higher sensitivity to an environmental exposure (Zare Jeddi et al., 2021).

1.2.1.2 High-throughput platforms

Biomarkers have been applied in the epidemiology field over the last decades, and its use has been widespread throughout the 21st century due to the development of high-throughput omics technologies available for generating large-scale molecular-level measurements (Paniagua - Michel and Olmos Soto, 2016). The opportunity to measure different components of a biological system from an omics scale approach have totally transformed the way in which we understand networks and systems involved in the biological processes (Goerdten and Floegel, 2021). These techniques are allowing us to develop more accurate biomarkers to enhance the existing ones. Furthermore, their study could provide a better interpretation on the underlying molecular mechanisms through which the environmental exposures can be involved in the development of disease or impaired health conditions. In addition, these altered molecular markers might be more sensitive than the final clinical endpoints, therefore sensitivity of environmental epidemiologic studies could improve (Mayeux, 2004).

1.2.1.3 The epigenome and other molecular layers

High-resolution omics platforms allow us to measure different molecular layers: the epigenome, the transcriptome, the proteome or the metabolome (Kim and Hong, 2017) (Figure 7).

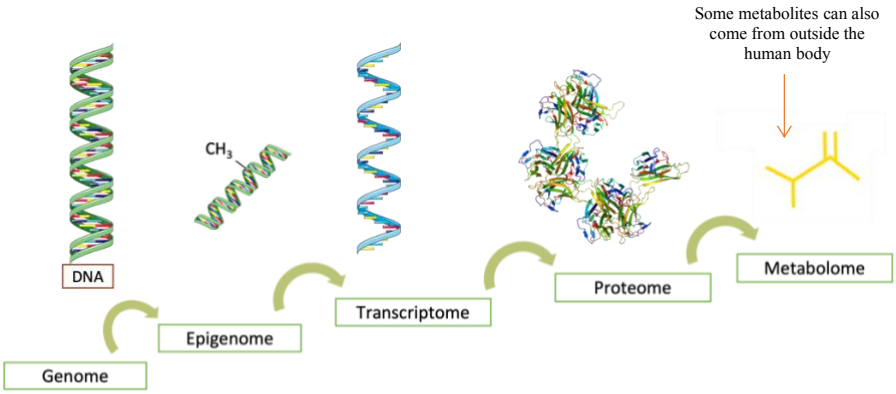

Figure 7. Molecular layers potentially influenced by the exposome.

The epigenome involves all the chemical modifications that alter the expression of genes within the genome without changing the DNA sequence (Morgensztern et al., 2018). These chemical modifications are known as *epigenetic marks* since when they are attached to the DNA sequence, they "mark" the genome changing the way in which the DNA instructions will be read. These marks can pass from cell to cell. The most investigated mark is called DNA methylation (DNAm) and it consists of a methyl group onto the C5 position of a cytosine which leads to the formation of a 5-methylcystosine (Wang and Ibeagha-Awemu, 2021) (Figure 8). The addition of this methyl group mainly occurs in specific regions known as CpGs sites, which are regions of the genome with a cytosine followed by a guanine (Lim et al., 2019). Briefly, the addition of the methyl group to the DNA sequence helps to regulate gene expression as it promotes the recruitment or inhibition of transcription factors' binding to DNA. DNAm is a crucial process during the development and tissue differentiation, and chromosome X inactivation (Moore et al., 2013).

DNAm is determined by genetic and environmental factors, as summarized elsewhere (Everson et al., 2021; Nakamura et al., 2021).

The second group of epigenetic marks are the modifications occurring in the histones, structures responsible for packaging DNA (Figure 8). Histone marks include methylation, acetylation, phosphorylation, ADP-ribosylation and ubiquitylation (Stein, 2012). These modifications alter chromatin condensation, therefore, they can control chromatin structure and gene transcription (Molina Serrano et al., 2019).

The transcriptome is defined as "*all coding and non-coding RNAs, that can be present in various physiological conditions or transcribed at specific developmental stages in a cell type or a tissue*" (Gunes and Mahmutoglu, 2018). Therefore, it includes ribosomal RNA (rRNA), messenger RNA (mRNA), transfer RNA (tRNA) and non-coding RNA (ncRNA) (Hasin et al., 2017). ncRNAs are part of the epigenome (Figure 8) and they can be separated into two groups: housekeeping ncRNAs and regulatory ncRNAs. The later are divided depending on their size in short ncRNAs, which include small interfering RNAs (siRNAs), micro RNAs (miRNAs), and piwi-interacting RNAs (piRNAs), and long-coding RNAs (Wei et al., 2017).

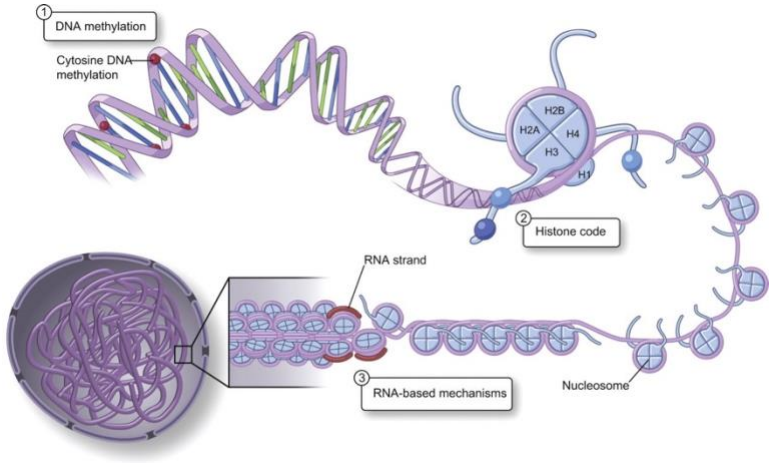

Figure 8. Epigenetic mechanisms that regulate gene expression (Yan et al., 2010).

The proteome corresponds to the complete set of proteins expressed by an organism, it can also be used to describe the number of proteins that are produced in a specific period of time in a particular cell or tissue by an individual (Wecker and Krzanowski, 2007). The proteome informs about the levels, modifications, or structures of the proteins in response to internal and extern stimuli.

Finally, the metabolome refers to the set of metabolites which can be divided into exogenous or endogenous. The former are metabolic compounds that can be acquired from the exposome (i.e., diet or cotinine from smoking exposures) and the later are produced through metabolic processed conducted in the human body.

Overall, the aim of studying molecular profiles is to identify, characterize and quantify the biological molecules that are comprehended in the structure, function and dynamics of the cell,

tissues, and the whole organism. Nonetheless, their study is challenging because they are dynamic over time, as they regulate developmental and physiological processes, they are tissue-dependent, they rely upon the genetic background and they also respond to environmental factors, which as we exposed before, are changeable.

1.2.2 Influence of environmental factors on molecular profiles and biological functions

As mentioned in previous sections, environmental exposures can impact different molecular layers leading to activation or deactivation of biological functions. In this Thesis we will focus on two biological pathways that have been proposed to mediate the effects of environmental factors on adverse health outcomes: aging and inflammation (Figure 9). They are two of the "hallmarks of environmental insults" among other cellular and molecular processes such as endocrine disruption, mitochondrial dysfunction, altered microbiome and intercellular communication, impaired nervous system function, genomic alterations and mutations, oxidative stress, and epigenetic alterations (Peters et al., 2021). Below we introduce the main evidence about the alteration of these biological pathways as well as about the epigenome in response to the exposome.

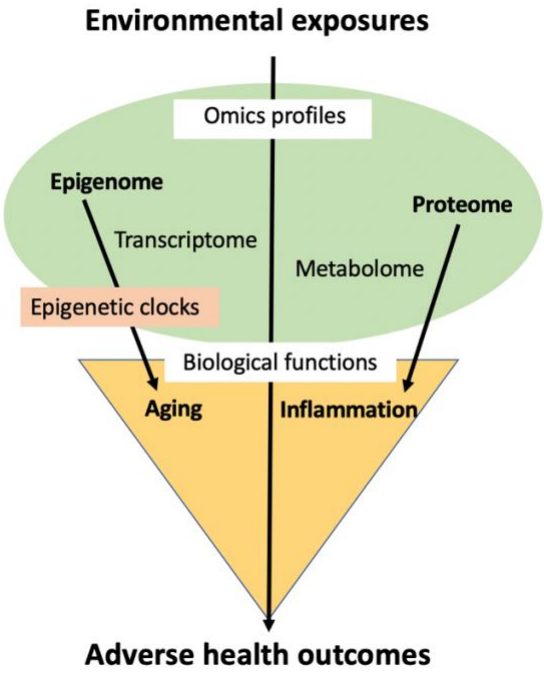

Figure 9. Molecular layers and biological pathways investigated in this Thesis.

1.2.2.1 Epigenome

Previous studies have shown that exposure to some environmental factors produces changes in the epigenome which may mediate specific mechanisms of toxicity (Baccarelli and Bollati, 2009). Within the framework of the Pregnancy and Childhood Epigenetics (PACE) consortium several studies have been conducted in relation to the epigenome. One found that maternal smoking was associated with substantial changes of DNAm in cord blood of the offspring (Joubert et al., 2016). These associations have been seen to persist into childhood, adolescence and even adulthood (Vives-Usano et al., 2020). A more recent one showed that maternal smoking affects

placental DNAm, however the overlap of smoking-sensitive CpGs between cord blood and placenta was low (Everson et al., 2021). In contrast, both tissues highlighted a series of common biological processes altered in response to tobacco smoke, which were related to proinflammatory response and growth factor signalling (Joubert et al., 2016).

Air pollution exposure has also been seen to be associated with alterations in DNAm patterns. A study from PACE found that exposure to PM_{10}, $PM_{2.5}$ and NO_2 during pregnancy was significantly associated to differentiated DNAm of CpGs in new-borns which were annotated to genes previously associated with lung-related outcomes or antioxidant defence pathways (Gruzieva et al., 2017b, 2019). Recently, air pollution has also been seen to affect placental DNAm (Abraham et al., 2018).

Many other environmental factors have also been associated with alterations in DNAm patterns such as metals (Dolinoy et al., 2007b; Fry et al., 2007; Hu et al., 1997), endocrine disruptors (Dolinoy et al., 2007a; Rusiecki et al., 2008), diet, social factors or lifestyle (Lee et al., 2009; McGowan et al., 2011; Roth et al., 2011; Weaver et al., 2004; Xiang et al., 2008; Yan et al., 2011). An inventory of these associations can be found in online databases such as the EWAS Atlas (https://ngdc.cncb.ac.cn/ewas/atlas) or the EWAS Catalog (http://ewascatalog.org/).

1.2.2.2 Inflammation

Inflammation has been proposed as one of the underlying biological mechanisms behind the association between several families of environmental factors and adverse health outcomes. This mechanism is a biological response triggered by an infection or an injury, hence it is one of the defence mechanisms of the human body to harmful stimuli (Chen et al., 2018). In addition, it is closely related to oxidative stress, which results of the presence of reactive oxygen species (ROS) (Peters et al., 2021; Salminen et al., 2012). ROS are involved in the initiation, progression and resolution of the inflammatory process (Chelombitko, 2018). Both inflammation and oxidative stress, are considered as necessary mechanisms for the adequate human body functioning, however when they are persistent, they can lead to tissue damage and disease. It has been proposed that when being exposed to detrimental factors, the cell response in the following phases: tolerance, adaptation, inflammation, and cell death (Peters et al., 2021) (Figure 10). Inflammatory response can be assessed through the analyses of different markers such as chemokines, cytokines, T helper cells and oxidative stress markers.

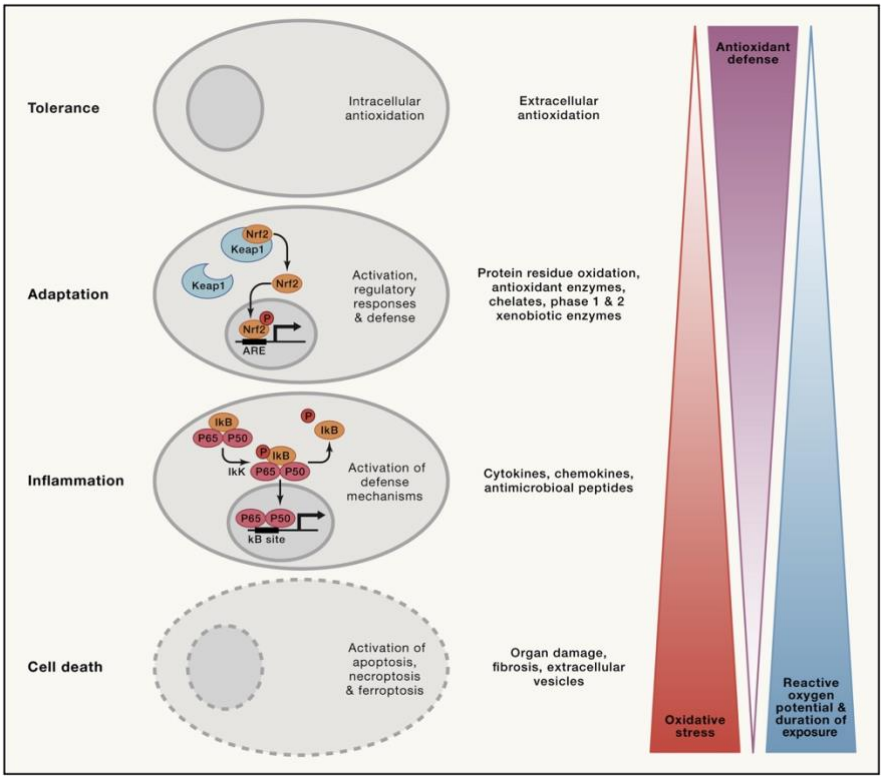

Figure 10. Proposed cell phases occurring when being exposed to environmental factors (Peters et al., 2021).

One of the top health menacing exposures known to produce inflammation is air pollution. For instance, previous evidence have revealed that inflammation might contribute to the development of cardiovascular, respiratory and central nervous system affections attributed to this detrimental exposure (Arias-Pérez et al., 2020). However, the specific mechanisms occurring are not yet entirely understood (Arias-Pérez et al., 2020). Most of the epidemiological studies have investigated a few specific inflammatory proteins such as interleukins (interleukin 1 (IL1), interleukin 6 (IL6), interleukin 8 (IL8), or interleukin 10 (IL10)), tumour necrosis factor-alpha (TNF-

α), C-reactive protein (CRP) (Yang et al., 1017), and adipokines (leptin or adiponectin, which are produced by the adipose tissue) (Dauchet et al., 2018). Furthermore, most of the studies have investigated either short- or long-term exposure to air pollution, but not both, and the majority of the evidence of biological mechanisms refers to the adult population (Elbarbary et al., 2021; Fiorito et al., 2018; Pilz et al., 2018; Riggs et al., 2020; Su et al., 2017; Sun et al., 2020; Tsai et al., 2019; Zhang et al., 2020), with only a few studies available in children (Alderete et al., 2018; Gruzieva et al., 2017a; Li et al., 2019). Thus, there is a paucity of studies considering multiple windows of exposure, different air pollutants, and multiple inflammatory proteins in children.

1.2.2.3 Aging

Aging, is a natural process characterized by a progressive decrease in physiological capacity and a reduction of the ability to respond to environmental factors, that leads to an increased susceptibility and vulnerability to diseases (Troen, 2003). Aging leads to changes in body composition, imbalance between energy availability and demand, dysregulated signalling networks and neurodegeneration with impaired neuroplasticity (Bektas et al., 2018).

The biology of aging is incredibly complex as it is the centre of a large network of processes that can influence on the molecular, cellular, organismal and even at population levels (Poole et al., 2020). Recently, a set of fundamental and connected biological processes

involved in aging has been recently described as the “hallmarks of aging” (Figure 11). They include oxidative stress and inflammation which is known as “inflammaging”, and some other critical processes such as genomic instability, epigenetic alterations such as DNAm changes, loss of proteostasis, altered metabolism, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication, and telomere attrition (López-Otín et al., 2013). In relation to the latter one, telomeres are specialized structures located at the ends of the human chromosomes to protect their integrity and avoid loss of genetic information (O’Sullivan and Karlseder, 2010). Their shortening is associated with age and with a higher risk of developing different adverse health outcome and age-related diseases (Haycock et al., 2014).

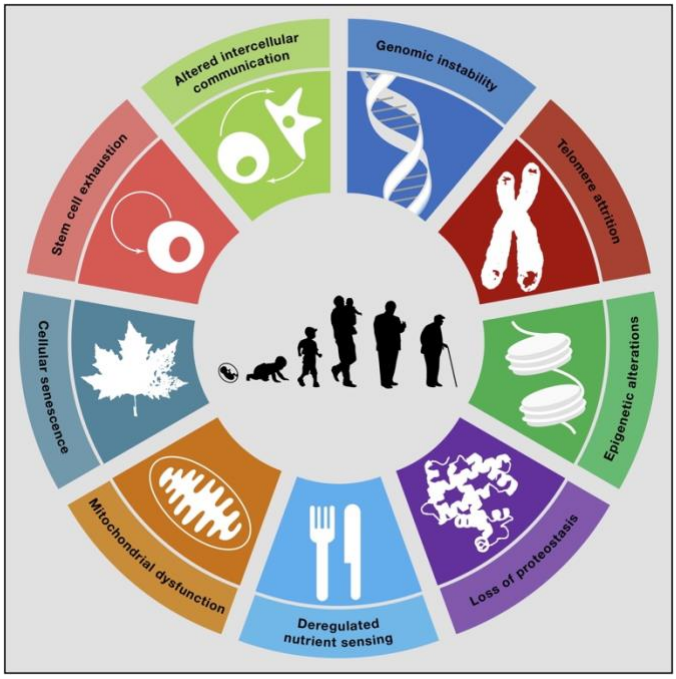

Figure 11. Hallmarks of biological aging (López-Otín et al., 2013).

Previous studies have observed that being exposed to environmental stressors, mainly those affecting inflammation and metabolism, is associated with an increased risk of accelerated aging (Franceschi et al., 2018). The most studied and established environmental factors related with aging are air pollution, tobacco smoking, heavy metals, and pesticides. All of them have been linked to different aging related adverse health outcomes such as cancer (Nawrot et al., 2006), neurological disorders (Power et al., 2014), cardiometabolic diseases (James et al., 2015; Lentini et al., 2017) or all-cause mortality (Gellert et al., 2012).

As shown in Figure 11, epigenetic alterations are considered as one of the biological processes that can change with age. They can also be affected by environmental exposures and influence health and lifespan (Malecki et al., 2022). A previous study have found that changes in DNAm patterns can occur throughout lifetime, even before birth, so they can be used to quantify age (Jones et al., 2015). Based on this, in the last decade, different epigenetic clocks have been developed to predict biological age using DNAm information. These clocks are built based on an informative and sparse set of methylation values of tens to hundreds of CpGs identified through supervised machine learning methods such as penalized regression (e.g., lasso or elastic net) and trained against chronological age (Bell et al., 2019; Oblak et al., 2021). Subsequently, the coefficients obtained in the prediction models can be used to calculate DNAm age and epigenetic age acceleration, which is a measure of whether the individuals' are biologically younger or older than their

chronological age in other datasets (Gibson et al., 2019; Horvath and Raj, 2018; White et al., 2019). However, it is still unclear which aspects of the aging process are captured with these epigenetic clocks. Previous evidence have observed that epigenetic age was associated to nutrient sensing, mitochondrial activity and stem cell composition, but not with telomere attrition, genomic instability or cellular senescence (Breitling et al., 2016; Kabacik et al., 2022; Vetter et al., 2022)

There are a number of epigenetic clocks available (Hannum et al., 2013; Horvath, 2013; Horvath et al., 2018; Levine et al., 2018) with a few ones suitable for children (McEwen et al., 2020; Wu et al., 2019a) or applicable to predict gestational age (Bohlin et al., 2016; Knight et al., 2016; Lee et al., 2019; Mayne et al., 2017). Each clock has been trained with samples from different tissues and considering different age ranges (Table 1).

Previous evidence has linked epigenetic age acceleration to age-related conditions such as cancer (Ambatipudi et al., 2017; Dugué et al., 2018; Zheng et al., 2016) or mortality (Chen et al., 2016; Christiansen et al., 2016; Horvath and Raj, 2018; Perna et al., 2016). Moreover, recent evidence has shown that different environmental factors, such as air pollution (Nwanaji-Enwerem et al., 2017, 2016; White et al., 2019), tobacco smoke (Yang et al., 2019) or cadmium exposure (Demanelis et al., 2017), can increase epigenetic age acceleration (Martin and Fry, 2018; Simpkin et al., 2016). However, the evidence available is still scarce and not consistent, and most of

the studies evaluated the impact on adults (Gao et al., 2016; Wu et al., 2019b) and elderly populations (Ward-Caviness et al., 2016; Yang et al., 2019), with few studies available on such an impact on children (Javed et al., 2016; Simpkin et al., 2017).

Table 1. Summary of the DNAm estimated biological clocks available.

Paper	Number of CpGs	Tissue	Variable	Age range / Weeks of gestation	Comments
Horvath et al., 2013	352	Multi-tissue	DNAmAge	0-centenarians	-
Hannum et al., 2013	71	Blood	DNAmAge	19 – 101 years	-
Levine et al., 2018	513	Blood	DNAmAge	21 – 100 years	-
Horvath et al., 2018	391	Skin and blood	DNAmAge	0-85 years	-
McEwen et al., 2019	84	Buccal epithelium	DNAmAge	0-20 years	-
Wu et al., 2019	111	Blood	DNAmAge	1-18 years	-
Knight et al., 2016	148	Cord blood and blood spot	DNAmGA	24-44 weeks	-
Bohlin et al., 2016	353	Cord blood	DNAmGA	-	-
Mayne et al., 2017	62	Placenta	DNAmGA	8-42 weeks	-
Lee et al., 2019	558	Placenta	DNAmGA	5-42 weeks	Robust placental clock (RPC): placental samples from a variety of pregnancy complications in the training data (e.g., hypertension or diabetes) or congenital abnormalities (e.g., trisomy 13, 18 and 21).
Lee et al., 2019	546	Placenta	DNAmGA	5-42 weeks	Control placental clock (CPC), tailor-made for measuring GA in normal pregnancies.
Lee et al., 2019	396	Placenta	DNAmGA	36-42 weeks	Refined robust placental clock (refRPC) which is like RPC but only including uncomplicated term pregnancies.

Note: GA = Gestational age.

2. RATIONALE

In the last decades, the world has been experiencing two major demographic changes: increase of urban areas and population aging. The process of urbanization implies that a large percentage of the world's population is now exposed to different environmental stressors than before. In addition, the aging of the population makes the promotion of healthy aging a public health priority, not only in the elderly population, but also in children as this process begins in early-life. Indeed, pregnancy and first years of life are considered critical periods for development and growth, so that some exposures can induce short- and long-term biological disturbances, which can lead to increased risk for diseases throughout adulthood. Therefore, understanding the influence that different environmental factors may have on population health throughout life, especially in *in utero* and early life, is essential to promote public policies and raise social awareness.

Recently, the exposome has emerged as a more holistic view of how to assess the impact of a wide range of environmental exposures across the life. It involves the study of both external and personal exposures throughout life, and of the underlying biological mechanisms. The recent development of high-throughput technologies provides the opportunity to measure different components of a biological system from an omics scale approach. They have highly impacted the field of the exposome adding

biological plausibility to the epidemiological studies and helping to create new biomarkers of effect and exposure.

Inflammation and aging are two of the main biological processes that are affected across diseases in response to diverse environmental factors. Both processes have already been associated with the exposure to air pollution and tobacco smoking, considered two of the top health menacing exposures worldwide. However, most of the studies linking these processes to exposures and diseases have been conducted in adult and elderly population, with a few studies available in children. Moreover, there is a paucity of studies considering the exposome, including a wide range of environmental exposures and multiple windows of exposure. Consequently, further research is needed to elucidate the role of exposure to different environmental factors during pregnancy and early-life, especially air pollution and maternal tobacco smoking, on markers related to inflammation and aging.

3. OBJECTIVES

The overall aim of this Thesis is to investigate how early life environmental exposures affect molecular markers in children.

This is addressed through the following specific objectives:

1. To assess the association between the early life exposome and epigenetic age acceleration in children from the Human Early-Life Exposome (HELIX) project (Paper I).
2. To evaluate the association of prenatal ambient air pollution exposure to NO_2 and $PM_{2.5}$ and active maternal smoking during pregnancy with placental epigenetic age acceleration in the *INfancia y Medio Ambiente* (INMA – Environment and Childhood) cohort (Paper II).
3. To assess the relationship of residential and school short- and medium- term (1 day, 1 week, and 1 year) outdoor air pollution exposure to NO_2, $PM_{2.5}$, PM_{10}, and indoor particulate matter absorbance (PM_{abs}) with 36 plasmatic protein levels and their mediating roles on blood pressure in children from the HELIX project (Paper III).
4. To conduct a descriptive analysis of the NO_2 concentrations during pregnancy, including, indoor, personal, and outdoor levels, and to investigate determinants of indoor and personal NO_2 levels in the BiSC cohort (Paper IV).

4. METHODS

This section provides a general overview of the study design, study population, and the exposure and outcome assessment used in this Thesis. A more detailed and specific description of the methods used, and the analyses followed is given in each of the papers included in section 5.

4.1 Study design and population

This Thesis has used data from different population-based birth cohorts in Europe. The Human Early-Life Exposome (HELIX) project that includes 1,301 children from six European on-going cohorts, the Sabadell, Valencia and Gipuzkoa subcohorts from the *INfancia y Medio Ambiente – Environment and Childhood (INMA*) project, and the Barcelona Life Study Cohort (BiSC) (Figure 12).

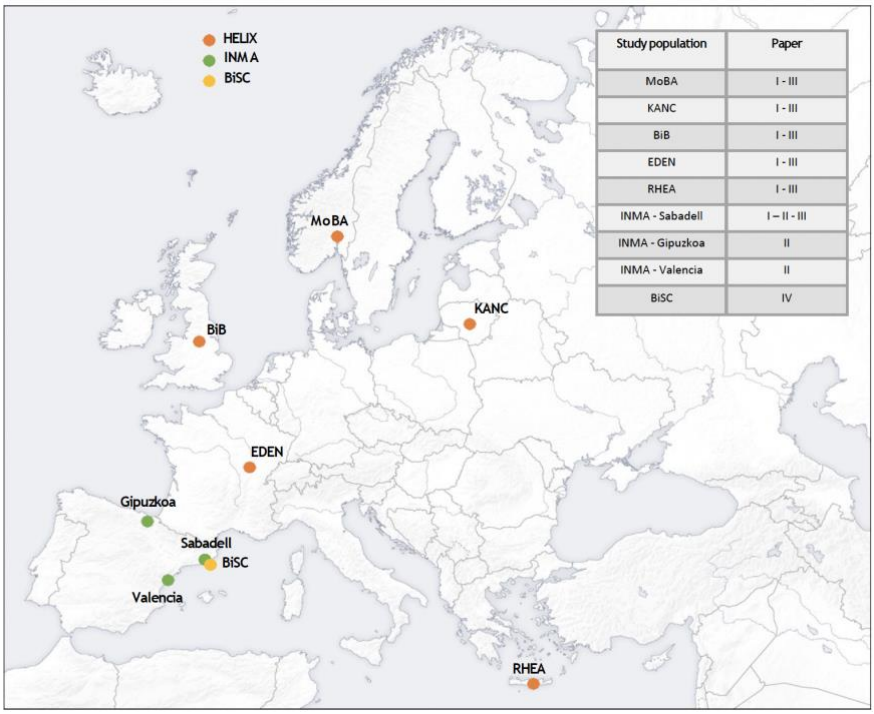

Figure 12. Study populations included in each paper of this Thesis.

4.1.1 The Human Early – Life Exposome (HELIX)

The HELIX project (http://www.projecthelix.eu/index.php/en) is a collaborative project that comprises six established ongoing longitudinal population-based birth cohort studies from six different European countries (Greece – *RHEA Mother Child cohort study*, Lithuania – *Kaunus cohort* (*KANC*), Norway – *The Norwegian Mother and Child Cohort Study (MoBA)*, Spain – *INfancia y Medio Ambiente (INMA)*, UK – *Born in Bradford (BiB)* and France - *Étude des Déterminants pré et postnatals du développement et de la santé de l'Enfant (EDEN))*. The aim of the project was to assess and

describe multiple environmental exposures during pregnancy and first years of life and relate them with different molecular omics signatures and health outcomes. The project uses a multilevel study design, with an entire study population of 31,472 mother-child pairs recruited during pregnancy, a subcohort of 1,301 mother-child pairs in which the measurement of biomarkers, omics signatures and health outcomes was obtained at age 6-11 years and, repeat-sampling panel studies with around 150 children and pregnant women with personal exposure data. Recruitment of pregnant women was conducted between 1999 and 2010. Specifically, INMA, KANC and RHEA recruited pregnant women during the first trimester of pregnancy between 2003 to 2008, EDEN and MoBA through the first and second trimester through 1999 to 2008, and BiB between weeks 26 and 28 of gestation between 2007 and 2010 (Maitre et al., 2018). For the HELIX subcohort (N=1,301) the six cohorts applied common standardized protocols for collecting biological samples, measuring exposure biomarkers and omics signatures and for assessing child health (Maitre et al., 2018). In this Thesis we used data from HELIX to elaborate papers I and III.

4.1.2 *INfancia y Medio Ambiente* – Environment and Childhood (INMA)

The INMA project (http://www.proyectoinma.org) is an ongoing population-based birth cohort study that was created with the aim of evaluating the role of different environmental pollutants in water, air, and diet during pregnancy and first years of life in relation to child

development and growth. Nowadays, INMA project includes more than 3,000 mother-child pairs from seven Spanish cohorts located in different geographical areas: Gipuzkoa, Asturias, Granada, Menorca, Ribera d'Ebre, Valencia and Sabadell (Guxens et al., 2012). Pregnant women were recruited between 2003 and 2008 at first prenatal visit in the main health centre or public hospital of their region. Different follow-ups were carried out at birth, 1.5, 4, 7, 9 and 11 years. In this Thesis we used data from three cohorts: Valencia, Gipuzkoa and Sabadell, as they had information on the exposure and the outcome assessed in paper II and III.

4.1.3 Barcelona Life Study Cohort (BiSC)

The BiSC project (https://www.projectebisc.org/en/home/) is an ongoing population-based prospective birth cohort study of pregnant women, their offspring, and partners in Barcelona city, Spain, including three of the major hospitals of the area; "*Sant Joan de Déu*", "*Maternitat-Clínic*", and "*Santa Creu i Sant Pau*". The aim of the study is to evaluate the influence of prenatal exposures on child health, with a special focus on urban air pollution exposure and neurodevelopment during pregnancy and first years of life. Finally, a total of 1,086 pregnant women and their offspring were recruited from October 2018 to April 2021. In paper IV of this Thesis, we used data from BiSC.

4.2 Exposure assessment

4.2.1 Early-life exposome in HELIX (Paper I)

We evaluated a broad range of environmental exposures, including 83 during the pregnancy period and 103 during childhood in paper I. Environmental exposures were encompassed in four groups of exposures: (1) outdoor and urban exposures, (2) water disinfection by-products (DBPs) and indoor air pollution, (3) contaminant exposure biomarkers and, (4) lifestyle and others. Below we briefly describe the exposure assessment of these families, but an extensive explanation can be found in the Supplementary Material of this Thesis (found in the following link), in Supplementary Material of paper I and in previous HELIX publications (Tamayo-Uria et al., 2019; Warembourg et al., 2021).

A. Outdoor and urban exposures

Urban exposures include air pollution, natural and blue spaces, built environment, traffic and road traffic noise, and they were assessed using geographic information systems (GIS) and land use regression (LUR) models. Exposures were assessed at the geocoded residential address at recruitment of each pregnant woman and at the time of the subcohort visit.

- Air pollution

Briefly, different atmospheric pollutants were evaluated: NO_2, PM_{10}, $PM_{2.5}$ and PM_{abs}. They were assessed using land use regression (LUR) modelling. LUR is a popular method used to estimate outdoor pollution concentrations based on participants addresses in large epidemiological studies. They are developed based on regression models in which a link is established between the air pollution concentrations observed and the most predictive environmental characteristics mainly derived from geographic information systems (GIS) (Eeftens et al., 2016). In the HELIX cohort, the models were temporally adjusted to measurement made in local background monitoring stations and afterwards averaged over the periods of interest. Site-specific LUR models developed in the context of the European Study of Cohort of Air Pollution Effects (ESCAPE) project were used in most of the cohorts (Beelen et al., 2009; Cyrys et al., 2012; Eeftens et al., 2012a, 2012b; Sellier et al., 2014). Estimates on air pollutants were assigned to each subcohort individual within GIS techniques considering their residential geocoded addresses. For paper I we selected for the pregnancy analyses the averaged measurement over pregnancy, and for the childhood period the averaged measurement over the year before childhood follow-up.

- Built environment

A wide range of built environment indicators were calculated from topological maps acquired from the local authorities or from Europe-

wide sources: building density, population density, street connectivity, facility richness index, facility density index, and the land use Shannon's Evenness Index (SEI) (Shannon, 1948), which is an indicator of walkability and accessibility.

- Traffic

Different density indicators such as inverse distance to nearest road, total traffic load of all roads in 100 m buffer, total traffic load of major roads in 100 m buffer (only in childhood) and traffic density on nearest road from home address, were calculated from road network maps following the ESCAPE protocol (Beelen et al., 2013; Eeftens et al., 2012a).

- Natural and Blue Spaces

Surrounding greenness was abstracted as the average of normalized differentiation vegetation index (NDVI) within buffers of 100 m around residential geocoded addresses. The presence of a major green spaces such as grass, trees or vegetation, and the presence of a blue space (i.e., visible water) within 300 m from the residential address was also evaluated. NDVI measures used to determine the surrounding greenness were obtained from the Landsat 4–5 Thematic Mapper (TM), Landsat 7 Enhanced Thematic Mapper Plus (ETM+), and Landsat 8 Operational Land Imager (OLI)/Thermal Infrared Sensor (TIRS) with a 30x30m resolution.

- Traffic noise

Noise levels were assessed based on day-evening-night noise level (Lden) and night noise (Ln) indicators. Both indicators were obtained from a different noise map produced in each local municipality under the European Noise Directive (EC Directive 2002/49/EC)(EUR-Lex, n.d.). In this Thesis Lden during pregnancy and childhood was assessed at residential addresses, and Ln was only evaluated during childhood period.

B. Contaminant exposure biomarkers

In HELIX, different chemical contaminants were evaluated within the framework of the early life exposome investigated in paper I. For the pregnancy period, several chemicals were already measured in some cohorts before HELIX project was created and their results were used. In the childhood period, the sample collection was performed based in a harmonized and standard protocol in all the six cohorts. Biological sample collection consisted in urine and blood samples. Briefly, two spot urine samples were collected (one first morning and one before bedtime), and they were stored at -4ªC and afterwards aliquots were made, and samples were frozen at -80ªC. Moreover, a total of 18 ml of blood were collected at the end of the clinical examination using a 'butterfly' vacuum clip and processed into a variety of sample matrices. It included EDTA Vacutainers, tempus tubes for RNA isolation, and plastic silica Vacutainers. Once processed, samples were frozen at -80ªC and after performing the

analyses, samples remained in storage for the subcohort children. The determination of the biomarkers was performed at the Department of Environmental Exposure and Epidemiology at the Norwegian Institute of Public Health, in Norway or in their contract laboratories. A summary of the assessment methods can be found in the table attached below (Table 1).

Table 2. Summary of the assessment of contaminant exposure biomarkers during pregnancy and childhood

Contaminant exposure biomarkers	Maternal samples previously analyzed in other labs*	Biological matrices of maternal and child samples
Metals and Essential minerals		
Arsenic, Cadmium, Cesium, Cobalt, Mercury, Selenium, Thallium, Zinc, Lead, Manganese, Molybdenum, Potassium, Magnesium, Sodium	N=223 of INMA cohort	Concentrations determined in **whole blood** according to Rodushkin et al., 2000.
Organochlorine compounds (OCs) and Polybrominated diphenyl ethers (PBDEs)		
Dichlorodiphenyldichloroethylene (DDE), Dichlorodiphenyltrichloroethane (DDT), Hexachlorobenzene (HCB), Polychlorinated biphenyl -118 (PCB – 118), Polychlorinated biphenyl -138 (PCB – 138), Polychlorinated biphenyl-153 (PCB – 153), Polychlorinated biphenyl-170 (PCB – 170), Polychlorinated biphenyl-180 (PCB – 180)	OCs → N = 223 (INMA) and 198 (RHEA) PBDEs → N= 198 (RHEA) only PBDE-47	The determination of OCs and PBDEs concentrations was determined in **serum** (maternal samples in EDEN, INMA, RHEA and BiB, and children's samples) or **plasma** (BiB and MoBA maternal samples) according to Caspersen et al., 2016, and adjusted for **lipids**
Organophosphate pesticides (OPs), Phenols, Phthalates		
Diethyl phosphate (DEP), Diethyl thiophosphate (DETP), Dimethyl phosphate (DMP), Dimethyl thiophosphate (DMTP), Dimethyl dithiophosphate (DMDTP) Bisphenol A (BPA), N-Butyl paraben (BUPA), Ethyl paraben (ETPA), Methyl paraben (MEPA), Oxybenzone (OXBE), Propyl paraben (PRPA), Triclosan (TCS) Mono benzyl phthalate (MBzP), Mono-2-ethyl 5-carboxypentyl phthalate (MECPP), Mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), Mono-2-ethylhexyl phthalate (MEHP), Mono-2-ethyl-5-oxohexyl phthalate (MEOHP), Monoethyl phthalate (MEP), Mono-iso-butyl phthalate (MiBP), Mono-n-butyl phthalate (MnBP), Mono-4-methyl-7-hydroxyoctyl phthalate (oh-MiNP), Mono-4-methyl-7-oxooctyl phthalate (oxo.MiNP)	Phenols → N = 62 (EDEN) Phtalate → N = 914 (INMA)	OPs, phenols and phtalates were determined in **urine** according to Cequier et al., 2016, Sakhi et al., 2018 and Sabaredzovic et al., 2015, respectively. Concentrations were adjusted for **creatinine**.
Per- and polyfluoroalkyl substances (PFASs)		
Perfluorohexane sulfonate (PFHxS), Perfluorononanoate (PFNA), Perfluorooctanoate (PFOA), Perfluorooctane sulfonate (PFOS), Perfluoroundecanoate (PFUnDA)	N = 208 (INMA)	Concentrations were determined in **whole blood**, **serum** or **plasma** for the pregnancy period and in **plasma** in childhood period. Haug et al., 2009 method was used for serum or plasma samples and the method presented by Poothong et al., 2017 was applied for the whole blood samples.

*The rest of the maternal samples were analysed in the NIPH as part of HELIX as children's samples.

C. Water disinfection by-products (DBPs) and indoor air pollution

- Water disinfection by products

For all the cohorts of HELIX, we obtained data on routine measurements of DBPs in water from water companies for the pregnancy period. A previous project called the HiWate project (Jeong et al., 2012) modelled exposure levels in the water supply of the residence of each participating mother-child pair for the KANC, BiB, INMA and RHEA cohorts. New DBP measurements were acquired for MoBA and EDEN following the protocol developed for HiWate project (Jeong et al., 2012). In conclusion, total concentration of trihalomethanes (THMs), and chloroform and brominated THMs was estimated for the pregnancy period.

- Indoor air

A subgroup of HELIX children was selected as the panel study (N=157), and measurements of indoor NO_2, benzene and toluene, ethylbenzene, and xylene (TEX) was conducted in their homes for all the cohorts except MoBA. The sampling for NO_2, benzene and TEX lasted 7 days, however for $PM_{2.5}$ and PM_{abs} the sampling lasted 24 hours. The combination of the previous measurements with questionnaire data obtained from the children encompassed in the panel study was used to create a prediction model to estimate indoor air concentrations of the mentioned pollutants in the whole subcohort. The variables that were included were related to number

of people living at home, presence of a garage or car property, exposure to environmental tobacco smoke, cooking and heating methods at home, cleaning products.

D. Lifestyle and other exposures

Different variables related with lifestyle were assessed within the exposome context mainly via questionnaires. Within the context of the exposome, information on diet, breastfeeding duration, alcohol intake, physical activity and allergens exposure was assessed. Data on different variables related with socio-economic status were included in the childhood exposome such as Family Affluence Score (FAS), house crowding (number of persons living in the house with the child), social participation (membership of organization), contact with friends and family, and maternal stress.

Finally, for tobacco smoking exposure, the assessment was conducted during pregnancy and childhood via questionnaires to collect active and passive smoking, and via cotinine measurements. Concentrations of cotinine were measured in urine and the limit of detection (LOD) was 3.03µg/L and 1.21µg/L for cotinine in maternal urine samples from INMA. For maternal smoking a categorical variable was created based on the urinary cotinine levels (non-smokers, second hand-tobacco smokers and smoker), and in children samples a dichotomic variable was created (detected and non-detected) based on the LOD. The following variables were created based on questionnaire data: any maternal smoking during pregnancy

(Yes/No), childhood exposure to SHS based on paternal smoking during childhood (Neither/One parent/Both parents) and maternal tobacco smoking during pregnancy in five categories (No exposure/ Only passive exposure/ Non-sustained smokers/ Sustained smokers at low dose (=< 9 cigarettes)/ Sustained smokers at high dose (>9)) to evaluate dose and duration.

4.2.2 Ambient air pollution and active maternal tobacco smoking exposure during pregnancy in INMA (Paper II)

- Ambient air pollution

In INMA cohort exposure levels of NO_2 and $PM_{2.5}$ were predicted from land use regression (LUR) models developed within the framework of the European Study of Cohorts for Air Pollution Effects (ESCAPE). To obtain the estimates a temporal adjustment was conducted using information from the background routine monitoring stations. For each study participant, exposure levels to each air pollutant was temporally adjusted following ESCAPE guideline, by which the LUR spatial estimates of pollutants for their residential geocode was combined with a temporal adjusting factor obtained from the monitoring stations (Beelen et al., 2013; Eeftens et al., 2012a). An average value (extrapolated back in time using ratio method) during pregnancy, first trimester, second trimester and third trimester was obtained for each pollutant. Within GIS tools an estimate was assigned to each pregnant woman based on their geocoded residential addresses collected at each period of interest.

- Tobacco smoke

In relation to active maternal tobacco smoking exposure, data was obtained through questionnaires. A total of four variables related to maternal tobacco smoking have been evaluated: active maternal smoking at week 12 of pregnancy (Yes/No), active maternal smoking at week 32 of pregnancy (Yes/No), any active maternal smoking during pregnancy (Yes/No) defined as smoke at any time point during pregnancy (beginning, 12weeks or 32 weeks), and active maternal sustained smoking (No smoking/Non-sustained smoking/Sustained smoking) which was defined as smoking at 12 weeks and 32 weeks.

4.2.3 Ambient air pollution childhood exposure in HELIX (Paper III)

In paper III the following atmospheric pollutants (NO_2, $PM_{2.5}$ and PM_{10}, and PM_{abs}) were assessed in HELIX subcohort during childhood. Briefly, outdoor air pollution exposures were assessed using estimates based on LUR modelling approach developed within the framework of the ESCAPE project (Beelen et al., 2009; Cyrys et al., 2012; Eeftens et al., 2012a, 2012b; Sellier et al., 2014). Estimates on air pollutants were assigned to each individual using GIS techniques considering their residential and school geocoded addresses, which was collected through the last available follow-up survey for each cohort. Different time windows were calculated for the evaluated air pollutants by averaging them over 1 day, 1week and,

1 year before the clinical and molecular assessment. A more extensive explanation of the air pollution exposure assessment can be found in Supplementary material (Appendix A, section S1) from paper III.

In HELIX cohorts that had air pollution measurements, missing values were imputed following a process previously described (Tamayo-Uria et al., 2019). Imputed values represented a maximum of 2% of the values within each cohort.

4.2.4 NO_2 concentrations during pregnancy in BiSC and its determinants (Paper IV)

In paper IV, we measured home-indoor, home-outdoor, and personal NO_2 concentrations during one week at first trimester (approximately week 12 of pregnancy) and third trimester (approximately week 32) with Gradko Environmental passive dosimeters. All the measurements obtained were subjected to a quality control and assurance procedure. In addition, to compare the concentrations of the pollutants with those from the NO_2 monitors operated by the Department of the Environment of Catalonia we installed one week per month a tube at the background reference station of *Palau Reial*, which is in the southwest of Barcelona.

The determinants of indoor and personal NO_2 levels were evaluated in paper IV. Data on socioeconomic, behaviour and home

characteristics was gathered throughout several questionnaires which were self-reported or conducted by the fieldworkers at 12 or 32 weeks of gestation. For those individuals that changed residence at 2nd or 3rd trimester data was collected twice, for the rest of the individuals we assumed that they did not made changes at their home.

Due to the Covid-19 pandemic we were forced to change the assessment protocols and the collection of information. A more detailed explanation can be found in the section of Materials and Methods of paper IV.

4.3 Outcome assessment

4.3.1 Epigenetic age acceleration (Paper I and II)

Epigenetic age was estimated using different clocks implemented in the *methylclock* R package (Gonzalez and Pelegí-Sisó, 2021; Pelegí-Sisó et al., 2020), as described below. We estimated blood epigenetic age in HELIX children (paper I) and placental epigenetic gestational age in INMA samples (paper II) based on DNAm data. A detailed explanation can be found in the materials and methods section of paper I and II. Briefly, for each clock we obtained different measures: i) DNA methylation predicted age (DNAm age) in years, ii) ageAcc, difference between DNAm and chronological age in years; iii) ageAcc2, residuals obtained after regressing chronological age on DNAm age, and iv) ageAcc3, residuals obtained after regressing chronological age and blood cell type proportions on DNAm age. We

estimated blood cell type proportion (CD4T, CD8T, Monocytes (Mono), B lymphocytes (Bcell), Natural killer (NK), Neutrophil (Neu) and Eosinophil (Eos)) using the Reinius et al. (2012) reference panel as implemented in *meffil* package (Min et al., 2018) for blood epigenetic clocks. When evaluating placental epigenetic clocks we estimated cell type composition from placental DNAm array data using the R package *planet*, which includes placental cells from third trimester (trophoblasts, stromal, Hoffbauer, endothelial, nucleated red blood cells (nRBC) and syncytiotrophoblast) (Yuan, 2022; Yuan et al., 2021).

4.3.1.1 Blood epigenetic clocks in HELIX (Paper I)

In HELIX subcohort children blood samples were collected at a mean age of 8.1 years (Maitre et al., 2018), and DNA was extracted from buffy coat. DNAm was measured using the Illumina Infinium HumanMethylation450 beadchip at the University of Santiago de Compostela – Spanish National Genotyping Center (CeGen-USC, Spain). Within each batch (slide), all the samples were randomized and balanced by cohort and sex. Then, data was normalized and we checked sex consistency (Fortin et al., 2014), genetic consistency of technical/biological duplicates and other samples making use of the genotype probes of the array and the genome-wide genotyping data when available. Batch effect (slide) was corrected using the *ComBat* R package (Johnson et al., 2007). Control probes, probes in sexual chromosomes, probes designed to detect single nucleotide polymorphisms (SNPs) and probes to measure methylation levels at

non-CpG sites were removed, giving a final number of 386,518 probes. Finally, CpGs were annotated using the IlluminaHumanMethylation450kanno.ilmn12.hg19 R package (Hansen and Aryee, 2012).

A total of four blood epigenetic clocks were used: Horvath's All Tissue clock (Horvath, 2013), Horvath's Skin and Blood clock (Horvath et al., 2018), the Paediatric-Buccal-Epigenetics' (PedBE) clock (McEwen et al., 2020)) and Wu's methylation-based age prediction model (Wu et al., 2019a).

4.3.1.2 Placental epigenetic clocks in INMA (Paper II)

Briefly, biopsies of approximately 5 cm^3 were obtained from the inner region of the placenta, approximately 1.0-1.5 cm below the foetal membranes, corresponding to the villous parenchyma, and at ~5 cm from site of cord insertion. Genomic DNA from placenta was isolated using the DNAeasy® Blood and Tissue Kit, (Qiagen, CA, USA). DNAm was assessed with the Infinium MethylationEPIC BeadChip from Illumina, following manufacturer's protocol in the Erasmus Medical Centre core facility. Three technical duplicates were included. Samples were randomized considering cohort and sex. The methylation data was pre-processed using the PACEAnalysis R package (v.0.1.7). Dye-bias and Noob background correction, implemented in *minfi* R package, were applied followed by normalization of the data with the functional normalization method. To correct for the bias of type-2 probes values the beta-

mixture quantile normalization was applied. SentrixID (array) batch effect was controlled with the ComBat method (Johnson et al. 2007). Finally, to correct for the possible outliers, we winsorized the extreme values to the 1% percentile (0.5% in each side), The final dataset consists of 379 samples and 811,990 probes.

A total of four placental epigenetic clocks were used: control placental clock (CPC), robust placental clock (RPC), refined robust placental clock (refRPC) (Lee et al., 2019) and Mayne's clock (Mayne et al., 2017). The CPC was constructed using placental samples (N=963) from pregnancies that did not have any placental pathology, the RPC (N=1,102) was constructed considering pregnancy conditions as preeclampsia or gestational age, and the refRPC was specifically trained (N=733) for uncomplicated term pregnancies. The clock by Mayne et al. was trained on a small set (n=170), and it seems that it under/overestimated gestational age (GA) according to pregnancy conditions (Mayne et al., 2017).

4.3.2 Plasmatic proteins in HELIX (Paper III)

During the clinical examination, blood samples were collected from HELIX subcohort children at a mean age of 7.4 years (Maitre et al., 2018). Plasma was obtained through blood centrifugation. Three Luminex kits commercially available from Life Technologies and Millipore, were selected to assess plasma proteins: Cytokines 30-plex (Cat #. LHC6003M), Apolipoprotein 5-plex (LHP0001M), and Adipokine 15-plex (LHC0017M). Plasma analyses were performed

following the standard protocol defined by the vendor. Raw intensities obtained with the xMAP and Luminex system for each plasma sample were converted to pg/ml (5, 15 and 30-plex kits) using the calculated standard curves of each plate and accounting for the dilutions that were made prior measurement. Among the quality control process, the % of coefficients of variation for each protein were estimated by plate and then averaged. The LOD was determined for each protein as well as the lower and upper quantification limit. Seven proteins were measured in two different plex and the measure with the lower quality was excluded. Those samples that were below the LOD or above the upper limit of quantification were excluded. Finally, for those proteins that passed the quality control mentioned above, data was log2 transformed to reach normal distribution. A final dataset with the log2-transformed, imputed, and normalized levels for 36 proteins of the HELIX subcohort was obtained. A more detailed explanation can be found in the materials and methods section and, in the Supplementary material (Appendix B) of paper III.

4.3.3 Blood pressure in HELIX (Paper III)

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were assessed using a standardized protocol during the clinical examination in HELIX subcohort (mean age 7.4 years). After 5 minutes of rest in the sitting position, 3 consecutive measurements, separated by 1-min intervals, were taken using an oscillometer device (OMRON 705-CPII, Omron, Kyoto, Japan). The children were in a pre-defined posture and the right arm was used preferably. The cuff

sizes were chosen considering each child's arm length and circumference. Each measurement of SBP and DBP was recorded, and the mean of the second and third measurements was calculated.

Table 3. Summary of study populations, exposure assessments, and outcome assessments in papers I to IV

Paper	Study population	Exposure	Exposure period	Exposure assessment	Outcome	Outcome assessment	Age of outcome assessment
I	HELIX • EDEN • RHEA • INMA • MoBA • KANC • BiB	Early life exposome • Outdoor and urban exposures • Water disinfection by-products and indoor air pollution • Contaminant exposure biomarkers • Lifestyle and others	Pregnancy and childhood	A total of 83 prenatal and 103 childhood exposure variables following different methods	Horvath's Skin and Blood clock to measure children epigenetic age and age acceleration	Blood samples	7-11 years
II	INMA • Valencia • Gipuzkoa • Sabadell	NO_2 PM_{10} $PM_{2.5}$	Pregnancy	LUR models and questionnaires	Control placental clock (CPC) to measure placental epigenetic age and age acceleration	Placental samples	Birth
III	HELIX • EDEN • RHEA • INMA • MoBA • KANC • BiB	NO_2 PM_{10} $PM_{2.5}$ PM_{abs}	Childhood	LUR models and questionnaires	36 plasmatic proteins Systolic and diastolic blood pressure	Blood samples Physical examination	7-11 years
IV	BiSC	NO_2	Pregnancy	Personal, indoor and outdoor measurements	- -	-	-

5. RESULTS

Paper I: The early-life exposome and epigenetic age acceleration in children.

Paper II: Association between ambient air pollution and active maternal smoking exposure during pregnancy and placental epigenetic age acceleration.

Paper III: Short- and medium-term air pollution exposure, plasmatic protein levels and blood pressure in children.

Paper IV: Determinants of indoor and personal NO_2 concentrations during pregnancy in BiSC cohort.

5.1 Paper I

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de Prado-Bert P, Ruiz-Arenas C, Vives-Usano M, Andrusaityte S, Cadiou S, Carracedo A, Casas M, Chatzi L, Dadvand P, González JR, Grazuleviciene R, Gutzkow K.B, Haug LS, Hernandez-Ferrer C, Keun H.C, Lepeule J, Maitre L, McEachan R.R.C, Niewenhuijsen M-J, Pelegrí D, Robinson O, Slama R, Vafeiadi M, Sunyer J, Vrijheid M, Bustamante M.

The early-life exposome and epigenetic age acceleration in children

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The early-life exposome and epigenetic age acceleration in children

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ABSTRACT

The early-life exposome influences future health and accelerated biological aging has been proposed as one of the underlying biological mechanisms. We investigated the association between more than 100 exposures assessed during pregnancy and in childhood (including indoor and outdoor air pollutants, built environment, green environments, tobacco smoking, lifestyle exposures, and biomarkers of chemical pollutants), and epigenetic age acceleration in 1,173 children aged 7 years old from the Human Early-Life Exposome project. Age acceleration

Abbreviations: [1]HNMR, Hydrogen Nuclear Magnetic Resonance; BC, Black Carbon; BiB, Born in Bradford; BMI, Body Mass Index; BMIQ, Beta-Mixture Quantile; BUPA, N-Butyl Paraben; CI, Confidence Interval; CpGs, Cytosine-phosphate-Guanine Sites; DBPs, Disinfection By-Product; DEP, Diethyl phosphate; DETP, Diethyl Thiophosphate; DMDTP, Dimethyl Dithiophosphate; DNA, Deoxyribonucleic acid; DNAm, DNA methylation; EC, Elemental Carbon; EDEN, *Étude des Déterminants Pré et Postnatals du Développement et de la Santé de l'Enfant*; eQTM, Expression Quantitative Trait Methylation; ExWAS, Exposome-Wide Association Study; GIS, Geographic Information System; HCB, Hexachlorobenzene; HELIX, Human Early-Life Exposome; INMA, *Infancia y Medio Ambiente*; IQR, Interquartile Range; KANC, Kaunas Cohort; KEGG, Kyoto Encyclopedia of Genes and Genomes; MoBA, Norwegian Mother, Father and Child Cohort Study; NO_2, Nitrogen Dioxide; OCs, Organochlorine Compounds; OPs, Organophosphate Pesticides; OXBE, Oxybenzone; PBDEs, Polybrominated Diphenyl Ethers; PCB-138, Polychlorinated Biphenyl-138; PCB-153, Polychlorinated Biphenyl-153; PCB-170, Polychlorinated Biphenyl-170; PCB-180, Polychlorinated Biphenyl-180; PCBs, Polychlorinated Biphenyls; PedBE, Paediatric-Buccal-Epigenetics; PFASs, Per- and Polyfluoroalkyl Substances; PFOS, Perfluorooctane Sulfonate; PM_{10}, Particulate Matter of <10μm in aerodynamical diameter; $PM_{2.5}$, Particulate Matter of <2.5μm in aerodynamical diameter; PM_{abs}, Absorbance of $PM_{2.5}$ filters, measurement of the blackness of $PM_{2.5}$ filters used as a proxy for elemental/black carbon; RHEA, Mother-Child Cohort in Crete; SHS, Second-hand smoke; SNPs, single nucleotide polymorphisms; TCs, Transcript Clusters; TEX, Toluene-Ethylbenzene-x-Xylene.

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was calculated based on Horvath's Skin and Blood clock using child blood DNA methylation measured by Infinium HumanMethylation450 BeadChips. We performed an exposure-wide association study between prenatal and childhood exposome and age acceleration. Maternal tobacco smoking during pregnancy was nominally associated with increased age acceleration. For childhood exposures, indoor particulate matter absorbance (PM_{abs}) and parental smoking were nominally associated with an increase in age acceleration. Exposure to the organic pesticide dimethyl dithiophosphate and the persistent pollutant polychlorinated biphenyl-138 (inversely associated with child body mass index) were protective for age acceleration. None of the associations remained significant after multiple-testing correction. Pregnancy and childhood exposure to tobacco smoke and childhood exposure to indoor PM_{abs} may accelerate epigenetic aging from an early age.

1. Introduction

Current evidence shows that early-life, including prenatal and early postnatal periods, could be considered as an important window of susceptibility to environmental exposures (Wright, 2017). Being exposed during these stages might permanently change the body's structure, metabolism, and physiology, and hence promote health or diseases in later stages of life (Barouki et al., 2012). Determining which exposures could be beneficial or detrimental for human health, and identifying the underlying biological mechanisms, could provide important evidence for reducing or enhancing exposure to them during early life (Buck Louis et al., 2017; Logan et al., 2018).

Aging is a gradual and multifactorial process, which is characterized by the physiological deterioration of the human body over time (López-Otín et al., 2013). At the molecular level, aging is described as the accumulation of cellular damage, which leads to structural and functional abnormalities and the decrease in the regenerative capacity of the cells (Carmona and Michán, 2016). Biological aging is reported to be a risk factor for the development of age-related diseases such as cancer, diabetes, cardiovascular, and neurodegenerative diseases as well as increased mortality (Kumar et al., 2017). In this context, aging could be considered as a continuous process already starting in early-life. Consequently, evaluating aging during this period might provide new evidence to slow down this process from the beginning and, prevent or delay the development of adverse health outcomes during adulthood and elderly (Benetos et al., 2013; Martens et al., 2019).

The evaluation of aging at the molecular level can be carried out using a series of biomarkers including epigenetic clocks (Horvath, 2013; Horvath et al., 2018; McEwen et al., 2019; Wu et al., 2019a), which predict DNA methylation age of an individual from its DNA methylation levels (Horvath and Raj, 2018). The property of DNA methylation to change with age is used by these clocks to identify a subset of cytosine-phosphate-guanine sites (CpGs) that can predict chronological age. There are a number of epigenetic clocks available with a few ones applicable to children (McEwen et al., 2019; Wu et al., 2019a). From these clocks, we can calculate epigenetic age acceleration, a measure of whether the individuals' are biologically younger or older than their chronological age (Gibson et al., 2019; Horvath and Raj, 2018; White et al., 2019). Although, DNA methylation is just one of the pathways by which epigenetics affect gene expression, besides histone modification or non-coding RNA, we will be referring to the rest of the text to DNA methylation age as epigenetic age. Epigenetic age acceleration has been linked to age-related conditions such as cancer (Ambatipudi et al., 2017; Dugué et al., 2018; Zheng et al., 2016), cellular senescence (Lowe et al., 2016), and mortality (Chen et al., 2016; Christiansen et al., 2016; Perna et al., 2016), among others (Horvath and Raj, 2018).

Recent evidence has shown that different environmental factors such as air pollution (Nwanaji-Enwerem et al., 2017; 2016; White et al., 2019), tobacco smoke (Yang et al., 2019) or cadmium exposure (Demanelis et al., 2017) can increase epigenetic age acceleration (Martin and Fry, 2018; Simpkin et al., 2016). However, the evidence available is still scarce and not consistent, and most of the studies evaluated the impact on adults (Gao et al., 2016; Wu et al., 2019b) and elderly populations (Ward-Caviness et al., 2016; Yang et al., 2019), with few studies available on such an impact on children (Javed et al., 2016; Simpkin et al., 2017). Moreover, most of the studies have investigated one environmental exposure, and there is a paucity of studies considering multiple exposures. This study aimed to investigate the association between the early life exposome (covering prenatal and childhood period) and epigenetic age acceleration in children from the Human Early-Life Exposome (HELIX) project.

2. Materials and methods

2.1. Study population

This study was conducted in the context of the HELIX project, which was based on six on-going longitudinal population-based birth cohorts established in six countries across different parts of Europe (Born in Bradford [BiB; UK], Étude des Déterminants Pré et Postnatals du Développement et de la Santé de l'Enfant [EDEN; France], Infancia y Medio Ambiente [INMA; Spain], Kaunas Cohort [KANC; Lithuania], Norwegian Mother, Father and Child Cohort Study [MoBa; Norway], and Mother-Child Cohort in Crete [RHEA; Greece]) (Magnus et al., 2016; Maitre et al., 2018; Vrijheid et al., 2014). Prior to the start of HELIX, all six cohorts had undergone the required evaluation by national ethics committees and obtained all the required permissions for their cohort recruitment and follow-up visits. Each cohort also confirmed that relevant informed consent and approval were in place for secondary use of data from pre-existing data. The work in HELIX was covered by new ethic approvals in each country and at enrolment in the new follow-up, participants were asked to sign a new informed consent form. For this study we sub-selected 1,173 children which had information on the exposome, and blood DNA methylation.

2.2. Exposome assessment during prenatal and early childhood

A broad range of environmental exposures was evaluated (Tamayo-Uria et al., 2019), including 83 prenatal and 103 childhood exposure variables (Table S1). Detailed exposure assessment was previously explained elsewhere (Tamayo-Uria et al., 2019). Briefly, geospatial models, monitoring stations, satellite data and land use databases were used to estimate the urban exposome and air pollution. An estimated exposure value was assigned to each study participant separately for their geocoded addresses of home through GIS platforms (Robinson et al., 2018). During pregnancy, at birth or during childhood, maternal and children blood and urine samples were collected by each cohort to assess chemical exposures: organochlorine compounds (OCs), organophosphate pesticides (OPs) metabolites, polybrominated diphenyl ethers (PBDEs), per- and polyfluoroalkyl substances (PFASs), essential minerals, non-essential minerals, phenols, phthalate metabolites, and cotinine (Robinson et al., 2018). During pregnancy, exposure to water disinfection by-product (DBPs) was also assessed based on models for the water supply according to their residency (Jeong et al., 2012). Indoor exposure to air pollutants including particulate matter absorbance (PM_{abs}), which is a proxy of elemental/black carbon as it measures the blackness of $PM_{2.5}$ filters, NO_2, benzene and TEX-toluene, ethylbenzene and xylene, was estimated through a prediction model trained in a

subgroup of children (n = 157) using home personal measurements and questionnaire data including parental smoking behaviour, among other variables (Tamayo-Uria et al., 2019). Questionnaires were used to gather information on active and/or passive tobacco smoking, socio-economic capital of the family, social participation, social contact, house crowding, and other lifestyle factors as maternal and children diet, physical activity and sleep duration.

An imputation process was performed for missing data of all exposures and covariates, resulting in 20 imputed databases (Tamayo-Uria et al., 2019). For comparability, continuous exposure variables were standardised by the interquartile range (IQR).

2.3. Methylation data collection

Blood samples were collected from HELIX subcohort children at a mean age of 8.1 years (Maitre et al., 2018). DNA was extracted from buffy coat using the Chemagen kit (Perkin Elmer). DNA concentration was determined in a NanoDrop 1000 UV–Vis Spectrophotometer (Thermo Fisher Scientific) and with Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies). Blood leukocyte DNA methylation was measured using the Illumina Infinium HumanMethylation450 beadchip at the University of Santiago de Compostela – Spanish National Genotyping Center (CeGen-USC, Spain). Briefly, 700 ng of DNA were bisulfite-converted using the EZ 96-DNA methylation kit following the manufacturer's standard protocol, and DNA methylation measured using the Infinium protocol. Within each batch (slide), all the samples were randomized and balanced by cohort and sex. DNA methylation data was pre-processed using *minfi* R package (Aryee et al., 2014). Two samples were filtered due to overall quality: one had a call rate < 98% at detection p-value threshold < $1.0x10^{-16}$ (Lehne et al., 2015) and the other did not pass general quality control parameters (Van Iterson et al., 2014). Then, data was normalized with the functional normalization method with Noob background subtraction and dye-bias correction (J.-P. Fortin et al., 2014). Then, we checked sex consistency (J. P. Fortin et al., 2014), genetic consistency of technical/biological duplicates and other samples making use of the genotype probes of the array and the genome-wide genotyping data when available. In total four samples were excluded, two with discordant sex and two with discordant genotypes. Batch effect (slide) was corrected using the *ComBat* R package (Johnson et al., 2007). Control probes, probes in sexual chromosomes, probes designed to detect single nucleotide polymorphisms (SNPs) and probes to measure methylation levels at non-CpG sites were removed, giving a final number of 386,518 probes. Finally, CpGs were annotated using the IlluminaHumanMethylation450kanno.ilmn12.hg19 R package (Hansen and Aryee, 2012).

2.4. Calculation of epigenetic age with different clocks

Child epigenetic age based on different clocks (Horvath's All Tissue clock (Horvath, 2013), Horvath's Skin and Blood clock (Horvath et al., 2018), the Paediatric-Buccal-Epigenetics' (PedBE) clock (McEwen et al., 2019)) and Wu's methylation-based age prediction model (Wu et al., 2019a) was calculated using the *methylclock* R package (Gonzalez and Pelegí-Sisó, 2021; Pelegí-Sisó et al., 2020). Briefly, from normalized and batched corrected methylation data, the package extracts methylation levels of CpGs included in each clock (*Table S2*). Subsequently, the coefficients obtained through elastic net in the prediction models of each of the clocks in the original papers are used to calculate DNA methylation age and epigenetic age acceleration. Therefore, for each clock we obtained: i) DNA methylation predicted age (DNAm age) in years, ii) ageAcc, difference between DNAm and chronological age in years; iii) ageAcc2, residuals obtained after regressing chronological age on DNAm age, and iv) ageAcc3, residuals obtained after regressing chronological age and blood cell type proportions on DNAm age. We estimated blood cell type proportion (CD4T, CD8T, Mono, Bcell, NK, Neu and Eos) using the Reinius et al. (2012) reference panel as implemented in *meffil* package (Min et al., 2018).

Additionally, we retrieved the same variables for the Horvath All Tissue clock through the Horvath's online calculator (Horvath, n.d.), which starts from raw data (IDAT files) and implements a normalization based on re-purposing the beta-mixture quantile (BMIQ) method (Teschendorff et al., 2013).

2.5. Gene expression data collection

RNA was extracted from whole blood collected in Tempus tubes with the MagMAX for Stabilized Blood Tubes RNA Isolation Kit (Termo-Fisher). The quality of RNA was evaluated with a 2100 Bioanalyzer (Agilent) and the concentration with a NanoDrop 1000 UV–Vis Spectrophotometer. Gene expression was assessed with the Affymetrix Human Transcriptome Array 2.0 ST arrays (HTA 2.0) at the University of Santiago de Compostela (USC, Spain), following manufacturer's recommendations. Samples were processed in two different rounds. In each round, several batches of 24–48 samples were processed. Samples were randomized per batch taking into account sex and cohort. Raw data were extracted with the AGCC software (Affymetrix) and stored into CEL files. The GCCN (SST-RMA) algorithm was applied to normalize data at gene level. After normalization four samples with discordant sex were excluded. The HTA-2_0 Transcript Cluster Annotations Release na36 (hg19) was employed to annotate transcript clusters (TCs) to genes. A TC is defined as a group of one or more probes covering a region of the genome reflecting all the exonic transcription evidence known for the region and corresponding to a known or putative gene. Control probes and probes in sexual chromosomes or probes without chromosome information were excluded. Probes with a DABG (Detected Above Background) p-value < 0.05 were considered to have an expression level different from the background, and they were defined as detected. Probes with a call rate < 1% were excluded from the analysis. The final dataset consisted of 58,254 TCs. Gene expression values were log2 transformed and batch effect was controlled by residualizing the effect of surrogate variables calculated with the sva method (Leek and Storey, 2007) while protecting for main variables in the study (cohort, age, sex, and blood cellular composition).

2.6. Covariates

During pregnancy and in the childhood follow-up examination information on the following key covariates was collected: cohort (BIB, EDEN, INMA, MOBA, KANC and RHEA), self-reported maternal education (primary school, secondary school and university degree or higher), maternal age at conception (continuous in years), self-reported ancestry (European, Asian and Pakistani, or other), self-reported maternal pre-pregnancy body mass index (BMI) (continuous in kg/m^2), birth weight (<2500 g, >=2500–3500 g, >=3500–4000 g, or >=4000 g), gestational age at delivery (continuous in weeks) and child's BMI z-score (continuous in kg/m^2) (De Onis et al., 2007; "WHO | BMI-for-age (5–19 years)," n.d.). A bivariate analysis was conducted through linear regression models to determine the crude relationship between the covariates and the outcome of our study (Table S3),

2.7. Statistical analyses

2.7.1. Descriptive analyses and correlations

For continuous variables, we calculated median and interquartile range (IQR) and for categorical variables, frequency and percentage. Pearson's correlation was used to test the correlation between DNA methylation age, calculated with different epigenetic clocks, and chronological age.

2.7.2. Exposome-wide association study of epigenetic age acceleration

To assess the association between the prenatal and childhood exposome and age acceleration we performed an exposome-wide association

study (ExWAS) using the *rexposome* R package (Hernandez-Ferrer and Gonzalez, 2019). The ExWAS approach consists of an exposure-by-exposure estimation of the association between each exposure and the outcome adjusting for potential confounders through independent linear regression models (Patel et al., 2010). It was performed separately for the prenatal and childhood exposome. Results from the 20 imputed datasets were aggregated as described before (Hernandez-Ferrer and Gonzalez, 2019). Results of the ExWAS analyses were expressed as β coefficients and 95% confidence intervals (CIs), that were reported for each IQR increase for continuous exposures or relative to the reference category for binary and categorical exposures. Nominal significance was established at nominal p-value < 0.05. For multiple hypothesis testing correction, we adapted the Bonferroni procedure to handle correlated exposures: we estimated the number of truly independent tests observed according to the correlation structure of the prenatal and childhood exposome (ENT), as $ENT = \sum_{i=1}^{M}[I(\lambda i > 1)(\lambda i - 1)]$, where I(x) is an indicator function and λ_i are the eigenvalues of the matrix of correlations between M exposures (adapted from Li et al. 2012 and Li, 2005 (Li and Ji, 2005; Li et al., 2012)) and then we divided the nominal significance by these calculated effective number of tests. This gave the following p-value thresholds (TEF): $1.01x10^{-3}$ and $8.39x10^{-4}$ for the prenatal and childhood exposome, respectively).

In the main analyses we evaluated the association between prenatal or childhood exposome and age acceleration adjusted for blood cell type proportions. Models for both periods were adjusted for a common set of confounders identified *a priori* based on literature and covariate selection through the DAGitty tool (Textor and Hardt, 2011) (Figs. S1 and S2): (i) child's sex, (ii) cohort, (iii) self-reported maternal education, (iv) self-reported ancestry and (v) maternal age at conception. We also fitted models further adjusted for maternal pre-pregnancy BMI, birthweight (grams) and gestational age at delivery (weeks) in pregnancy exposome models, and maternal pre-pregnancy BMI, birthweight (grams) and child's BMI z-score (De Onis et al., 2007; "WHO | BMI-for-age (5–19 years)," n.d.) in childhood models.

2.7.3. Additional insights on main exposure – epigenetic age acceleration associations

For nominally significant exposure - epigenetic age acceleration associations we did further analyses. Firstly, we investigated dose and duration of the maternal smoking exposure during pregnancy and parental smoking behaviour in childhood. Second, to assess the potential window of susceptibility for smoking effect, we ran additional mutually adjusted models: maternal tobacco smoke during pregnancy adjusted for parental smoking in childhood, and vice-versa. The correlation between exposure to tobacco smoke in both exposure periods was calculated using the polychoric correlation test (Revelle, 2017). Third, we evaluated the association between childhood exposure to indoor PM_{abs} and age acceleration adjusted for maternal tobacco smoke during pregnancy or for parental smoking in childhood. Forth, we tested the association between log2 concentration levels of urinary hippurate obtained by ^{1}HNMR spectroscopy, which is a metabolite marker of fruits and vegetables consumption (Lau et al., 2018), and epigenetic age acceleration to determine whether the potential association found between OP pesticides and epigenetic age acceleration could be explained by a high fresh fruit and vegetables consumption (Papadopoulou et al., 2019). Furthermore, we ran additionally adjusted models: OP pesticides (undetected/detected) adjusted for fruit intake, vegetable intake or hippurate concentrations, besides the common set of confounders identified a priori.

2.7.4. Sensitivity analyses

We repeated the main and further adjusted models of the ExWAS evaluating the association between the prenatal and childhood exposures and age acceleration non-adjusted for blood cell type proportions. Moreover, we repeated the analysis restricted to European ancestry children (n = 1,048) to determine if the ancestry had any influence. We also conducted a cohort-by-cohort analysis for each association with a nominal p-value < 0.05, in the main model, to check the pattern of association within each cohort. The *meta* R package (Schwarzer, 2007) was used to conduct the fixed effect meta-analyses based on the estimates and standard errors of the associations. We looked at the I^2 statistics to describe the percentage of variation across the different cohorts that is due to heterogeneity.

2.7.5. Expression quantitative trait methylation (eQTM)

To provide further biological insight into the "Horvath's Skin and Blood clock", we performed pathway enrichment analyses with the genes associated with the methylation levels of the CpGs used to construct the clock. We conducted a cis eQTM analysis using data from 874 HELIX children of European ancestry (https://helixomics.isglobal.org/) (Ruiz-Arenas et al., 2020). First, we linked each one of the 391 CpGs in the "Horvath's Skin and Blood clock" to the nearby genes (1 Mb window from the CpG and the transcription start site). Then, we tested the association between DNA methylation and gene expression levels of the 12,208 identified CpG-gene pairs through linear regression models adjusted for cohort, child's age and sex. After, multiple-testing correction through a permutation processes, we identified 129 unique genes associated with the methylation of 72 of the 391 CpGs in the "Horvath's Skin and Blood clock". Finally, we performed a pathway enrichment analysis of these 129 genes using the over-representation method of the *ConsensusPath* tool (Kamburov et al., 2011) and three different databases (Kyoto Encyclopedia of Genes and Genomes (*KEGG*), *Reactome* and *BioCarta*). We accepted as significant those pathways with a minimal overlap of 2 genes and a cut-off at q-value of 0.025.

The statistical framework R (version 3.6.0) was used to perform all the analyses (R Core Team, n.d.).

3. Results

3.1. Study population

The study included 1,173 children from the HELIX project, aged between 6 and 11 years old that had information on DNA methylation and on the exposome. Of these children 89.3% were of European ancestry, 54.9% were males, a 20.9% were overweight or obese, and 50.7% were born from mothers with a university degree or higher education level (Table 1).

3.2. Selection of the best epigenetic clock for children

We calculated epigenetic age using different clocks: "Horvath's All Tissue clock" (with *methylclock* R package and Horvath's online calculator), "Horvath's Skin and Blood clock", "PedBe's clock" and Wu's methylation-based age prediction model (with *methylclock* R package) (Table S2). The correlations of the epigenetic age (DNAmAge) calculated from each clock vs. chronological age measured in years are presented in Fig. 1. We found that "Horvath's Skin and Blood clock" showed the strongest correlation with chronological age ($R = 0.85$, $p < 2.2x10^{-16}$). The methylation-based age prediction model by Wu et al., and "Horvath's All Tissue clock", obtained from *methylclock* R package showed a slightly weaker correlation ($R = 0.75$, $p < 2.2x10^{-16}$; $R = 0.72$, $p < 2.2x10^{-16}$, respectively). However, slightly stronger than the one obtained from "Horvath's online calculator" ($R = 0.61$, $p < 2.2x10^{-16}$). The "PedBE's clock", although trained in DNA methylation from buccal cells in children (from 0 years to 20 years), showed the lowest correlation ($R = 0.53$, $p < 2.2x10^{-16}$).

Considering that DNAm data was obtained from blood and observing Pearson's correlation results between epigenetic age and chronological age in our study population, we decided to continue the analyses with the "Horvath's Skin and Blood epigenetic clock", using age acceleration adjusted for blood cell type proportion as the main outcome. This clock

Table 1
Characteristics of the study population (n = 1,173).

Variable	n (%) or median (IQR)
Cohort	
BIB	203 (17.3)
EDEN	146 (12.4)
INMA	215 (18.3)
KANC	198 (16.9)
MOBA	212 (18.1)
RHEA	199 (17)
Self-reported ancestry	
Asian and Pakistani	98 (8.4)
European	1047 (89.3)
Other	27 (2.3)
Maternal age (years)	31 ± 6.8
Maternal education	
Primary school	176 (15)
Secondary school	402 (34.3)
University degree or higher	595 (50.7)
Maternal pre-pregnancy BMI (kg/m^2)	24.1 ± 5.9
Sex of the child	
Female	529 (45.1)
Male	644 (54.9)
Birthweight	
<2500 g	40 (3.4)
>=2500–3500 g	662 (56.4)
>=3500–4000 g	357 (30.4)
>=4000 g	114 (9.7)
Gestational age (weeks)	40 ± 2
Child z-score BMI (kg/m^2)	0.3 ± 1.5
Child BMI (WHO categorization)	
Grade 1/2/3 thinness and Normal weight	927 (79.1)
Overweight or Obese	246 (20.9)
Age at blood collection (years)	7.2 ± 2.4

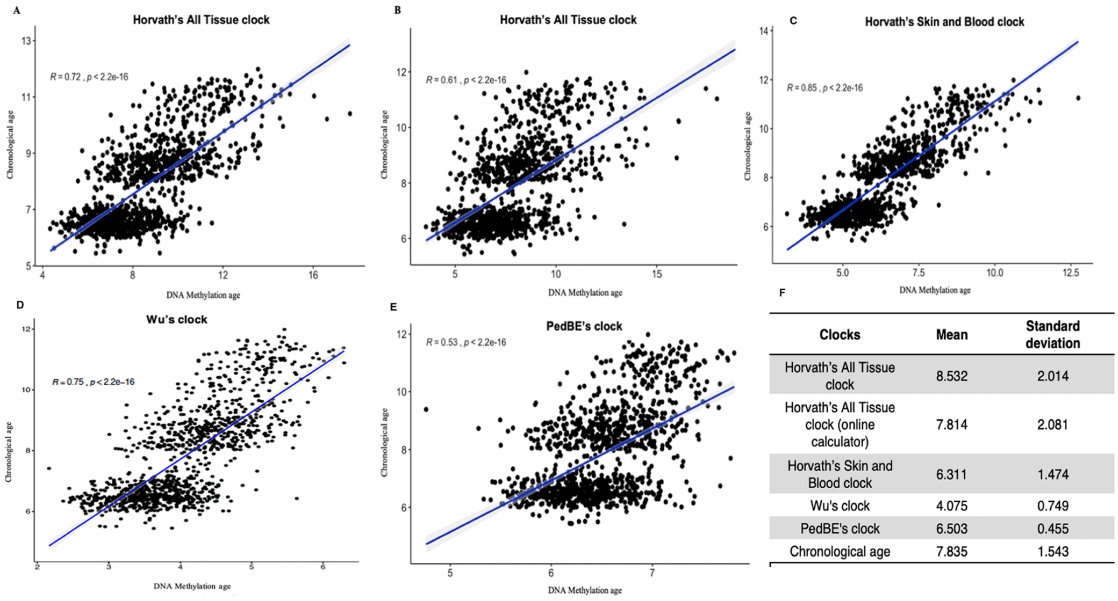
Note: BIB = Born in Bradford; EDEN = Étude des Déterminants Pré et Postnatals du Développement et de la Santé de l'Enfant; INMA = Infancia y Medio Ambiente; KANC = Kaunas Cohort; MoBa = Norwegian Mother, Father and Child Cohort Study; RHEA = Mother-child Cohort in Crete; BMI = Body Mass Index.

was created and trained on a sample size of 1206 individuals from 0 to 75 years old and it is widely used in the aging field as marker of biological aging.

3.3. Exposome-wide association study (ExWAS)

For the prenatal exposome, the ExWAS identified that maternal tobacco smoke during pregnancy was associated with increased epigenetic age acceleration (β = 0.14, 95% CI = 0.02 to 0.26) although it did not pass multiple testing correction ($p < 1.01x10^{-3}$ for the pregnancy exposome) (Table 2, Fig. S4A). Moreover, this association remained similar when the model was further adjusted for maternal pre-pregnancy BMI, birthweight, and gestational age at delivery (no vs. yes; β = 0.13, 95% CI = 0.01 to 0.25) (Table S4 and Fig. S5A; Supplementary Material 2 for the full set of results: Excel Tables S1-S2).

With regard to the childhood exposome, the ExWAS identified two exposure variables that were associated with an increase in age acceleration ($p < 0.05$): indoor particulate matter absorbance (PM_{abs}) (β = 0.07, 95% CI = 0.02 to 0.12) and parental smoking (neither vs. both parents; β = 0.15, 95% CI = 0.01 to 0.29) (Table 2, Fig. S4B). Moreover, two other variables were inversely associated with age acceleration ($p < 0.05$): the organic pesticide (OP) dimethyl dithiophosphate (DMDTP) (undetected vs. detected; β = −0.13, 95% CI = −0.24 to −0.02) and of the persistent pollutant polychlorinated biphenyl-138 (PCB-138) (β = −0.07, 95% CI = −0.14 to −0.01) (Table 2, Fig. S4B). None of these associations passed multiple testing correction ($p < 8.39x10^{-4}$ for the childhood exposome). After further adjustment of the models for maternal pre-pregnancy BMI, birthweight and child's z-score BMI, indoor PM_{abs} (β = 0.06, 95% CI = 0.01 to 0.11) and DMDTP (undetected vs. detected; β = −0.12, 95% CI = −0.24 to −0.02) were the only two exposures that remained significant ($p < 0.05$) (Table S4 and Fig. S5B). Furthermore, in the fully adjusted model, the N-butyl paraben (BUPA) was also nominally associated with age acceleration ($p < 0.05$) (β = 0.04, 95% CI = 0.01 to 0.08) (Table S4 and Fig. S5B; Supplementary



Clocks	Mean	Standard deviation
Horvath's All Tissue clock	8.532	2.014
Horvath's All Tissue clock (online calculator)	7.814	2.081
Horvath's Skin and Blood clock	6.311	1.474
Wu's clock	4.075	0.749
PedBE's clock	6.503	0.455
Chronological age	7.835	1.543

Fig. 1. Pearson's correlations between DNA methylation age, calculated with different clocks, and chronological age. Each graph shows a different epigenetic clock: (A) Horvath's All Tissue clock (*methylclock*. R package); (B) Horvath All Tissue clock (Horvath's online calculator); (C) Horvath's Skin and Blood clock (*methylclock* R package); (D) Wu's clock (*methylclock* R package); (E) PedBE's clock (*methylclock* R package); (F) Summary table of the mean and standard deviation (sd) of the clocks evaluated.

Table 2
ExWAS* of prenatal and childhood exposures vs. age acceleration adjusted for blood cell type proportions (main model).

				ExWAS*	
	Exposure	**Exposure family**	**Units**	**Estimate (95% CI) [a]**	**P-value**
Prenatal	Maternal tobacco smoking	Tobacco smoke	No vs. Yes	0.14 (0.02, 0.26)	0.025
Childhood	Indoor PM_{abs}	Indoor air	ug/m^3	0.07 (0.02, 0.12)	0.003
	Parental smoking	Tobacco smoke	Neither vs. Both	0.15 (0.01, 0.29)	0.036
	Dimethyl dithiophosphate (DMDTP)	OP Pesticides	Undetected vs. Detected (adjusted for creatinine)	−0.13 (−0.24, −0.02)	0.017
	Polychlorinated biphenyl-138 (PCB-138)	OCs	ng/g (adjusted for lipids)	−0.07 (−0.14, 0.01)	0.037

Note: ExWAS = exposome-wide association study; PM_{abs} = Particulate Matter Absorbance, DMDTP = Dimethyl dithiophosphate; OP Pesticides = Organophosphate Pesticides; PCB-138 = Polychlorinated biphenyl-138; OCs = Organochlorine compounds; IQR = Interquartile range. *Results are presented only for the exposures with nominal significance (p value < 0.05) in the ExWAS adjusted for: child's sex, cohort, self-reported maternal education, self-reported ancestry and maternal age in years. The analyses were conducted in 1,173 children from the HELIX subcohort. [a]Coefficient estimates are given in age acceleration effect change for each IQR increase in continuous exposure variables, or relative to the reference category in binary and categorical variables.

Material 2 file for the full set of results: Excel Tables S1 and S2).

3.4. Additional insights on main exposure – epigenetic age acceleration associations

Firstly, we tested the effect of dose and duration of the maternal exposure to tobacco smoking during pregnancy, through linear regression models (Table S5), that suggested a slightly positive trend. Childhood exposure to second-hand tobacco smoke due to parental smoking (classified as: none, one parent, or both parents) also showed a linear trend (Table S5). Secondly, we assessed the effect of the exposure window to tobacco smoke, by running mutually adjusted models. The effect estimates of maternal tobacco smoke during pregnancy adjusted for childhood parental smoking were smaller (28.57% β reduction) (Table S6). A similar pattern was observed when adjusting parental smoking in childhood for maternal tobacco smoke during pregnancy (40% β reduction) (Table S6). To determine if we could disentangle prenatal and childhood smoking exposure, we calculated their correlation. Indeed, a strong positive correlation was shown between exposure to tobacco smoke in both exposure periods (polychoric coefficient = 0.72, prenatal vs. childhood exposure).

In regards to indoor PM_{abs}, we evaluated the influence of parental smoking behaviour and also maternal smoking during pregnancy on the association between indoor PM_{abs} and epigenetic age acceleration as indoor PM_{abs} was estimated through a prediction model trained in a subgroup of children (n = 157) using home personal measurements and questionnaire data including parental smoking behaviour, among other variables (Tamayo-Uria et al., 2019). After adjustment for prenatal or childhood exposure to tobacco smoke, no differences in the estimate coefficients of PM_{abs} were observed (0% β change), suggesting independent effects (Table S7).

Furthermore, given that OP pesticides are present in fruit and vegetables, we speculated that they could be a proxy of fresh fruit and vegetable intake in the study. However, fruit and vegetable intake, were not associated with age acceleration (Supplementary Material 2 file for the full set of results: Excel Table S2) and the effect size of the models of DMDTP additionally adjusted for vegetable or fruit intake did not change substantially (7.14% β increase for vegetables and 0% β change for fruit) (Table S9). Given that food frequency questionnaire data has some misclassification issues, we also adjusted the model of DMDTP for urinary hippurate, which is a metabolite marker of fruits and vegetables consumption (Lau et al., 2018; Pallister et al., 2017). Again, the effect size did not change (0% β change) (Table S9), although hippurate was higher in those children with DMDTP over the limit of detection (mean difference = 0.211, p-value < $2x10^{-16}$).

Finally, when evaluating the childhood model further adjusted for birthweight, maternal pregnancy body mass index and child z-score body mass index, the effect size of the association between PCB-138 and epigenetic age acceleration was drastically attenuated (53.16% β reduction) (Supplementary Material 2 file for the full set of results: Excel Table S2). In our data, as in other studies (Huang et al., 2019), we observed a positive association between child z-score BMI with age acceleration (β = 0.08, 95% CI = -0.05 to 0.12, not adjusted for cell type proportions; β = 0.07, 95% CI = -0.03 to 0.10, adjusted for cell type proportions). Thus, the association of child's BMI with PCB-138 and with epigenetic age acceleration could explain this reduction.

3.5. Sensitivity analyses

When assessing the association between the prenatal and childhood exposures and epigenetic age acceleration non-adjusted for blood cell type proportions we detected all the associations described above for the main model (Fig. S7). However, additional childhood exposures of the same exposure families identified in the main model reached nominal significance (PCB-153, PCB-170, PCB-180, Diethyl thiophosphate (DETP)), as well as of new exposure families (Perfluorooctane sulfonate (PFOS), Hexachlorobenzene (HCB) and Oxybenzone (OXBE)) (Fig. S7B). None of these associations passed multiple testing correction (Supplementary Material 2 file for the full set of results: Excel Tables S1 and S2).

We repeated the prenatal and childhood ExWAS restricting the analyses to European ancestry children and similar results were obtained (Supplementary Material 2 file for the full set of results: Excel Tables S3 and S4). For the significant exposure – epigenetic age acceleration associations described above, the absolute percent change in the coefficients (β) between the whole study sample and the subset of European ancestry sample was < 12%.

Finally, we conducted fixed effects inverse variance weighted meta-analysis of results by cohort of the top exposure – epigenetic age acceleration associations (Fig. S8). For maternal tobacco smoking during pregnancy and childhood indoor PM_{abs} estimated effects were consistent across cohorts (Figs. S8A, S8D). For the other associations the pattern was slightly more heterogeneous with some cohorts going in the opposite direction (Figs. S8B, S8C, and S8E). The statistic I^2 was lower than was 41% for all exposures.

3.6. eQTM analyses

To interpret the biological meaning of epigenetic age, we searched the genes whose expression was associated with the methylation levels of the CpGs included in the "Horvath's Skin and Blood clock" in HELIX. 72 CpGs out of the 391 (18.41%) in "Horvath's Skin and Blood clock" were associated with the expression of 151 unique transcript clusters (TCs, or genes), which were annotated to 129 unique gene symbols (Supplementary Material 2 file: Excel Table S5). 122 out of 129 genes were mapped in ConsensusPathDB and were enriched for the following biological pathways (q-value < 0.025): i) Adaptive and innate immune system, ii) Apoptosis, cell cycle and cancer, and iii) Detoxification of xenoestrogens (Supplementary Material 2 file: Excel Tables S6 and S7).

4. Discussion

This is the first study to evaluate associations between a wide range of prenatal and childhood environmental exposures (the early-life exposome) and the epigenetic age acceleration in children.

We observed a positive association between maternal tobacco smoke during pregnancy and exposure to parental smoke through childhood and age acceleration in childhood, in line with previous studies (Javed et al., 2016; Simpkin et al., 2016; Wu et al., 2019b; Yang et al., 2019). For instance, in adult and elderly populations, active smoking has been correlated with increased epigenetic age acceleration (Gao et al., 2016). Others, using the "Horvath's All tissue clock" clock, have found that maternal smoking increases epigenetic age acceleration as early as at birth (Javed et al., 2016) and that effect is persistent at least until childhood (Simpkin et al., 2016). We also observed that childhood exposure to second-hand smoke (SHS) was associated with increased age acceleration in children. Adjustment for maternal smoking during pregnancy attenuated the association, and considering that pregnancy active smoking implies a higher dose than childhood passive smoking and that smoking effects on blood DNA methylation seem to be persistent (Vives-Usano et al., 2020), these results suggest that the SHS association may have been partly confounded by the exposure during the pregnancy period. Prior research described that maternal tobacco smoking was associated with cord blood DNA methylation at >6,000 CpG sites (Joubert et al., 2016). However, none of these CpGs overlapped with the CpGs used in the Horvath's Skin and Blood clock, thus suggesting different mechanisms. Moreover, we observed a dose dependent effect regarding exposure to tobacco smoke during pregnancy and childhood, in which a higher dose or a longer duration of the exposure was related with increased estimates. What we observed is biologically plausible and is consistent with previous evidence related to other health outcomes (Banderali et al., 2015; Vives-Usano et al., 2020; Zhuge et al., 2020).

In relation to air pollution, we found a positive association between epigenetic age acceleration and childhood indoor PM_{abs}, which is used as a proxy of elemental/black carbon (EC or BC) in the HELIX project. Both pollutants are particles coming from the incomplete combustion of fossil fuels, biofuels, and biomass (Briggs and Long, 2016). Adjustment for childhood SHS exposure did not change this association suggesting that the association observed was led by other sources of PM. Two longitudinal cohort studies, one in adult women and the other in adult men, found that exposure to outdoor BC and ambient $PM_{2.5}$ were associated with increased age acceleration (Nwanaji-Enwerem et al., 2016; Ward-Caviness et al., 2016). Also, a recent study observed that different clusters defined by outdoor $PM_{2.5}$ component profiles were related to accelerated aging in women (White et al., 2019). As far as we know there are no studies of the air pollution effects on epigenetic age in children, neither for indoor nor outdoor levels. Thus, further exploration in children is needed.

In this study, we observed an association between higher DMDTP exposure and decreased age accelerations, which is contrary to what we would expect as DMDTP exposure could be considered as a risk factor for age-related diseases (mainly neurodegenerative (Hayden et al., 2010)). We tried to explain the results that we obtained by looking at the possible association reported before between DMDTP exposure and fruit/vegetable intake (Pallister et al., 2017), in which a higher consumption of fruit/vegetable intake was associated with higher concentrations of DMDTP. As our data on fruit and vegetable intake was obtained by a food frequency questionnaire in which we might be facing misclassification issues, we evaluated a urinary metabolite called hippurate, which is accepted as a metabolite marker of fruits and vegetables consumption. Thus, here hippurate is a biomarker of fruit and vegetables intake, and it might be associated with OPs. However, the role of DMDTP reflecting the effects of fruit and vegetable intake needs further investigation in other studies.

PCBs are widely present in the environment, although whose production was banned in 2001 due to their toxicity and persistence in health (Sun et al., 2005). In our study, we observed a protective effect of PCB-138 on epigenetic age acceleration. When we additionally adjusted the model for child BMI z-score, the association was largely attenuated, suggesting that z-score BMI could explain a notable part of the association. It has been previously reported an inverse association between PCB-138 and child z-score BMI or BMI as these chemicals are highly lipophilic and are stored in fat tissues (Agudo et al., 2009; Dirinck et al., 2011; Domazet et al., 2020; Vrijheid et al., 2020). We thus hypothesize that the association we observed for PCBs might be capturing the relationship between epigenetic age acceleration and body mass index, instead of PCBs exposure. Future studies should address this issue by considering adipose tissue and BMI distinctly, and incorporating toxicokinetic models of PCBs during childhood (Cadiou et al., 2020; Jackson et al., 2017; Vrijheid et al., 2020; Wood et al., 2016).

When evaluating epigenetic age acceleration non-adjusted for blood cell type proportions we detected the same associations as in the main model adjusted for cellular composition plus additional associations with lipophilic chemical compounds. These compounds tend to accumulate in lipid-rich tissues, and their serum levels depend on child's adipose tissue content. At the same time child's adipose tissue content (i.e., obesity) leads to an inflammatory state and an imbalance of the blood cellular percentages. Thus, we hypothesize that when not considering blood cell type proportions we might be capturing the effects of BMI on epigenetic age acceleration, and in turn confounded associations with the lipophilic chemical compounds. In this sense, most of these associations disappeared in models further adjusted for child's BMI.

Aging is a multi-factorial process which involves multiple and complex interactions between biological mechanisms (Borup et al., 2008; Weinert and Timiras, 2003). Aging is related to increased oxidative stress and inflammation, increased DNA damage due to reduced DNA repair, and decreased immune response to external agents and tumorigenic cells (Franzke et al., 2015; Lovell and Markesbery, 2007; Sadighi Akha, 2018). We found that part of the CpGs of the Horvath's Skin and Blood clock were related to the expression of genes involved in pathways relevant for aging processes such as immune response, cell cycle and apoptosis, and detoxification, suggesting that they might mediate the effects of exposure to tobacco smoke, indoor PM_{abs}, and BMI (Camous et al., 2012; Horstman et al., 2012; Kuba and Raida, 2018; Leandro et al., 2015; Weng, 2006).

Major strengths of the present study encompass detailed and comprehensive assessment of the early-life exposome in six populations across Europe with different cultures and settings. We were able to characterize a broad range of environmental exposures for a relatively large sample size in two separate periods of time, pregnancy and childhood, which can be considered as critical periods of vulnerability for children's development (Wright, 2017). Moreover, the environmental exposures that shown an association with epigenetic age acceleration were not correlated among them revealing a non-linear relationship. We have conducted a screening analyses of single exposures using the ExWAS approach, which was characterized by its high sensitivity, but also high false positive rate (Holme et al., 2016). In addition, we have published all the estimates obtained for each exposure-epigenetic age acceleration association to avoid selective reporting bias (Reid et al., 2015). Finally, we have conducted several sensitivity analyses, which did not result in a notable change in our findings.

Nevertheless, our results should be interpreted in the context of its limitations. First, the "Horvath's Skin and Blood clock" used in this study was trained considering all ages and it is not specific for children, which could lead to less precision in assessing epigenetic age. However, one of the known epigenetic clocks based on children population used buccal cells, which gave lower correlations with chronological age likely due to the major differences between tissues than among age ranges. A more robust and improved epigenetic clock within the range of age of children based on blood cells is needed as the one evaluated in this study (Wu

et al., 2019a) was as based on a smaller sample size and it was not widely validated. Second, we acknowledge that we cannot directly contrast the effect size and significance levels between exposures as the exposures evaluated in this study were measured with different measurement errors. Third, when evaluating the childhood exposome our study had a cross-sectional design, and we could not establish a causal link between the environmental exposures and the epigenetic age acceleration. Moreover, although we have adjusted the models for several variables, there can still be residual confounding. In our analyses we evaluated a wide range of prenatal and childhood environmental exposures, however we could not include all factors affecting age acceleration and cover a complete exposome. Therefore, we encourage future studies to further investigate the factors not considered in our analyses. Fourth, the statistical power of our study was relatively limited, because of our sample size.

5. Conclusions

In summary, our study found that prenatal and childhood exposure to tobacco smoke and childhood indoor PM_{abs} are associated with accelerated epigenetic aging. Epigenetic modifications in pathways involved in inflammation, detoxification and cell cycle control may be mechanisms by which these environmental exposures can impact human health from early life onward. As aging is considered a public health issue worldwide, new evidence in child populations might drive new policies to reduce environmental exposures and promote a "healthy aging" from early stages of life.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author's contributions

PdP-B, MB and MV designed the study. MV is the coordinator of the HELIX project, with the help of LM and OR. SA, SC, MC, LC, RG, KBG, JL, RMc, RS, MV and JS are the PIs of the cohorts, recruited participants or obtained biological samples. LSH, MN and PD were involved in the measurements of environmental or chemical pollutants or gave advise about them. AC and MB produced DNA methylation data; MV-U and MB obtained gene expression data. HCK and LM obtained urinary metabolomics data. CR-A and CH-F performed the quality control of the methylation and expression data. JRG, CH-F, and DP developed the *rexposome* and *methylcock* R packages. PdP-B, under the supervision of MB, MV and JS, performed the statistical and bioinformatics analyses. PdP-B and MB wrote the manuscript and all others approved it.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106683.

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5.2 Paper II

de Prado-Bert P, Cilleros A, Fernandez-Jimenez N, Fernández M, Santa Marina Rodríguez L, Llop Pérez S, Vrijheid M, Bustamante M, Sunyer J.

Association between ambient air pollution and active maternal smoking exposure during pregnancy and placental epigenetic age acceleration

In preparation

*Supplementary material of this manuscript can be found in the following link.

Association between ambient air pollution and active maternal smoking exposure during pregnancy and placental epigenetic age acceleration

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Abstract

Air pollution exposure and active maternal tobacco smoke during pregnancy are considered as two of the top health menacing exposures worldwide which might be linked to pregnancy complications, such as lower birth weight or reduced gestational age. The human placenta is a complex organ with a key role during pregnancy. It has been found to be susceptible to maternal environmental factors which can alter its formation, functioning and biological aging. Here, we investigated the association between exposure to ambient air pollution and active maternal tobacco smoking during pregnancy and placental epigenetic age acceleration in 379 individuals of the INfancia and Medio Ambiente (INMA) cohort. Different air pollutants (NO_2 and $PM_{2.5}$) were estimated for the pregnancy period based on residential addresses. Active maternal tobacco smoking was self-reported at 12 and 32 weeks of gestation. Placental DNA methylation was measured with the Illumina EPIC array. Epigenetic age acceleration was calculated with four different clocks. For downstream analysis we used the control placental clock previously developed by Lee et al., as it showed the highest correlation with chronological age. We fitted linear regression models for each exposure (air pollutant or maternal tobacco smoking) and each outcome (birth weight, gestational age or and placental epigenetic age acceleration), adjusting for covariates. Sustained maternal smoking was related to lower birth weight, but not with gestational age. Air pollution variables were not related to any reproductive outcome. None of the pregnancy exposures, air pollution or maternal smoking, were associated with epigenetic age acceleration in the main analysis or in the several sensitivity analyses. As healthy aging is considered a public health issue worldwide, further research with a more accurate exposure assessment in larger samples sizes is needed to elucidate which is the role of environment in placental biological aging.

Keywords

Aging; Placental epigenetic age; DNA methylation; Pregnancy; Environmental exposures; Maternal smoking; Air pollution

INTRODUCTION

The human placenta is a multifunctional organ with a key role during pregnancy as it acts as the interface between the mother and the foetus, and it serves as a physical barrier for toxic compounds. It is responsible for a variety of critical functions such as gas exchange, transfer of nutrients and waste products between the mother and the foetus, transfer of immune factors, and secretion of hormones essential for the foetal growth and development (Griffiths and Campbell, 2015). It has been observed that during pregnancy, placenta experiences physiologic aging: the organ grows rapidly throughout the period of gestation and matures within a limited time (Cox and Redman, 2017). An adequate timely ageing is needed for the placenta to reach an optimal foetal growth and development as it has been seen that accelerated placental aging could be a risk factor for a variety of adverse health outcomes such as early onset preeclampsia (Mayne et al., 2017), late-onset foetal smallness (small for gestational age (SGA) and foetal growth restriction (FGR)) (Paules et al., 2019) or higher risk of still birth (Maiti et al., 2017).

During development, the placenta is the first complex organ to form, therefore, it is susceptible to maternal environmental factors from earliest stages, which can influence its formation, functioning and aging (Marsit, 2015). Two of the top health menacing worldwide exposures, which are air pollution and maternal tobacco smoking, have already been related with adverse pregnancy and birth outcomes such as lower birthweight, reduced gestational age at delivery, higher risk for small for gestational age or foetal growth restriction (Glassman et al., 2020; Ko et al., 2014; Leonardi-Bee et al., 2008; Maisonet et al., 2004; Marufu et al., 2015; Qiu et al., 2020; Salmasi et al., 2010; Taylor et al., 2021; Yuan et al., 2020, 2019). Previous studies have detected the presence of air pollution particles and toxic compounds from tobacco in placental samples (Bongaerts et al., 2020; Bové et al., 2019; Mohammadi et al., 2017; Raia-Barjat et al., 2020; Suter et al., 2019).

Besides direct translocation of toxic particles into the placenta and fetus, these exposures also produce systemic and placental inflammation and oxidative stress (Romero et al., 2007) (Bangma et al., 2021; Núñez Estevez et al., 2020; Zavatta et al., 2022). In turn, both processes can interfere with the physiological aging process of the placenta (Manna et al., 2019; Menon, 2014). Finally, it has been seen that exposure during pregnancy to environmental risk factors such as air pollution or tobacco smoking are related to placental DNA methylation alterations (Abraham et al., 2018; Everson et al., 2021; Saenen et al., 2019; Suter and Aagaard, 2020). DNA methylation is considered as an epigenetic mark that consists of transferring a methyl group onto the C5 position of a cytosine which leads to the formation of a 5-methylcystosine (Wang and Ibeagha-Awemu, 2021) which helps to regulate gene expression (Moore et al., 2013). In most tissues, DNAm is relatively stable within cell types, however during gestation placental DNAm is subject to extensive alterations due to changes in cell composition to start the formation of the placenta, the correct functioning of this tissue and the need to adapt to in utero stressors (Del Gobbo et al., 2019; Hogg et al., 2012; Novakovic et al., 2011; Rondinone et al., 2021). Differently from adult somatic tissues which the vast majority of the genome is highly methylated (>70%), 37%

of the placenta genome is covered by partially methylated domains (PMDs), regions with low methylation levels (Schroeder et al., 2013). PMDs are stable through gestation and between individuals, and the expression of the genes in these regions tend to be repressed.

Chronological aging of the placenta can be measured as gestational age which is estimated from ultrasound measures or the last menstrual period (Lynch and Zhang, 2007). However, new molecular estimators have been proposed to evaluate biological GA, in contraposition to chronological GA. Some of them, known as placental epigenetic clocks, are based on DNA methylation data from placental tissue, and they can be used to predict GA and the difference between placental epigenetic age and chronological GA at delivery, which is known as placental epigenetic age acceleration. When placenta ages faster than expected, this age acceleration takes positive values, compared to when the organ ages slower that the measure is negative. To our knowledge, two different placental epigenetic clocks with high placental age prediction accuracy exist (Lee et al., 2019; Mayne et al., 2017). One of them has already been associated with early onset preeclampsia (Mayne et al., 2017). Although, DNA methylation is just one of the pathways by which epigenetics affect gene expression, besides histone modification or non-coding RNA, we will be referring to the rest of the text to placental DNA methylation age as placental epigenetic age.

In this study we aimed to evaluate the association between exposure to ambient air pollution and active maternal tobacco smoking during pregnancy and placental epigenetic age acceleration in the INfancia and Medio Ambiente (INMA) cohort.

MATERIALS AND METHODS

Study population

This study was conducted in the context of the INMA - INfancia y Medio Ambiente - (Environment and Childhood) Project, which is an ongoing population-based birth cohort established in different cities of Spain. The project includes more than 3,000 mother-child pairs from seven Spanish cohorts located in different geographical areas: Granada, Menorca, Ribera d'Ebre, Gipuzkoa, Asturias, Valencia, and Sabadell. The last four cohorts shared common protocols based on the experience in Granada, Menorca, and Ribera d'Ebre, and their recruitment was done between 2003 and 2008 (Guxens et al., 2012). Pregnant women were recruited at first prenatal visit at primary health care centres or public hospital of their region if they fulfilled the following inclusion criteria: singleton pregnancy, intention to deliver at the reference hospital, ≥16 years of age, no problems of communication and no assisted conception. Afterwards, different follow-ups of the offspring were carried out at birth, 1.5, 4,7,9, and 11 years of age. The work in INMA was covered by a study approval obtained from the ethics committees of each participating centre and informed consents from the mothers were collected.

The present analysis uses data from three INMA cohorts: Valencia (n=67), Gipuzkoa (n=155) and Sabadell (n=157), of which information on the exposure and the outcome assessed was available (total = 379).

Exposure assessment

Ambient air pollution exposure assessment

The following atmospheric pollutants were assessed for different time windows in all the cohorts: NO_2 and $PM_{2.5}$. A detailed exposure assessment on NO_2 exposure was previously explained elsewhere (Iñiguez et al., 2009). Briefly, passive samplers were used to measure NO_2 levels. In four sampling campaigns, one-week measurements were carried out at each sub-cohort area according to geographic criteria, expected pollution gradients and population density. Afterwards, temporally adjusted land-use regression (LUR) models were developed to estimate exposure to NO_2 in different time windows: trimester 1 (1–13 weeks), trimester 2 (14–28 weeks), trimester 3 (29 weeks to delivery), and for the entire pregnancy. LUR models temporally adjusted to measurements of local background monitoring stations and averaged over the trimesters of pregnancy and the whole pregnancy period were used to assess $PM_{2.5}$ exposure. The site-specific LUR model developed in the context of the ESCAPE project (Eeftens et al., 2012) was used for Sabadell, and the ESCAPE European-wide LUR model was applied for Gipuzkoa and Valencia (Wang et al., 2014). In this study the main analyses have been conducted evaluating the NO_2 and $PM_{2.5}$ exposure regarding the entire pregnancy.

Active maternal tobacco smoking exposure assessment

Information on active maternal smoking during pregnancy was collected via questionnaires administered by trained interviewers and answered by the pregnant women at 12 and 32 gestational weeks. A total of four variables have been evaluated: active maternal smoking at week 12 of pregnancy (Yes/No), active maternal smoking at week 32 of pregnancy (Yes/No), any active maternal smoking during pregnancy (Yes/No), and active sustained maternal smoking (Non-smokers/Non-sustained smoking/Sustained smoking), where sustained means that the pregnant woman had smoked, at least, in the 1st and in the 3rd trimester. In this study the main analyses have been conducted evaluating the variables related to any active maternal smoking during pregnancy and active sustained maternal smoking.

Outcome assessment

Reproductive outcomes

The self-reported last menstrual period collected via questionnaires was used to define gestational age in weeks and it was confirmed using ultrasound examination in 12 weeks of gestation. We performed a rank-based inverse normal transformation of gestational age in weeks to evaluate its relationship with air pollution and active maternal tobacco exposure. Different anthropometric measurements were done at birth, such as birthweight, which was measured by midwifes and is evaluated in grams.

Placental methylation data collection

Briefly, biopsies of approximately 5 cm^3 were obtained from the inner region of the placenta, approximately 1.0-1.5 cm below the foetal membranes, corresponding to the villous parenchyma, and at ~5 cm from site of cord insertion. Genomic DNA from placenta was isolated using the DNAeasy® Blood and Tissue Kit, (Qiagen, CA, USA). DNA methylation was assessed with the Infinium MethylationEPIC BeadChip from Illumina, following manufacturer's protocol in the Erasmus Medical Centre core facility. Three technical duplicates were included. Samples were randomized considering cohort and sex. The methylation data was pre-processed using the PACEAnalysis R package (v.0.1.7). Dye-bias and Noob background correction, implemented in minfi R package, were applied followed by normalization of the data with the functional normalization method. To correct for the bias of type-2 probes values the beta-mixture quantile (BMIQ) normalization was applied. SentrixID (array) batch effect was controlled with the ComBat method (Johnson et al. 2007). Finally, to correct for the possible outliers, we winsorized the extreme values to the 1% percentile (0.5% in each side), The final dataset consists of 379 samples and 811,990 probes.

Calculation of epigenetic age with different clocks

Placental epigenetic age and age acceleration through different clocks were calculated with *methylclock* R package (Pelegí-Sisó et al., 2020) based on placental DNA methylation data using the Lee's clock and the Mayne's clock: control placental clock (CPC), robust placental clock (RPC), refined robust placental clock (refRPC) (Lee et al., 2019), and Mayne's clock (Mayne et al., 2017). Briefly, from normalized and batched corrected methylation data, the package extracts methylation levels of CpGs included in each clock (Table S1). Subsequently, the coefficients obtained through elastic net in the prediction models of each of the clocks in the original papers are used to calculate placental epigenetic age and age acceleration. Therefore, for each clock we obtained different measures: i) DNA methylation predicted age (DNAm age) in weeks, ii) difference between DNAm and chronological age in weeks (ageAcc); iii) residuals obtained after regressing chronological age on DNAm age (ageAcc2), and iv) residuals obtained after regressing chronological age and placental cell type

proportions on DNAm age (ageAcc3). We estimated cell type composition from placental DNA methylation array data using the R package *planet*, which uses as reference DNA methylation levels from cells isolated from third trimester placentas (trophoblasts, stromal, Hoffbauer, endothelial, nucleated red blood cells (nRBC) and syncytiotrophoblast) (Yuan, 2022; Yuan et al., 2021).

Covariates

Detailed information about covariates was obtained through questionnaires administered at week 12 and 32 of pregnancy, except for BMI that was collected at 12 weeks, and it refers to the pre-pregnancy period. The following key covariates were considered to gain precision or as potential confounders a priori: cohort (Gipuzkoa, Valencia and Sabadell), child's sex (female, male), child's ethnicity (European, other), maternal age at recruitment in years, maternal education (primary school secondary school, university degree or higher), maternal pre-pregnancy BMI in kg/m^2. Other covariates were used to describe the study population or for the sensitivity analyses: gestational age (in weeks), preterm delivery, type of delivery (vaginal, caesarean), labour initiation (spontaneous, caesarean elective, induced), type of delivery in three categories (eutocic, caesarean, instrumental), preterm birth (Yes/No), birthweight in grams, parity (0,1, 2 or more), ever active maternal smoking during life (Yes/No), active maternal smoking at the beginning of pregnancy (Yes/No) and paternal smoking during pregnancy (Yes/No).

Statistical analyses

Descriptive analyses and correlations

For categorical variables, we calculated frequency and percentage; and for continuous variables, we calculated median and interquartile range (IQR). We used Spearman's correlation coefficients to quantify the correlation between chronologic GA and placental epigenetic age, the correlation between NO_2 and $PM_{2.5}$ exposure, including 1st trimester, 2nd trimester, 3rd trimester and the whole pregnancy period by cohort.

Main analyses

Ambient air pollution exposures and active maternal tobacco smoking and reproductive outcomes

To assess the association between ambient air pollution exposures during pregnancy and active maternal tobacco smoking with gestational age and birthweight we applied linear regression models adjusting for potential confounders using the lm function from the *stats* R package. Each air pollutant exposure (NO_2 and $PM_{2.5}$) during the whole period of pregnancy and each variable related to active maternal tobacco smoking was tested versus gestational age (weeks) and birthweight (grams). Models were adjusted for a common set of covariates identified a priori based on literature and its availability in INMA cohort: child's sex, maternal

education, maternal age, cohort, and child's ethnicity. We conducted complete case analyses and the results of the linear regression models were expressed as β coefficients and p-value, that were reported for each IQR increase of air pollutant exposure and relative to non-smokers when evaluating maternal tobacco smoking. Nominal significance was established at nominal p-value < 0.05.

Ambient air pollution exposures and placental epigenetic age acceleration

To assess the association between the ambient air pollution exposure during pregnancy and placental epigenetic age acceleration we applied linear regression models adjusting for potential confounders using the lm function from the *stats* R package. Each air pollutant exposure (NO_2 and $PM_{2.5}$) during the whole period of pregnancy was tested versus placental epigenetic age acceleration adjusted for placental cell type proportions. In the main analyses, models were adjusted for a common set of covariates identified a priori based on literature and its availability in INMA cohort: child's sex, maternal education, maternal age, cohort, and child's ethnicity. We conducted complete case analyses and the results of the linear regression models were expressed as β coefficients and p-value, that were reported for each IQR increase of the exposure. Nominal significance was established at nominal p-value < 0.05.

Active maternal tobacco smoking exposure and placental epigenetic age acceleration

To assess the association between active maternal tobacco smoking during pregnancy and placental epigenetic age acceleration we fitted linear regression models adjusting for potential confounders using the lm function from the *stats* R package. Each variable related to active maternal tobacco smoking was tested versus placental epigenetic age acceleration adjusted for placental cell type proportions. In the main analyses, models were adjusted for a common set of covariates identified a priori based on literature and its availability in INMA cohort: child's sex, maternal education, maternal age, cohort, and child's ethnicity. We conducted complete case analyses and the results of the linear regression models were expressed as β coefficients and p-value, that were reported relative to non-smokers.

Sensitivity analyses

We conducted several sensitivity analyses. First, we further adjusted main models for maternal pre-pregnancy BMI or mean temperature during pregnancy. Second, we repeated the main analyses evaluating the association between air pollution exposure or active maternal tobacco smoking and placental epigenetic age acceleration non-adjusted for placenta cell type proportions. Third, we also evaluated the different time windows of ambient air pollution exposure and active maternal tobacco smoking exposure (1^{st}, $2^{nd,}$ and 3^{rd} trimester of pregnancy). Fourth, as tobacco smoking can be considered as one of the compounds of ambient air pollution exposure, we further adjusted the models of air pollution for the variable of any smoking during pregnancy. Fifth, for air

pollution and maternal tobacco smoking exposures we repeated the analyses stratifying by cohort. Finally, we restricted the analyses considering not preterm deliveries.

Pathway enrichment analyses

To provide further biological insight into the control placental clock, we performed pathway enrichment analyses with the genes associated with the methylation levels of the CpGs used to construct the clock. We annotated each one of the 548 CpGs in the clock to the nearby genes through the library from the *IlluminaHumanMethylation450kanno.ilmn12.hg19* R package (Ref.). The CpGs used for developing the placental epigenetic clock were mapped to 431 unique genes. Finally, we performed a pathway enrichment analysis of these 431 genes using the over-representation method of the *ConsensusPath* tool (Kamburov et al., 2011) and three different databases (Kyoto Encyclopaedia of Genes and Genomes (*KEGG)*, *Reactome* and *BioCarta*), and 382 out of the 431 genes were identified. We accepted as significant those pathways with a minimal overlap of 2 genes and a cut-off at q-value of 0.025.

The statistical framework R (version 4.1.1) was used to perform all the analyses (R Core Team, 2021).

RESULTS

Study population

The study included 379 pregnant women from the INMA project that had information on the exposures and the outcome. Of these women, 40.9% were from Gipuzkoa cohort, 41.4% from Sabadell and 17.7% from Valencia, and 34.8% had a university degree or higher. The median gestation age among the study population was 39.9 weeks, 3.2 % were preterm births, and an 13.5 % had a caesarean delivery (Table 1).

A total of 111 of the pregnant women in our study (29.3%) had smoked during the pregnancy period, and 53 (14.2%) were classified as sustained smoking (Table 1). In relation to air pollution, NO_2 exposure during pregnancy was slightly correlated with $PM_{2.5}$ during pregnancy (Figure S3) (R = 0.72). A graphical display of the correlations between air pollution exposures is shown in Figure S3 considering both pollutants and each time window. Higher levels of NO_2 during pregnancy were found in Sabadell (median = 39.4 ug/m^3) and Valencia (median = 26.5 ug/m^3) compared to Gipuzkoa (median = 14.5 ug/m^3). However, levels of $PM_{2.5}$ were similar in the three cohorts and the median ranged between 12.9 to 15.1 ug/m^3 (Table S4).

Table 1. Baseline characteristics of the study population (N=379)

Variable	n (%) or median (IQR)
Cohort	
Gipuzkoa	155(40.9 %)
Sabadell	157(41.4 %)
Valencia	67(17.7 %)
Sex of the child	
Female	191(50.4 %)
Male	188(49.6 %)
Child's ethnicity	
European	344(90.8 %)
Other	30(7.9 %)
Missings, N	5(1.3 %)
Gestational age, in weeks	39.9±1.6
Type of delivery	
Vaginal	326(86 %)
Cesarean	51(13.5 %)
Missings, N	2(0.5 %)
Labour initiation	
Spontaneous	282(74.4 %)
Elective cesarean	18(4.7%)
Induced	67(17.7 %)
Missings, N	12(3.2 %)
Type of delivery	
Eutocic	249(65.7 %)
Cesarean	51(13.5 %)
Instrumental	77(20.3 %)
Missings, N	2(0.5 %)
Preterm birth	
No	366(96.6 %)
Yes	12(3.2 %)
Missings, N	1(0.3 %)
Birthweight, in grams	3255±532.5
Low weight at birth (<2500 g)	
No	361(95.3 %)
Yes	18(4.7 %)
Maternal pre-pregnancy BMI	22.6±4.2
Maternal age at recruitment, in years	31±5
Maternal education	
Primary school	70(18.5 %)
Secondary school	177(46.7 %)
University degree or higher	132(34.8 %)
Parity	
0	213(56.2 %)
1	138(36.4 %)
2 or more	28(7.4 %)
Ever active maternal smoke during life	
No	178(47 %)
Yes	196(51.7 %)
Missings, N	5(1.3 %)
Active maternal smoke at the beginning of pregnancy	
No	268(70.7 %)
Yes	106(28 %)
Missings, N	5(1.3 %)
Active maternal smoke at week 12 of pregnancy	
No	315(83.1 %)
Yes	59(15.6 %)
Missings, N	5(1.3 %)
Active maternal smoke at week 32 of pregnancy	
No	321(84.7 %)
Yes	53(14 %)
Missings, N	5(1.3 %)
Any active maternal smoking during pregnancy	
No	268(70.7 %)
Yes	106(28 %)

Missings, N	5(1.3 %)
Maternal sustained smoking	
No smoking	268(70.7 %)
Non sustained smoking	53(14 %)
Sustained smoking	53(14 %)
Missings, N	5(1.3 %)
Paternal Smoking during pregnancy	
No	268(70.7 %)
Yes	106(28 %)
Missings, N	5(1.3 %)

Note: BMI = Body Mass Index

Association of ambient air pollution and active maternal tobacco smoking with reproductive outcomes

Results from linear and logistic regression models with ambient air pollution during pregnancy or active maternal tobacco smoking during pregnancy as exposure, and reproductive outcomes can be found in Table S1. One statistically significant association was observed between maternal sustained smoking during pregnancy and lower birthweight (β = -231.48, p value = 0.001). None statistically significant associations were found for the other exposures and outcomes.

Selection of the best placental epigenetic clock

We calculated placental epigenetic age with the control placental clock (CPC), robust placental clock (RPC), refined robust placental clock (refRPC) (Lee et al., 2019), and Mayne's clock (Mayne et al., 2017). The Spearman's correlations between the placental epigenetic age (DNAmGA) calculated from each clock and chronological GA measured in weeks are presented in Figure 1 (lower panel). The distribution of each clock through histograms and the correlation plots by cohort can be also found in Figure 1. We found that "Mayne's clock" showed the lower correlations with GA measured in weeks (R= 0.22) and with the rest of the placental epigenetic clocks (vs. RPC: R = 0.33, vs CPC: R = 0.35 and, vs. refRPC: R= 0.29). All clocks obtained from Lee et al., 2019 showed strong correlations among them that ranged between 0.79 to 0.89. However, the CPC clock showed the strongest correlation with GA (R = 0.57) compared to RPC (R = 0.56) or refRPC (R = 0.51).

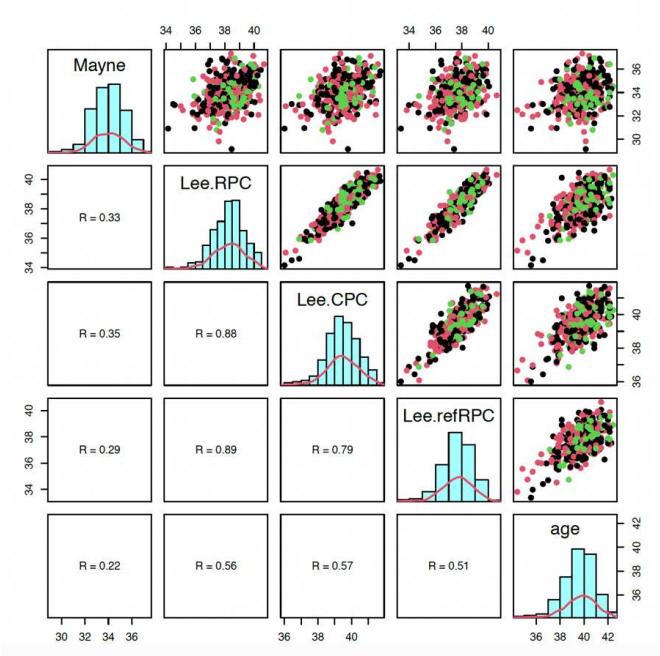

Figure 1. Spearman's correlation between gestational age chronological and placental epigenetic age by cohort. Note: The lower panel shows the Spearman's correlation results, the upper panel shows the correlation plot between each variable of epigenetic gestational age by cohort and the diagram panel shows the histogram of each variable and the density line in red.

Considering this correlation and that INMA is a birth cohort from the general population, we decided to continue the analyses with the CPC clock. This clock was trained on placental samples of pregnancies that did not have any placental pathology such as gestational diabetes, preeclampsia or chorioamnionitis (Lee et al., 2019). Among the different epigenetic age acceleration variables, we focused on the one adjusted for placental cell type proportions as the main outcome.

Association of ambient air pollution and active maternal tobacco smoking with placental epigenetic gestational age

Results from linear regression models with ambient air pollution during pregnancy or active maternal tobacco smoking during pregnancy as exposure, and placental epigenetic age acceleration are shown in Table 2. No statistically significant associations were found between any of the models (Table 2). However, we observed that increased placenta epigenetic age acceleration was linked to exposure to NO_2 during pregnancy ($\beta = 0.0151$, p value = 0.618) and maternal sustained tobacco smoking during pregnancy (No smoking vs. non-sustained smoking, $\beta = 0.0149$, p value = 0.896). In contrast, the other exposures were related to decreased placental epigenetic age acceleration: $PM_{2.5}$ during pregnancy ($\beta = -0.0044$, p value = 0.939), any maternal tobacco smoking during pregnancy ($\beta = -0.0041$, p value = 0.964) and maternal sustained tobacco smoking during pregnancy (No smoking vs. sustained smoking, $\beta = -0.0252$, p value = 0. 832).

Table 2. Linear regression main models of the association between ambient air pollution and maternal active tobacco smoking and placental epigenetic age acceleration adjusted for placental cell type proportions.

Exposure	Units	Estimate	SE	P-value	N
NO_2 during pregnancy	ug/m^3	0.0151	0.1031	0.884	374
$PM_{2.5}$ during pregnancy	ug/m^3	-0.0044	0.0581	0.939	374
Any active maternal tobacco smoking during pregnancy	Non-smokers vs. Any smokers	-0.0041	0.0894	0.964	374
Maternal sustained tobacco smoking during pregnancy	Non-smokers vs. Non-sustained smoking	0.0149	0.1139	0.896	369
	Non-smokers vs. Sustained smoking	-0.0252	0.1191	0. 832	369

NO_2 = Nitrogen dioxide; $PM_{2.5}$ = Particulate matter with an aerodynamic diameter of less than 2.5 μm. The main model was adjusted for: child's sex, maternal education, maternal age, cohort, and child's ethnicity. In each model the samples sizes differ as there are missing in some of the covariates by which the model was adjusted.

Sensitivity analyses

We run several sensitivity analyses. Models further adjusted for pre-pregnancy BMI or for mean ambient temperature during pregnancy, resulted in slightly similar β estimates with no statistically significant results (see Supplementary excel, Table S2). Moreover, models not adjusted for placental cell type proportions, gave a similar trend on the size and direction of the β estimates in most of the associations (see Supplementary Excel, Table S3).

When evaluating different time windows of exposure, including NO_2 exposure in 1st, 2nd and 3rd trimester or active maternal smoking at 12 and 32 weeks, none of the associations were statistically significant (see Supplementary Excel, Table S3). When further adjusting the air pollution models for any active maternal smoking during pregnancy, β estimates remained similar. In addition, when stratifying the analyses by cohort we found inverse results when comparing Gipuzkoa and Sabadell with Valencia for $PM_{2.5}$ and maternal tobacco smoking exposures (see Supplementary Excel, Table S6). Maternal tobacco exposure in Valencia seemed to be positively associated with placental age acceleration contrary to the results obtained for Gipuzkoa and Sabadell. Moreover, in Gipuzkoa and Sabadell $PM_{2.5}$ exposure was related with an increased placental epigenetic age

acceleration which was contrary to the results obtained for Valencia that show a negative β coefficient (see Supplementary Excel, Table S6).

Finally, we restricted the main analyses to non-preterm birth and contrary to the analyses with the whole sample population, any maternal smoking during pregnancy and sustained smoking shown positive estimates on placental epigenetic age acceleration (see Supplementary Excel, Table S7). Overall, none of the sensitivity analyses results in substantial changes compared to the main analysis.

Pathway enrichment analyses

To interpret the biological meaning of placental epigenetic age, we annotated CpGs of the clock to a total of 431 unique genes. Functional enrichment through ConsensusPathDB identified 23 biological pathways related to embryonic and perinatal development, neuronal system and neurodegenerative diseases, signal transduction, gene expression or diseases of programmed cell death. However, none of the associations surpassed the multiple testing (see Supplementary Excel, Table S8).

DISCUSSION

To our knowledge, this is the first study on the association between ambient air pollution and active maternal tobacco smoking during pregnancy and placental aging using an epigenetic clock based on placental DNAm data. In both the main analyses and the sensitivity analyses, we did not observe any statistically significant associations between the exposures of interest and placental epigenetic age acceleration. However, NO_2 provided positive associations and $PM_{2.5}$ and smoking inverse associations. Placental epigenetic age was estimated using the two existing placental clocks: Mayne's clock (Mayne et al., 2017) and Lee's clock (Lee et al., 2019). From the second one we selected the control placental clock (CPC) as it was trained on placental samples that were designated as "control" without placental pathologies such as gestational diabetes, preeclampsia or chorioamnionitis, similarly to our study. Although not shown here, we also tested the other clocks, and no associations were observed.

We also tested the association of these exposures versus reproductive outcomes, and we did not observe any statistically significant relationship, except for an inverse association between sustained maternal smoking and birthweight. The association between maternal sustained smoking during pregnancy and lower birthweight has extensively been reported in the literature (Di et al., 2022; Pereira et al., 2017). In contrast, we have not been able to replicate the relationship between air pollution exposure during pregnancy and lower birth weight (Chen et al., 2018; Fleischer et al., 2014; Pedersen et al., 2013; Sarizadeh et al., 2020; Stieb et al., 2012). Moreover, we did not find any significant association between air pollution or maternal tobacco smoking and gestational

age. Lack of association could be due to the small sample size of our study in combination with the relatively small effects of the exposures, except for sustained smoking.

Due to the lack of studies on the association of environmental factors with placental epigenetic age, we cannot compare our null findings with other studies. So far, only one study has evaluated the association between residential NO_x exposure during 1st trimester of pregnancy and placental aging (Engström et al., 2021). This study included placentas from a total of 111 women, of which 29 were preeclamptic (PE) cases and 82 were controls, and they have observed that early exposure to high levels of NO_x on PE cases was associated with placental deceleration, however non conclusive results were found in control cases (Engström et al., 2021).

Differently from our findings in placenta, other studies have found that these exposures are related to increased epigenetic age acceleration in blood at birth. There are two epigenetic clocks designed to predict epigenetic gestational age in neonates, which are Bohlin's clock (based on cord blood) and Knight's clock (based on cord blood and neonatal blood) (Bohlin et al., 2016; Knight et al., 2016). Gestational epigenetic age acceleration measured with these clocks have been related to different environmental factors (Wang and Zhou, 2021) including pregnancy air pollution levels and maternal tobacco smoking. In particular, outdoor exposure to NO_2 during the first trimester of pregnancy and any maternal smoking during pregnancy were associated with increased gestational epigenetic age (Dieckmann et al., 2021; Sbihi et al., 2019)

Other clocks have been develop to estimate epigenetic age from blood or buccal samples collected at later ages, from children to adulthood (Horvath, 2013; Horvath et al., 2018; McEwen et al., 2020; Wu et al., 2019). These include the paediatric clocks (0-20 years) by Wu's (trained in blood) (Wu et al., 2019) and the PedBe's clock (trained in buccal samples)(McEwen et al., 2020); and the all age range clocks by Horvath (trained using data from multiple tissues) (Horvath, 2013; Horvath et al., 2018). Within the framework of the Human Early-Life Exposome (HELIX) project we found that maternal smoking during pregnancy, exposure to second-hand smoke in childhood and indoor particulate matter absorbance (PM_{abs}) also in childhood were linked to an increase in epigenetic age acceleration in children's blood (de Prado-Bert et al., 2021). This goes in line with previous studies of maternal smoking during pregnancy and cord blood epigenetic age acceleration (Simpkin et al., 2017). Also in adults own smoking is related to accelerated epigenetic aging for several clocks (Cardenas et al., 2022; Gao et al., 2016). Accelerated aging in adults and elderly was also related with long-term exposure to different air pollutants such as $PM_{2.5}$ (Nwanaji-Enwerem et al., 2016; Ward-Caviness et al., 2016). Thus, overall exposure to tobacco smoke, either through maternal smoking in pregnancy, own smoking or second-hand smoking, prenatal and postnatal air pollution seem to affect blood epigenetic age at birth or later.

Several explanations can be found to explain the discrepancy between the significant associations of environmental exposures and epigenetic age acceleration in blood but not in placental tissue. One could be the limited statistical power of our study. Besides this, other explanations are plausible. First, we need to consider

that aging is a multifactorial process that involves a large network of interconnected processes including genomic instability, altered metabolism, mitochondrial dysfunction, telomere attrition, cellular senescence, macromolecular damage and inflammation (López-Otín et al., 2013). The placental epigenetic clock might be capturing information about specific biological processes related to aging, and it could be that the ones captured are not affected by our exposures of interest. Genes annotated to the CpGs included in the placenta epigenetic clock were related to development and neuronal process, which do not seem very relevant for aging. In contrast, CpGs used to develop blood epigenetic clocks have been shown to be linked to genes enriched for biological pathways related to immune response, detoxification, cell differentiation, cell aging and cellular senescence (de Prado-Bert et al., 2021; Wang and Zhou, 2021). Third, in this study epigenetic placental age acceleration was calculated based on placental DNAm data. It is known that placenta is a complex tissue that presents a unique methylation profile, in which the observed changes in DNAm may be attributed to variation in cell composition rather than changes happening in the constituent cell populations. Thus, differences between the cellular composition of the samples in the training clock and in the INMA study might have affected the estimation of epigenetic age. Moreover, a recent study found that epigenetic age differed between tissues (chorionic villi, placental tissue and cord blood) of the same person, which could indicate that epigenetic age is an specific characteristic of each tissue more than a general characteristic of the individual (Dieckmann et al., 2021). Forth, as the aging process might not happen simultaneously in all the zones of placenta, it could be important to know for future studies, from which side where placental samples taken to create the placental clocks and in the study population that is going to be evaluated. Indeed, in our study the correlation between placental epigenetic age with chronological gestational age was high, which may indicate that somehow the placental clock is related with placental age. Finally, within the biological interpretation we need to consider that there is still controversy whether the epigenetic clocks in general are the result of specific phenotypes, or they are the markers of specific health outcomes or phenotypes.

Our study also presents other limitations, besides the ones commented above. First, in our analyses we only evaluated ambient air pollution exposure based on LUR models and considering their residential addresses. Nonetheless, it has been seen that outdoor exposure do not highly correlated with the real exposure to which the individuals are exposed in their day-to-day life. Therefore, we encourage future studies to evaluate indoor and personal exposure as they could provide more precision on the exposure levels of the pregnant woman. Third, active maternal tobacco smoking was assessed based on questionnaire data which could lead to social desirability bias. Hence, evaluating maternal smoking based on biomarkers of exposure could be suggested for next studies. Finally, studying placenta is considered a high-top priority as it is a key organ during pregnancy which provides loads of information regarding the development process and the *in utero* environment. However, further studies are needed to determine how the placental aging process occurs, which are the most accurate and precise approaches to measure this complicated process, and which are the molecular mechanisms behind each marker of aging, especially for epigenetic clocks.

CONCLUSIONS

In summary, we have conducted one of the first studies that evaluated the influence of ambient air pollution exposure and active maternal tobacco smoking during pregnancy on placental epigenetic age acceleration based on DNAm data. We did not find any statistically significant results in our study population. Further research with a more precise exposure assessment in larger samples sizes is required to elucidate which is the role of environment in placental biological aging.

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5.3 Paper III

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Short- and medium-term air pollution exposure, plasmatic protein levels and blood pressure in children

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Short- and medium-term air pollution exposure, plasmatic protein levels and blood pressure in children

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ABSTRACT

Exposure to air pollution influences children's health, however, the biological mechanisms underlying these effects are not completely elucidated. We investigated the association between short- and medium-term outdoor air pollution exposure with protein profiles and their link with blood pressure in 1170 HELIX children aged 6–11 years. Different air pollutants (NO_2, PM_{10}, $PM_{2.5}$, and $PM_{2.5abs}$) were estimated based on residential and school addresses at three different windows of exposure (1-day, 1-week, and 1-year before clinical and molecular assessment). Thirty-six proteins, including adipokines, cytokines, or apolipoproteins, were measured in children's plasma using Luminex. Systolic and diastolic blood pressure (SBP and DBP) were measured following a standardized protocol. We performed an association study for each air pollutant at each location and time window and each outcome, adjusting for potential confounders. After correcting for multiple-testing, hepatocyte growth factor (HGF) and interleukin 8 (IL8) levels were positively associated with 1-week home exposure to some of the pollutants (NO_2, PM_{10}, or $PM_{2.5}$). NO_2 1-week home exposure was also related to higher SBP. The mediation study suggested that HGF could explain 19% of the short-term effect of NO_2 on blood pressure, but other study designs are needed to prove the causal directionality between HGF and blood pressure.

1. Introduction

Air pollution is extensively known as a key contributor to the global burden of mortality and disease (Cohen et al., 2017). Nowadays, approximately 91% of the worldwide population is living in places where the levels of air quality exceed guideline limits established by the WHO (World Health Organization, 2021). Air pollution comprises different types of pollutants such as particulate matter (PM) or gaseous

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pollutants. PM can be classified according to their size: (a) particulate matter with an aerodynamic diameter of fewer than 2.5 μm ($PM_{2.5}$); (b) particulate matter with an aerodynamic diameter of fewer than 10 μm (PM_{10}). Besides particulate matter, other air pollutants are considered harmful for human health, such as nitrogen dioxide (NO_2), which is a gaseous air pollutant mainly used as a marker for traffic-related air pollution (WHO, 2003). These air pollutants together with sulphur dioxide (SO_2), carbon monoxide (CO), ozone (O_3), and organic compounds are considered as the top health-menacing air pollutants.

Previous evidence has shown that short- (=< 1week), medium- (>1 week, but =<1year), and long-term (>1year) air pollution exposure is related to a wide range of acute and chronic adverse health effects such as cardiovascular diseases in adults (Rajagopalan et al., 2018). Nowadays, cardiovascular diseases are one of the leading causes of death, responsible for more than 18 million deaths each year (Roth et al., 2018). Different modifiable risk factors for cardiovascular diseases are known such as smoking, diabetes, lipid abnormalities, or hypertension, which is one of the major contributors to cardiovascular diseases (Fuchs and Whelton, 2020). Emerging evidence, mainly in adults, has shown that short- and long-term exposure to air pollution can lead to higher blood pressure (BP) (Brook et al., 2011; Choi et al., 2019; Foraster et al., 2014). The early-life period is an important window of susceptibility to environmental exposures, and any alterations during pregnancy and childhood might permanently change the body's structure, metabolism and physiology (Barouki et al., 2012; Wright, 2017). In children, only a few studies have evaluated both short-, medium- and long-term effects of air pollutants on BP. However, the available studies in children reach similar conclusions to studies in adults (Huang et al., 2021; Sanders et al., 2018; Zhang et al., 2019). A recent meta-analysis concluded that both short-term (5 studies) and long-term (10 studies) exposure to ambient air pollution exposure can be associated with elevated BP in children (Huang et al., 2021). These are important findings as recent evidence found that children with higher BP are more likely to develop cardiovascular diseases during adulthood (Lurbe et al., 2009; Yang et al., 2020). Moreover, hypertension in children is related to other risk factors for cardiovascular diseases such as insulin resistance or hyperlipidemia (Martino et al., 2013).

Different underlying biological mechanisms have been proposed to mediate the effect of air pollution and adverse health outcomes such as oxidative stress and systemic inflammation (Clemente et al., 2017; Johnson et al., 2021; Z. Li et al., 2019b). It is known that circulating proteins such as adipokines and cytokines are related to inflammation processes and their levels can be increased by air pollution exposure (Dadvand et al., 2014; Yang et al., 2017). In general, epidemiological studies have focused on a few specific proteins such as interleukins (IL1, IL6, IL8, or IL10), tumor necrosis factor-alpha (TNF-α), C-reactive protein (CRP) (Yang et al., 2017), and adipokines (leptin or adiponectin) (Dauchet et al., 2018). Moreover, most of the studies have investigated either short- or long-term exposure to air pollution. Thus, there is a paucity of studies considering multiple windows of exposure and air pollutants, and multiple plasmatic proteins. Finally, the majority of the evidence of biological mechanisms refers to the adult population (Elbarbary et al., 2021; Fiorito et al., 2018; Pilz et al., 2018; Riggs et al., 2020a; Su et al., 2017; Sun et al., 2020; Tsai et al., 2019; Zhang et al., 2020a), with only a few studies available in children (Alderete et al., 2018; Gruzieva et al., 2017; X. Li et al., 2019a).

Within the framework of the HELIX project, we have shown that short- and medium-term air pollution exposure during the childhood period was related to higher diastolic blood pressure (DBP) at age 4–5 years (Warembourg et al., 2021), and a similar not statistically significant trend was observed for systolic blood pressure (SBP) at the age of 8 years (Warembourg et al., 2019). In this study, we expanded previous association studies to additional exposure windows and locations in 1170 HELIX children aged 6–11 years and explored potential biological mechanisms. In particular, we aimed to investigate the relationship of residential and school short- and medium-term (1 day, 1 week, and 1 year) outdoor air pollution exposure to NO_2, $PM_{2.5}$, PM_{10}, and absorbance of PM2.5 filters (PM_{abs}) with 36 plasmatic protein levels (including cytokines, apolipoproteins, adipokines and other proteins such as growth factors) and blood pressure in HELIX children, and to evaluate the potential mediating role of selected proteins.

2. Materials and methods

2.1. Study population

This study was conducted in the context of the HELIX project, which was based on six on-going longitudinal population-based birth cohorts established in six countries across different parts of Europe (Born in Bradford [BiB; UK](Wright et al., 2013), Étude des Déterminants Pré et Postnatals du Développement et de la Santé de l'Enfant [EDEN; France] (Heude et al., 2016), Infancia y Medio Ambiente [INMA; Spain](Guxens et al., 2012), Kaunas Cohort [KANC; Lithuania](Grazuleviciene et al., 2009), Norwegian Mother, Father and Child Cohort Study [MoBa; Norway](Magnus et al., 2016), and Mother-Child Cohort in Crete [RHEA; Greece](Chatzi et al., 2017)). Before the start of HELIX, all six cohorts had undergone the required evaluation by national ethics committees and obtained all the required permissions for their cohort recruitment and follow-up visits. The work in HELIX was covered by new ethic approvals in each country and at enrolment in the new follow-up, participants were asked to sign a new informed consent form. The HELIX project included 31,472 mother-child pairs of which 1301 children, around 200 from each of the cohorts, were selected to create a subcohort based on some criteria of eligibility explained elsewhere (Warembourg et al., 2019). A clinical examination, a computer-assisted interview with the mother, and the collection of additional biological samples were carried out during the second follow-up in 2014–2015 of the HELIX subcohort. For this study, we sub-selected 1170 children from the subcohort aged between 6 and 11 years (mean age of 7.4 years) which had information on air pollution exposure, plasmatic proteins, and blood pressure (Figure A 1).

2.2. Childhood outdoor air pollution exposure assessment

The following atmospheric pollutants were assessed for different locations and time windows: NO_2, $PM_{2.5}$ and PM_{10}, and PM_{abs}. A detailed exposure assessment was previously explained elsewhere (Tamayo-Uria et al., 2019; Warembourg et al., 2019). Briefly, outdoor air pollution exposures were assessed using estimates based on land use regression (LUR) modelling approach developed within the framework of the European Study of Cohorts for Air Pollution Effects (ESCAPE) (Beelen et al., 2009; Cyrys et al., 2012; Eeftens et al., 2012a, 2012b; Sellier et al., 2014), which were temporally adjusted to measurements made in local background monitoring stations (Tamayo-Uria et al., 2019). Estimates on air pollutants were assigned to each subcohort individual within GIS techniques considering their residential and school geocoded addresses, which was collected through the last available follow-up survey for each cohort. Moreover, different time windows were calculated for the evaluated air pollutants by averaging them over 1 day, 1week and, 1 year before the clinical and molecular assessment (see Supplementary material for a more extensive explanation of the air pollution exposure assessment, appendix A, section S1).

Some air pollutants could not be assessed in some cohorts because land use regression (LUR) models were not available. In those cohorts that had air pollution measurements, missing values were imputed following a process previously described (Tamayo-Uria et al., 2019). Imputed values represented a maximum of 2% of the values within each cohort. Sample sizes after imputation were: (a) 1170 individuals for NO_2 and $PM_{2.5}$ models; (b) 1020 individuals for PM_{10} models (missing in EDEN cohort); and (c) 828 individuals for PM_{abs} models (missing in EDEN and RHEA cohorts). To enable the comparison of results between different pollutants, air pollution exposure variables were standardized

by their interquartile range (IQR).

2.3. Measurement of plasmatic proteins levels

Blood samples were collected from HELIX subcohort children at a mean age of 7.4 years during the clinical examination, thus simultaneously to the blood pressure measurement (Maitre et al., 2018). Plasma samples were analysed to detect and quantify a panel of relevant proteins. Three Luminex kits commercially available from Life Technologies and Millipore were selected, which assessed a total of 50 measurements (43 unique proteins): Cytokines 30-plex (Cat #. LHC6003M), Apolipoprotein 5-plex (LHP0001M), and Adipokine 15-plex (LHC0017M). Plasma analyses were performed following the standard protocol defined by the vendor. The % of coefficients of variation (% CV) for each protein estimated by plate and then averaged ranged from 3.4% to 36%. For each protein, the limit of detection (LOD) was determined and the lower and upper quantification limits (LOQ1 and LOQ2, respectively) were obtained from the calibration curves. For those proteins that passed the quality control, data were log2 transformed to reach normal distribution. Afterwards, the plate batch effect was corrected by subtracting for each individual and each protein the difference between the overall protein average minus the plate-specific protein average. Finally, values below LOQ1 and above LOQ2 were imputed using a truncated normal distribution implemented in the truncdist R package (Nadarajah and Kotz, 2006) (see details on the QC in the Supplementary material (Appendix A, section S2)) and a descriptive table of the proteins evaluated in the study in Supplementary Excel (Appendix B) file (Table B 1)). A final dataset with the log2-transformed, imputed, and normalized levels for 36 proteins of the 1170 individuals of the HELIX subcohort.

2.4. Blood pressure measurement

A standardized protocol was followed to measure BP during the clinical examination. After 5 min of rest in the sitting position, 3 consecutive measurements, separated by 1-min intervals, were taken using an oscillometric device (OMRON 705-CPII, Omron, Kyoto, Japan). The children were in a pre-defined posture and the right arm was used preferably. The cuff sizes were chosen considering each child's arm length and circumference. Each measurement of systolic blood pressure (SBP) and diastolic blood pressure (DBP) was recorded, and the mean of the second and third measurements was calculated and used in further analyses. In the following manuscript measures of 1167 individuals were evaluated.

2.5. Covariates

During pregnancy and in the childhood follow-up examination information on the following key covariates was collected: self-reported maternal education (primary school, secondary school and university degree or higher), self-reported ancestry (European, Asian and Pakistani, or other), child age at blood sample collection (continuous in years), self-reported maternal pre-pregnancy body mass index (BMI) (continuous in kg/m^2), child's BMI z-score (based on continuous BMI in kg/m^2) (De Onis et al., 2007; WHO), child's height (continuous in meters), maternal smoking during pregnancy (no smoker, only passive smoker, or smoker), smoking status of parents during childhood (none, one, or both), mean outdoors temperature (one day, one week and one month before blood and protein measurements) at residential and at school addresses (continuous in °C), exposure to outdoor air pollution during pregnancy (NO_2, PM_{10}, $PM_{2.5}$ and PM_{abs} as an average of the whole pregnancy period estimated at maternal residential addresses). Missing values in covariates (<3%) were imputed as described above.

2.6. Statistical analyses

2.6.1. Descriptive analyses and correlations

For categorical variables, we calculated frequency and percentage; and for continuous variables, we calculated median and interquartile range (IQR). We used Spearman's correlation coefficients to quantify the correlation between plasmatic proteins data and Pearson's correlation coefficient to quantify the correlation between the different air pollutant measurements.

2.6.2. Outdoor air pollution exposures and plasmatic proteins analyses

We assessed the association between childhood outdoor exposure to air pollution and protein levels using the *omicRexposome* R package (Bioconductor - omicRexposome,). Each air pollutant exposure (at different locations, windows, and pollutants) was related to the plasmatic levels of each protein through linear regressions adjusted for covariates using the *omicRexposome* based on *limma* R package (Ritchie et al., 2015). Models were adjusted for a common set of confounders identified a priori based on literature: child's sex, cohort, self-reported maternal education, self-reported ancestry child's age and mean outdoors temperature of each participant at residential or school addresses. The effect size was expressed as log2 fold change (log2FC) in protein levels per IQR change of the exposure. Nominal significance was established at nominal p-value <0.05. Multiple testing correction was addressed by applying the effective number of tests (ENT) (Li et al., 2012) method, which estimates the number of independent tests considering the correlation among proteins: ENT = 31.54, p-value threshold = 0.0016.

2.6.3. Sensitivity analyses

We conducted several sensitivity analyses. First, we run additional models adjusted for other covariables that could be confounding the associations. Models were further adjusted for: (i) maternal smoking and outdoor air pollution exposure during pregnancy, or (ii) for parental smoking during childhood and child BMI z-score. Second, for the air pollutants that survived multiple-testing correction, we ran mutually adjusted models (NO_2 models were further adjusted for $PM_{2.5}$, and vice versa) to determine if the estimated effects remained statistically significant. We selected NO_2 and $PM_{2.5}$ as they were not strongly correlated (Figure A 6) and had data available in the whole sample (n = 1170 individuals). Third, we conducted a cohort-by-cohort analysis for each statistically significant association in the main model, to check the pattern of association within each cohort. The *meta* R package (Schwarzer, 2007) was used to conduct the fixed-effects inverse variance weighted meta-analyses based on the estimates and standard errors of the associations. We looked at the I^2 statistics to describe heterogeneity across cohorts.

2.6.4. Mediation analyses

We hypothesized that part of the association between exposure to air pollution and blood pressure could be mediated by the change in protein levels. Therefore, first, linear regression models were conducted to examine the associations between each of the childhood outdoor air pollution exposures (different locations, windows, and air pollutants) and SBP and DBP, respectively. Models were adjusted for a common set of confounders identified a priori based on literature: child's sex, cohort, self-reported maternal education, self-reported ancestry, mean temperature of each participant at residential or school addresses, child's age, and child's height. Effect size is reported as the change in blood pressure (millimeters of mercury (mmHg)) by IQR of exposure levels. Then, we investigated the potential mediating role of selected proteins in the association between air pollutants and blood pressure. This was restricted to statistically significant associations between air pollutants and proteins and blood pressure. To do so, we conducted a formal mediation analysis using the function '*mediate*' from the R package *mediation* (Tingley et al., 2014). This package allows the calculation of various

quantities: the total effect, the average direct effect (ADE), indirect effect or average causal mediation effects (ACME), and the proportion mediated (Imai et al., 2010).

The statistical framework R (version 3.6.0) was used to perform all the analyses (R Core Team, 2021).

3. Results

3.1. Study population

Descriptive statistics of the sociodemographic characteristics of the study participants are presented in Table 1. From the 1170 participants included in the study, the median age at the clinical examination was 7.4 (2.4) years old. Of these children 89.6% were of European ancestry, 45.4% were female, and 50.6% were born from mothers with a university degree or higher education level. The median average SBP and DBP was 98 (15) and 57 (10) mmHg, respectively.

Within each pollutant and time window, correlations between home and school were very high ($r > 0.772$) (Figure A 2-A 5). Regarding time windows, higher correlations were detected between 1- day and 1-week than for 1-week and 1-year within each pollutant. For instance, for the exposure to $PM_{2.5}$ at home, the correlation between 1-day and 1-week was $r = 0.665$, while for 1-week and 1-year it was $r = 0.591$. Finally, we found higher correlations among $PM_{2.5}$, PM_{10} and lower correlations or no correlation between PMs subtypes and NO_2. For instance, the correlation between 1-week exposure at home to $PM_{2.5}$ and to PM_{10} was $r = 0.835$, however, its correlation with NO_2 was $r = -0.051$. A graphical display of the correlation matrix between all air pollution exposures is shown in Figure A 6.

Table 1
Characteristics of study population (N = 1170).

Variable	N (%) or median (IQR)
Cohort	
BIB	196 (16.8%)
EDEN	150 (12.8%)
INMA	210 (17.9%)
KANC	199 (17%)
MOBA	223 (19.1%)
RHEA	192 (16.4%)
Ethnicity	
Asian and Pakistani	95 (8.1%)
European	1.048 (89.6%)
Other	27 (2.3%)
Sex of the child	
Female	531 (45.4%)
Male	639 (54.6%)
Child age at blood collection, in years	7.4 ± 2.4
Child z-score BMI	0.3 ± 1.5
Child SBP, mmHg*	98 ± 15
Child DBP, mmHg*	57 ± 10
Maternal age, in years	31 ± 6.8
Maternal pre-pregnancy BMI	24 ± 5.9
Maternal education	
Primary school	172 (14.7%)
Secondary school	406 (34.7%)
University degree or higher	592 (50.6%)
Maternal smoking during pregnancy	
No smoker	633 (54.1%)
Only passive smoker	363 (31%)
Smoker	174 (14.9%)
Parental smoking during childhood	
Neither	721 (61.6%)
One parent	322 (27.5%)
Both parents	127 (10.9%)

Note: BIB = Born in Bradford; EDEN = Étude des Déterminants Pré et Postnatals du Développement et de la Santé de l'Enfant; INMA = Infancia y Medio Ambiente; KANC = Kaunas Cohort; MoBa= Norwegian Mother, Father and Child Cohort Study; RHEA = Mother-child Cohort in Crete; BMI = Body Mass Index; SBP = Systolic Blood Pressure; DBP = Diastolic Blood Pressure. *For systolic and diastolic blood pressure the sample size was 1167 individuals.

Plasmatic proteins were classified into 4 groups according to their function: adipokines, apolipoproteins, cytokines, and other proteins, including growth factors, hormones, and the C-reactive protein (CRP) (See Supplementary Excel (Appendix B) for further information, Table B 1). Their average concentrations can be found in Table B 1, and their pair-wise correlation is shown in Figure A 7. The heatmap suggests 4 clusters, which are mostly consistent with the four groups of proteins previously created based on their biological function. Higher correlations can be found within most of the cytokines, adipokine PAI1(Plasminogen activator inhibitor-1) and some growth factors (EGF (Epidermal growth factor), GSCF (Granulocyte colony-stimulating factor), and FGFBasic (Basic fibroblast growth factor)), all of them related with inflammatory processes. The group of apolipoproteins was correlated between them and with adiponectin and CRP. Moreover, leptin, interleukin-1 beta (ILbeta), interleukin-6 (IL6), all produced by the fat tissue, and insulin were quite correlated among them. Finally, we observed correlations within a smaller group of cytokines (Interleukin-8 (IL8), tumor necrosis factor alfa (TNF-α) and monocyte chemoattractant protein-1m (MCP1)), the B-cell activating factor (BAFF) which is an adipokine, the hepatocyte growth factor (HGF) and one hormone (Cpeptide), all of them with anti-inflammatory properties.

3.2. Outdoor air pollution exposures and plasmatic proteins analyses

Higher 1-week NO_2, $PM_{2.5}$, and PM_{10} home levels were associated with increased levels of HGF (Table 2). In addition, higher 1-week $PM_{2.5}$ and PM_{10} school levels were also related to increased levels of HGF. Finally, exposure to 1-week PM_{10} at home and school was associated with higher IL8 concentration (Table 2). The beforementioned associations are the ones that passed the multiple testing correction threshold ($p < 0.0016$) based on the effective number of tests (ENTs) considering

Table 2
Results of the association between outdoor air pollution exposures and plasmatic proteins levels (main model).

Exposure	Outcome	N	log2FC (95%CI) [a]	P-value	Adjusted p-value
NO_2 home exposure (1 week) (IQR = 0.57)	HGF	1170	0.06 (0.02, 0.10)	0.001	0.036
$PM_{2.5}$ home exposure (1 week) (IQR = 6.89)	HGF	1170	0.04 (0.01, 0.06)	0.001	0.035
$PM_{2.5}$ school exposure (1 week) (IQR = 6.78)	HGF	1170	0.04 (0.01, 0.06)	0.001	0.036
PM_{10} home exposure (1 week) (IQR = 15.28)	HGF	1020	0.04 (0.02, 0.06)	8.08×10^{-05}	0.003
PM_{10} school exposure (1 week) (IQR = 15.15)	HGF	1020	0.04 (0.01, 0.06)	0.001	0.027
PM_{10} home exposure (1 week) (IQR = 15.28)	IL8	1020	0.05 (0.02, 0.07)	1.74×10^{-04}	0.005
PM_{10} school exposure (1 week) (IQR = 15.15)	IL8	1020	0.05 (0.02, 0.08)	0.001	0.006

NO_2 = Nitrogen dioxide; $PM_{2.5}$ = Particulate matter with an aerodynamic diameter of less than 2.5 μm; PM_{10} = Particulate matter with an aerodynamic diameter of less than 10 μm; HGF = Hepatocyte growth factor; IL8 = Interleukin 8; log2FC = log2 fold change of protein levels by IQR or air pollutant; IQR = Interquartile range. Results are presented only for the exposure-protein associations that surpassed the multiple testing correction threshold considering correlated proteins (ENT = 31.54). The main model was adjusted for: child's sex, cohort, self-reported maternal education, self-reported ancestry, and mean temperature. The analyses were conducted in 1170 children from the HELIX subcohort for the NO_2 and $PM_{2.5}$ models and in 1020 for the PM_{10} models. exposure.

all the proteins. The rest of the associations are shown in the supplementary information (see Supplementary Excel for the full set of results, Table B 4-B.7).

When further adjusting the models for maternal smoking and outdoor air pollution exposure during pregnancy and, for parental smoking during childhood and child BMI z-score plus the main covariates considered before, the associations remained significant and still passed the multiple testing correction threshold (see Supplementary Excel (Appendix B) file for the full set of results, Table B 4-B 6). Then, in the mutually adjusted models, the associations were not statistically significant anymore, however effect sizes were only slightly smaller, and all maintained the same direction (see Supplementary Excel (Appendix B) file for the full set of results, Table B 4- B.5). Finally, we conducted fixed-effects inverse variance weighted meta-analyses of the results by the cohort of the exposure-omics associations that passed the multiple testing correction (Figure A 8). For 1-week NO_2 exposure estimated effects were consistent across cohorts (Figure A 8A). For the other associations the pattern was slightly more heterogeneous with some cohorts going in the opposite direction (Figure A 8B, A 8C, and A 8D). Nevertheless, the statistic I^2 was equal to 0 for all exposure variables.

3.3. Mediation analyses

Linear regression models adjusted for covariates showed marginally significant associations between 1-week exposure to NO_2 at home and school and higher SBP (beta = 1.21, p-value = 0.091; and beta = 1.24, p-value = 0.081, respectively), and for 1-year exposure to NO_2 and to PM_{abs} at school (beta = 1.77, p-value = 0.063; and beta = 1.91, p-value = 0.079, respectively). For the other air pollutants and regards to DBP non-significant associations were found (see Supplementary Excel (Appendix B) file for the full set of results, Table B 8-B 9).

Associations between air pollution and BP did not change substantially after further adjusting the models for (a) maternal smoking and outdoor air pollution exposure during pregnancy, and for (b) parental smoking during childhood and child BMI z-score (see Supplementary Excel (Appendix B) file for the full set of results, Table B 8-B 9). The only exception was the association between 1-week NO_2 exposure at home and SBP, where a statistically significant effect was observed when further adjusting the models for variables related to pregnancy (beta = 1.53, p-value = 0.040). In the mutually adjusted models, stronger estimates were observed between NO_2 exposure and higher SBP; on the contrary, $PM_{2.5}$ exposure was related to a decrease in SBP (see Supplementary Excel (Appendix B) file for the full set of results, Table B 8-B 9).

Finally, we conducted mediation analyses for those exposures, proteins, and outcomes that were involved in marginally significant associations, namely 1-week NO_2 exposure at home, HGF, and SBP. The results of the mediation analyses showed that 19% of the effect of the exposure to 1-week NO_2 levels at home on SBP could be partly mediated via the HGF concentrations (see Table B 10).

4. Discussion

To our knowledge, this is one of the first studies to simultaneously evaluate the possible influence of different time windows of air pollution exposure (1-day, 1-week, and 1-year) and various pollutants on the levels of various cytokines, apolipoproteins, adipokines, and other proteins such as growth factors in children, and their link with blood pressure. We showed that higher levels of 1-week exposure to NO_2, $PM_{2.5}$, and PM_{10} at home or school were associated with higher levels of HGF. A similar association, but only for PM_{10} was observed for IL8. Finally, higher levels of 1-week exposure to NO_2 were related to higher SBP, and the mediation analysis suggests that HGF might be implicated in this link.

Inflammation and oxidative stress are known to be the main biological mechanisms by which air pollution induces health effects, which might be translated to an inflammation cascade, and oxidation stress process in the lung, vascular, or heart tissue (Lodovici and Bigagli, 2011), together with dysfunction of vascular endothelium (Araujo and Nel, 2009; Brook et al., 2009; Zhong et al., 2015). In line with previous studies, we have shown a positive association between IL8 levels and PM, specifically, PM_{10}. IL8 is a chemotactic factor that can be produced by a wide range of cells such as epithelial, fibroblasts endothelial, macrophages, or lymphocytes in response to inflammation (Benakanakere et al., 2016). It is considered a pro-inflammatory mediator that intermediates in host responses to tissue damage and inflammation (Mehrbani et al., 2016). In particular, it is involved in mitogenesis, inhibition of angiogenesis, chemotaxis, neutrophil degranulation, calcium homeostasis, and leukocyte activation (Brennan and Zheng, 2007). Two observational studies in adults have shown that short-term exposure to $PM_{2.5}$ increased levels of circulating MCP1, IL8, and TNF-α (Zhang et al., 2020b), and also of IL6 (Pope et al., 2016). A study carried out with children (8- to 10-year-old) found higher levels of saliva IL8 in a region with higher air pollution (Mehrbani et al., 2016). In vitro studies using primary human bronchial epithelial cells (HBECs) exposed to PM_{10} have confirmed an increase in IL8 concentrations in 24h after exposure, which goes in line with our results (Fujii et al., 2001). Moreover, in response to air pollution exposure it has been seen that IL8 gene expression increases in the macrophages located in the pulmonary alveoli (Drumm et al., 1999). An elevated expression of this cytokine has been previously associated with some conditions as hypertension (Martynowicz et al., 2014), carcinogenesis (Gales et al., 2013) or chronic obstructive pulmonary diseases (Gilowska, 2014). Thus, elevated IL8 levels in response to air pollution might lead to other adverse health effects, besides blood pressure. However, in our study PM_{10} exposure, which was associated with IL8, was not related to BP, therefore, we did not conduct a formal mediation analysis between this exposure and BP.

In relation to HGF, we found that plasma levels of this protein were related to 1-week exposure to NO_2, PM_{10}, and $PM_{2.5}$. Results were consistent across cohorts and not modified when adjusting for other covariates, which suggests a robust association. However, there is scarce evidence regarding the influence of air pollution on HGF levels. One of the available studies so far, found a positive association between short-term exposure to NO_2 and HGF in the adult population (Dadvand et al., 2014). In contrast, another study in adults did not find any association between short-term exposure to $PM_{2.5}$ and HGF (Riggs et al., 2020b). HGF is not usually considered as an inflammatory marker, and it was first described as a liver-regenerative circulating factor. Currently, it is thought to be an angiogenic growth factor by its participation in the HGF/c-Met signaling cascade (Neuss et al., 2004). This cascade regulates proliferation, differentiation, survival, and mitogenesis of endothelial cells that are linked to the reparation of tissues in different organs such as the heart (Mungunsukh et al., 2014; Oliveira et al., 2018). Previous evidence has shown positive associations between HGF and BP(Hayash et al., 2002). In our study, we ran a mediation analysis between air pollution, HGF levels, and blood pressure. We found that 19% of the effect of air pollution on SBP could be mediated through HGF. However, we need to interpret the results cautiously as the direction of the relationship between HGF and BP is uncertain. In vitro models, suggest that HGF could be a downstream product of increased blood pressure (Nakamura et al., 1996). Other studies in humans suggest that HGF would be produced to counteract the endothelial damage induced by hypertension (Morishita et al., 1998, 2002; Shimizu et al., 2016) as HGF/c-Met pathway could have a role in cardiovascular remodeling after tissue injury (Gallo et al., 2015). Thus, further studies should address the causal connection between HGF and blood pressure in the context of air pollution.

Finally, we did not find any association between short- and medium-term exposure to air pollutants and CRP, PAI1, TNF-α, IL6, and IL10, previously related to air pollution in other studies (Liu et al., 2019; Tang et al., 2020; Wu et al., 2012). A meta-analyses of 40 studies conducted in adults confirmed a positive association between being exposed to $PM_{2.5}$

or PM_{10} and levels of circulating CRP, with stronger associations when considering long-term exposures (more than 6 months) (Liu et al., 2019). In children, it was found that exposure to traffic-related pollutants (PM_{10} and NO_2) during the first year of life was associated with the levels of IL6 and IL10 measured at 8 years of age (Gruzieva et al., 2017). Besides measurement error problems, which do not seem to be the case, the lack of replication of some air pollution-protein associations in our study might have other explanations. First, our levels of air pollution could be lower compared to other studies. Previous evidence have shown that the production of some pro-inflammatory mediators such as IL8 could be more sensitive to air pollution exposure than others (Mehrbani et al., 2016), which might explain why we found an association between PM and IL8, and not with IL6. Second, existing evidence have also found that exposure to PM was associated with an increased production of inflammatory mediators (IL6 or CRP) by stimulated immune cells, but not with their circulating levels (Tripathy et al., 2021), and in our study circulating protein levels in plasma were considered as the outcome. Additionally, the influence on protein concentrations might not be just affected by PM levels, but also by the proportion of each chemical component found in PM, as it has been seen that the effect of each component can differ (Li et al., 2020; Xu et al., 2020). According to it, future studies should determine the chemical composition of PM to clearly evaluate which are the components with a higher impact in protein levels. Third, most of the studies have been conducted in adults and our study is based on children, which might develop a different response to this risk factor as their inflammatory response could be lower due to the chronic exposure is lower compared to adults. Finally, based on our results, we observed that stronger associations are found within one-week of exposure before the clinical and molecular assessment, thus suggesting acute effects of air pollution on these traits. In the way our exposure was assessed, the 1-year average exposure to air pollution is not collecting information on the 1-day or 1-week peaks of air pollution through that period, which could be the main contributors to the increased levels of protein or BP measurements. Therefore, the evaluation of these peaks of air pollution and, the potential chronical influence on health outcomes would require further analyses based on longitudinal studies.

The main strengths of our study are the comprehensive assessment of the air pollution exposure in six populations across Europe with different cultures and settings, the evaluation of different air pollutants and time windows in the same analyses, the harmonized protocols used for the measurement of blood pressure and plasmatic proteins levels, and the adjustment of the statistical models for covariates. Moreover, the analyses investigated the influence of air pollution exposure in children, which are considered as one of the most vulnerable population groups. Finally, we reported the estimates obtained through the analyses of each exposure-protein association to avoid selective reporting bias.

However, our results should be interpreted in the context of its limitations. First, we were not able to consider children's behavior throughout day-to-day life as we have only estimated air pollution values at home and school. Second, we need to consider that the sources of the different air pollutants are unknown and might be different from cohort to cohort, and that we were not able to determine the chemical composition of PM. Moreover, we cannot clearly identify which are the most sensitive windows of exposure because of the low within-subject variability. Third, we have evaluated only thirty-six plasmatic proteins, which is limited considering all the circulating proteins that are present in the human body. However, within the sample of proteins investigated, we have considered acute phase proteins and the most involved in systemic inflammation. Finally, we acknowledge that our study had a cross-sectional design, and we cannot establish a causal link between protein levels and blood pressure. Importantly, the mediation analyses do not imply causality as the relationship could be due to reverse causation. We believe that future studies should investigate the molecular and cellular response to air pollution to elucidate underlying biological mechanisms involved in the relation between air pollution and health outcomes.

Overall, we found that short-term exposure to air pollutants was related to increased levels of HGF, IL8, and systolic blood pressure. HGF seems to be connected to higher blood pressure in the context of air pollution, but direct causation is not proven. These findings reinforce the adverse cardiovascular effects of air pollution in children, a potentially susceptible group. Moreover, considering that elevated blood pressure during childhood impacts on health across the lifespan, reducing the exposure to this environmental risk factor could be also an important prevention strategy. Considering all the above, this study might provide more evidence to promote and implement new strategies and public policies to reduce exposure to air pollution.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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5.4 Paper IV

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de Prado-Bert P, Foraster M, Dadvand P, Gasón M, Querol X, Jurado B, Persavento C, Miralles B, Sunyer J, Rivas I.

Determinants of indoor and personal NO_2 concentrations during pregnancy in BiSC cohort

In preparation

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*Supplementary material of this manuscript can be found in the following link.

Determinants of indoor and personal NO_2 concentrations during pregnancy in BiSC cohort

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Abstract

Nitrogen dioxide (NO_2) is known to be one of the main contributors to air pollution associated with a negative impact on health. Most of the studies have assessed outdoor levels, however it is essential to measure indoor and personal NO_2 concentrations as they might reflect individual's actual exposure more accurately. Within the framework of the BiSC cohort, we conducted the largest monitoring campaign of personal, home-indoor, and home-outdoor exposure to NO_2 in pregnant women, which included 1,086 participants and 4646 samples of NO_2. The main aim of the study is to evaluate indoor and personal NO_2 concentrations during pregnancy and its main determinants. To this end, passive dosimeters were used to measure home-indoor, home-outdoor, and personal NO_2 concentrations during one week at first trimester and third trimester. In addition, information on socioeconomic, maternal behaviour and home characteristics factors was collected through questionnaires. Higher concentrations of personal NO_2 were observed compared to indoor NO_2 (median = 27.23 vs. 22.57 $\mu g/m^3$), and concentrations before Covid-19 were higher to the ones observed after Covid-19 (indoor, median = 24.62 vs. 20.27 $\mu g/m^3$; personal, median = 29.77 vs. 24.29 $\mu g/m^3$; outdoor, median = 42.51 vs. 32.80 $\mu g/m^3$). Linear Mixed-Effects regression models showed that major determinants of indoor NO_2 concentrations were outdoor NO_2 concentrations, the use of gas for cooking and non-European ancestry, and of personal NO_2 were indoor and outdoor NO_2 concentrations. All the models were stratified by season (Spring-Summer vs. Autumn-Winter), as outdoor NO_2 concentrations are subjected to seasonal variability, and the ventilation rate and infiltration differs between seasons. A sensitivity analysis stratifying also for pre- or post-Covid-19 period showed different estimates between periods only for personal NO_2 models. Our findings reinforce the importance of studying personal and indoor determinants among vulnerable groups, and further studies considering other pollutants and their chemical composition, as well as the influence of Covid-19 in pollution levels and lifestyle are needed.

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Keywords

Pregnancy, Indoor NO_2, Personal NO_2, Exposure assessment, Determinants

INTRODUCTION

Nitrogen dioxide (NO_2) is known to be one of the main contributors to air pollution as a primary pollutant and as a precursor to ozone and fine particulate matter (PM) production (Cooper et al., 2022). Exposure to this air pollutant has been widely considered as an important worldwide public health issue (Hamra et al., 2015). Recent systematic reviews and meta-analyses have observed that short and long-term exposure to NO_2 can be related to all-cause and cause-specific mortality (Huangfu and Atkinson, 2020; Orellano et al., 2020). The impact on human health when being exposed to this air pollutant differs depending on the stage of life in which the individual is exposed. In addition, pregnancy has been considered as one of the most sensitive periods, as the embryo and the foetus are extremely susceptible to NO_2 exposure (Shang et al., 2020). Exposure to NO_2 during pregnancy has been related to different birth outcomes such as low birth weight, preterm birth, stillbirth, or intrauterine growth restriction (Chen et al., 2021; Glinianaia et al., 2004; Sarovar et al., 2020). It is well-known that NO_2 exposure has a negative impact on health, however the effects and threshold levels are still under debate. This uncertainty can be related with the issue of correctly estimating individual exposure to air pollutant in large-scale epidemiological studies, which are scarce, and also to the challenge of assessing health effects in a large number of people (Stroh et al., 2012).

NO_2 is a gaseous air pollutant, that can be released to the atmosphere from natural and human-generated sources. It is mainly created when oxygen combines with nitrogen during high-temperature combustion in the atmosphere (Brusseau et al., 2019). Based on this, outdoor NO_2 exposure is mainly consequence of combustion processes, and the principal source is known to be road traffic converting NO_2 into a good marker of traffic-related pollution (Yan et al., 2020). Even though outdoor air pollution has been widely studied and was the first to draw public attention to the health effects of air pollution, it has been observed that indoor exposure could have the greatest impact on children's health and be considered as a top public health problem (Morales et al., 2009). The most important known sources of indoor NO_2 include burning appliances as stoves, ovens, heaters and fireplaces, and tobacco smoke (Arbex et al., 2007). In addition, levels of NO_2 can be significantly influenced by outdoor NO_2 concentrations due to the exchange between the indoor and outdoor air through mechanical or natural ventilation and, infiltration (Hu and Zhao, 2020).

With the popularization of land use regression (LUR) models, that allow to estimate exposure levels for almost any individual based on their geocoded residential or working addresses, concentrations and determinants to outdoor NO_2 have been widely investigated (Yang et al., 2017). Those studies have been mainly conducted in urban areas as they are known to be regions with a higher population density closer to traffic and therefore, more exposed to traffic-related pollutants such as NO_2 (Degrauewe et al., 2019). However, since most of the people tend to spend an estimated 90% of their time indoors, it is

essential for the NO_2 assessment to measure indoor levels, as outdoor exposure might not accurately reflect individual's actual exposure (Klepeis et al., 2001). Besides indoor exposure, through personal monitoring techniques we can evaluate personal exposure to air pollutants. Measurements obtained through this techniques seem to produce less uncertainty in estimating concentrations over the time period of measurement (Cherrie, 2002). High correlations between levels of indoor and personal exposure have been observed in previous studies (Woo et al., 2011). However, sources of indoor and personal exposure to NO_2 may be distinct, therefore determinants of both exposures can vary. Published evidence considering adult population observed that indoor NO_2 exposure was linked to time spend at home, outdoor NO_2 levels, indoor NO2 sources such as cooking activities at home (time of cooking, presence of exhaust fan or type of stove), tobacco smoking, emissions from appliances or housing conditions (ventilation, infiltration, or fan use), and other variables as season of the year or socioeconomic status (SES) (Ferguson et al., 2021; Lai et al., 2006; Vardoulakis et al., 2020; Woo et al., 2011). Studies considering NO_2 indoor exposure during pregnancy and first year of life observed also a relation with parental tobacco smoking, country of origin and educational level (Esplugues et al., 2010; García Algar et al., 2004; Valero et al., 2009). Scarce evidence has been published in relation to personal NO_2 determinants. To our knowledge, only one study on pregnant women (N=108) found that outdoor NO_2, indoor NO_2, time spent in outdoor environment and time exposed to a gas cooker were strongly correlated with personal exposure (Valero et al., 2009).

Measuring indoor and specially personal exposure to air pollutants is challenging as it has a high cost of implementation and it is hard to collect repetitive measures on the same group of the population (Liang et al., 2019). Moreover, individual exposure in urban areas is the result of a dynamic process between the individual and urban air, and at the end of the day each individual have a unique personal exposure based on indoor and outdoor environments, which makes the quantifying process more complex (Dias and Tchepel, 2018). Previous studies investigating the concentrations and determinants of indoor and personal exposure to NO_2 examined small populations, with just one large-scale population-based study of urban adult population assessing 413 individuals within six European cities (Lai et al., 2006). Therefore, within the framework of Barcelona Life Study Cohort (BiSC) that involved 1086 participants and 4646 samples of NO_2, we conducted the largest monitoring campaigns of personal and home-indoor exposure to NO_2 in pregnant women. The aim of the following study is to evaluate indoor and personal NO_2 concentrations during pregnancy and its main determinants in BiSC cohort.

MATERIALS AND METHODS

Period, population, and area of study

The Barcelona Life Study Cohort (BiSC, www.projectebisc.org) is an ongoing mother-child cohort. A total of 1,086 participants were recruited during their first trimester of pregnancy and were followed-up twice during their pregnancy to assess their exposure to NO_2. Data was collected from October 2018 until September 2021. Participants of the BiSC study were recruited within the caption are of 3 major hospitals in the metropolitan area of Barcelona: the BCNatal consortia (Hospital Sant Joan de Déu and Hospital Clínic) and Hospital de la Santa Creu i Sant Pau. Thus, the area of study includes participants mostly from Barcelona, Esplugues de Llobregat, Cornellà de Llobregat, Hospitalet de Llobregat, Sant Just Desvern and Sant Joan Despí.

Home visits for NO_2 measurements

We measured home-indoor, home-outdoor, and personal NO_2 concentrations during one week at first trimester (approximately week 12 of pregnancy) and third trimester (approximately week 32) with Gradko Environmental passive dosimeters. Indoor NO_2 tubes were placed in the participant's room, in their bed's side area. The tube measuring home outdoor air was attached to the most exposed façade through a window or balcony. The tube for personal measurements were worn by the participants either in a necklace or attached to backpack straps close to the breathing zone. While at home, participants were allowed to leave the tube in the living room or bedroom (hanging at a minimum height of 1.5 m) and were asked to wear it any time they leave their home. The participants were informed about the importance of not covering the tube with their hair or clothes.

Before the lockdown derived from the Covid-19 pandemic, a trained fieldworker visited the participant's home and installed the indoor and outdoor tubes and instructed face-to-face the participant on how to wear the personal tube. During the partial lockdowns and during periods of high incidence of Covid-19 the fieldworkers did not enter the participant's home but, instead, delivered at their doors the NO_2 tubes with a very detailed instructions on how to install the three tubes. Once installed by the participants, the participants sent a picture to the fieldworker to ensure a proper installation of the tubes. In case of any doubt, the participants could easily contact our fieldworkers by phone call or through common messaging phone applications.

Quality control and assurance procedures were put in place. Besides the laboratory blank, we included at least 2 blank tubes in each batch sent to the laboratory. Those tubes were kept on the fridge properly

sealed in plastic bags. Moreover, one week per month we installed a tube at the urban background reference station of *Palau Reial*, located in the southwest of Barcelona city (41° 23′ 14″ N, 02° 06′ 56″ E, 80 m a.s.l.) to compare the concentrations with those from the NO_2 monitors (Thermo Scientific model 42i) operated by the Department of the Environment of Catalonia. We obtained a good agreement between the passive tubes and the reference NO_2 monitor, with an R^2=0.88.

Questionnaires and covariates

Several questionnaires were answered by the volunteers included in the study at approximately 12w and 32w of gestation. Throughout them we were able to collect information on socioeconomic, behaviour and home characteristics factors, that were used as probable determinants of indoor and personal NO_2 levels.

At 1st trimester information on maternal age (in years), self-reported ethnicity (European ancestry, Latin American and others), maternal education (primary, secondary and university or higher), maternal tobacco smoking use at 12w (does not smoke at present but before smoked, non-smokers, active smoking) and 32w (non-smokers and active smoking) was collected. Furthermore, some variables related to maternal behaviour were also gathered through self-reported questionnaires: : use of candles or incense at home (never, less than once a week and more than once a week), hours during the whole week inside home, hours during week inside home, hours during weekend inside home, age of the home (<30, 31-50, >50 years), hours per week with the windows of the parents' bedroom or kitchen opened, hours per day cooking, extractor in the kitchen (yes, no), use of the extractor while cooking (always, sometimes and never), opening the window while cooking (always, sometimes, never and there is no window), and time on foot, cycling, bus or tram, subway or train, car or moto during the week for go and to come from work. Finally, when fieldworkers were doing the home visit, they filled in a home characterization questionnaire in which they collect information on: location of the cuisine (interior and open, interior and separate and others), type of cuisine (gas, electric and others), central heating (yes, no), central water boiler (yes, no) type of water boiler (gas, electric and others), mother's room faces the street (not, yes directly on the street, yes it turns aside a street, yes but it's far), windows frames material (wood, synthetic material and metal), presence of gaskets (yes, no), quality of closing of the windows (okay, regular and bad), and type of window glass (single-glazed, double-glazed and triple-glazed). This information was only collected at 3rd trimester for those individuals that change residence at 2nd or 3rd trimester, for the rest of the individuals we assumed that they had the same data for each variable. Moreover, when fieldworkers were not available to carry out the home visit due to Covid-19, a short questionnaire was handled to the pregnant women, and they answered it.

When conducting the multivariate analyses, some of the variables included as predictors were recategorized due to a low number of individuals in each category, or to collect information from two variables in one:

- The use of candles or incense at home (yes, no) was based on the previous variable collected through the questionnaire. All the individuals which answered *more than once a week* or *less than once a week*, were included in the *yes* category, and the ones who answered *never* were included in the *no*.
- The use of the extractor (yes, no) was based on the variables of extractor in the kitchen and use of the extractor while cooking. All the individuals that answered *always* or *sometimes* were classified at the *yes* category, and all the individuals that answered *never* or that they do not have an extractor were classified in the *no* category.
- A new variable gathering information on the windows characteristics was created. It was obtained by mixing information on windows frames' material and presence of gaskets. New categories were synthetic material and gaskets, metal and gaskets or no gaskets, wood and gaskets and wood and no gaskets.

Finally, variables related to Covid-19, and season were created based on the assessment day of each NO_2 measurement. We considered pre-Covid-19 period all the measurements before March 2020, and post- Covid-19 to all the measurements after March 2020. A variable to identify if the measurement was conducted in the total lockdown was created (yes, no), we considered the total lockdown period from March 2020 to June 2020. Season variable was divided in two; Spring-Summer (for measurements done from March to August) and Autumn-Winter (from September to February).

Statistical analyses

Descriptive analyses and correlations

A descriptive analysis was carried out for the socioeconomic, demographic and behaviour factors, and for the characteristics of the home. For categorical variables, we calculated frequency and percentage; and for continuous variables, we calculated mean and standard deviation (SD). To describe NO_2 indoor, personal, and outdoor levels within the BiSC cohort, we obtained a boxplot of each exposure expressed as $\mu g/m^3$ by the season of the measurement assessment (Autumn and Winter vs. Spring and Summer) and differentiating between pre- or post-Covid-19 situation. We further used Spearman's correlation coefficients to quantify the correlation between the different air pollutant measurements.

Bivariate analyses

We also evaluated the distribution of NO_2 indoor and personal levels of pregnant women at 12 and 32 weeks of gestation in relation to socioeconomic and demographic factors, and for the characteristics of the home. A bivariate analysis was conducted to determine if the NO_2 levels were significantly different among the socioeconomic, demographic, and housing characteristics. For the categorical variables, the median of the exposure was expressed in $\mu g/m^3$ and to evaluate any statistically significant difference among groups, a t-test was used when the variable was dichotomous, and an ANOVA was used when the variables had more than two categories. For continuous variables, a linear regression model was developed between the exposure and the covariate.

Linear Mixed-Effects Regression models

To assess the effect on indoor and personal levels of NO_2 at 12 and 32 weeks of gestation of different socioeconomic, behaviour and home characteristics, different linear mixed-effects regression (LMM) models were performed to account for repeated NO_2 measurements (with the participant as random effect). Based on the bivariate analyses and previous literature the models constructed considered as dependent variables the indoor or personal NO_2 concentrations (both 12w and 32w) on continuous ($\mu g/m^3$) with the individual as a random effect. Furthermore, the models were made based on those factors that were found to be significant in the bivariate analyses (p-value < 0.001) and with less than 30% of missing values in the study population. Therefore, for the indoor NO_2 models the variables were outdoor NO_2 concentration ($\mu g/m^3$) at 12w and 32w, period of exposure assessment (pre- or post-Covid-19), location of the kitchen, type of kitchen, use of the extractor, use of incense or candles and ethnicity. For the personal NO_2, we considered as variables indoor NO_2 concentration at 12w and 32w, outdoor NO_2 concentrations at 12w and 32w, and period of exposure assessment. All the models were stratified by season (Autumn and Winter vs. Spring and Summer).

Sensitivity analyses

We conducted several sensitivity analyses; a) adding maternal education, and b) adding time inside home during the week (5 working days), type of water boiler and windows characteristics, besides the variables considered in the main model to the NO_2 indoor LMERs. Finally, we repeated the main models for both indoor and personal NO_2 levels but separately for those measurements that were done before or after March 2020, which was considered the beginning of the Covid-19 pandemics.

The statistical framework R (version 4.1.1) and the package lme4 (Pinheiro and Bates, 2022) was used to perform all the analyses (R Core Team, 2021).

RESULTS

Descriptive analyses and correlations

Descriptive statistics of the sociodemographic, behavioural, and home characteristics of the study participants are presented in Table 1. From the 919 pregnant women included in the study, the mean age at recruitment was 34.29 (4.6) years old, a 74.7% were European ancestry, and a 71.8% had a university degree or a higher educational level. Furthermore, at 12w and 32w a 45.2% and a 40.7%, respectively had a gas cooker, a 34.7% and a 29.6% always used the extractor while cooking, and 64.6% and a 57.9% had an interior and separate kitchen.

Table 1. Baseline socio-demographic, behaviour, and home characteristics of BiSC cohort (N=919).

Socio-demographic, behaviour, and home characteristics	**Categories**	**Mean (SD) or N (%)**
Age, in years (continuous)	-	34.29 (4.6)
Self-reported ethnicity	European ancestry	686 (74.7 %)
	Latin American	201 (21.9 %)
	Other (including Arabian, Sub-Saharan Africa, Far East Asia, South Asia and Other)	32 (3.5 %)
Maternal education	Primary or less	38 (4.1 %)
	Secondary	221 (24 %)
	University	659 (71.8 %)
	Missing values	1 (0.1%)
NO_2 samples at 12w collected during lockdown*	No	758 (82.5 %)
	Yes	61 (6.6 %)
	Missing values	100 (10.9%)
NO_2 samples at 32w collected during lockdown*	No	727 (79.1 %)
	Yes	54 (5.9 %)
	Missing values	138 (15.0%)
Maternal tobacco smoking at 12w	Does not smoke at present but before smoked (daily or not daily)	320 (34.8 %)
	Non smokers	397 (43.2 %)
	Active smoking	5 (0.5 %)
	Missing values	197 (21.5%)
Maternal tobacco smoking at 32w	Non smokers	603 (65.6 %)
	Active smoking	10 (1.1 %)
	Missing values	306 (33.3%)

		12w	32w
Season assessment	Autumn	244 (26.6 %)	192 (20.9 %)
	Winter	238 (25.9 %)	176 (19.2 %)
	Spring	155 (16.9 %)	166 (18.1 %)
	Summer	182 (19.8 %)	247 (26.9 %)
	Missing values	100 (10.9%)	138 (15.0%)
Outdoor NO_2 concentrations in $\mu g/m^3$	-	40.44 (12.81)	37.69 (13.12)
NO_2 samples collected pre or post the beginning of Covid-19 pandemics	Post-COVID[1]	353 (38.4 %)	431 (46.9 %)
	Pre-COVID[2]	466 (50.7 %)	350 (38.1 %)
	Missing values	100 (10.9%)	138 (15.0%)
Use candles or incense at home	Never	388 (42.2 %)	334 (36.3 %)
	Less than once a week	226 (24.6 %)	184 (20 %)
	More than once a week	63 (6.9 %)	58 (6.3 %)
	Missing values	242 (26 %)	343 (37.3%)
Use the extractor while cooking during pregnancy	Always	319 (34.7 %)	272 (29.6 %)
	Sometimes	279 (30.4 %)	235 (25.6 %)
	Never	75 (8.2 %)	64 (7 %)
	Missing values	246 (26.8 %)	348 (37.9%)
Location of cuisine	Interior and open	167 (18.2 %)	166 (18.1 %)
	Interior and separate	594 (64.6 %)	532 (57.9 %)
	Others	22 (2.4 %)	19 (2.1 %)
	Missing values	136 (14.8%)	202 (21.9%)
Type of cuisine	Gas	415 (45.2 %)	374 (40.7 %)
	Electric	358 (39 %)	336 (36.6 %)
	Others	6 (0.7 %)	5 (0.5 %)
	Missing values	140 (15.3%)	204 (22.2%)
Type of water boiler	Gas	346 (37.6 %)	361 (39.3 %)
	Electric	110 (12 %)	27 (2.9 %)
	Others	5 (0.5 %)	5 (0.5 %)
	Missing values	485 (49.8%)	526 (57.2%)
Windows frames material	Wood	272 (29.6 %)	248 (27 %)
	Synthetic material	81 (8.8 %)	70 (7.6 %)
	Metal	127 (13.8 %)	105 (11.4 %)
	Missing values	439 (47.8%)	496 (54.0%
Presence of gaskets in the window	Yes	364 (39.6 %)	325 (35.4 %)
	Not	110 (12 %)	93 (10.1 %)
	Missing values	445 (48.4%)	501 (54.5%)

*Levels measured at March, April, and June of 2020
[1]Post-COVID refers to the measurements done after March 2020.
[2]Pre-COVID refers to the measurements done before March 2020.

The distribution of indoor, personal, and outdoor NO_2 concentrations by season of assessment are reported in Figure 1. Considering the whole assessment period of BiSC, higher concentrations of personal NO_2 were observed compared to indoor NO_2 (median = 27.23 vs. 22.57 $\mu g/m^3$). Moreover, slightly higher concentrations of outdoor NO_2 can be observed during Autumn-Winter compared to Spring-Summer season (median = 40.65 vs. 34.16 $\mu g/m^3$), while indoor and personal levels remained similar across seasons (indoor, median = 22.21 vs. 23.01 $\mu g/m^3$; personal, personal, median = 27.84 vs. 26.63 $\mu g/m^3$). The boxplot is coloured to indicate if the concentration was assessed before or after Covid-19 pandemics started. For instance, concentrations before Covid-19 were higher to the ones observed after Covid-19 (indoor, median = 24.62 vs. 20.27 $\mu g/m^3$; personal, median = 29.77 vs. 24.29 $\mu g/m^3$; outdoor, median = 42.51 vs. 32.80 $\mu g/m^3$). Finally, spearman's correlations can be found in Figure S1, in which a higher correlation can be observed between indoor and personal concentrations in both 12w and 32w assessment (12w indoor vs personal: r = 0.75; 32w indoor vs. personal; r = 0.77) than between indoor or personal with outdoor concentrations ($r \leq 0.41$).

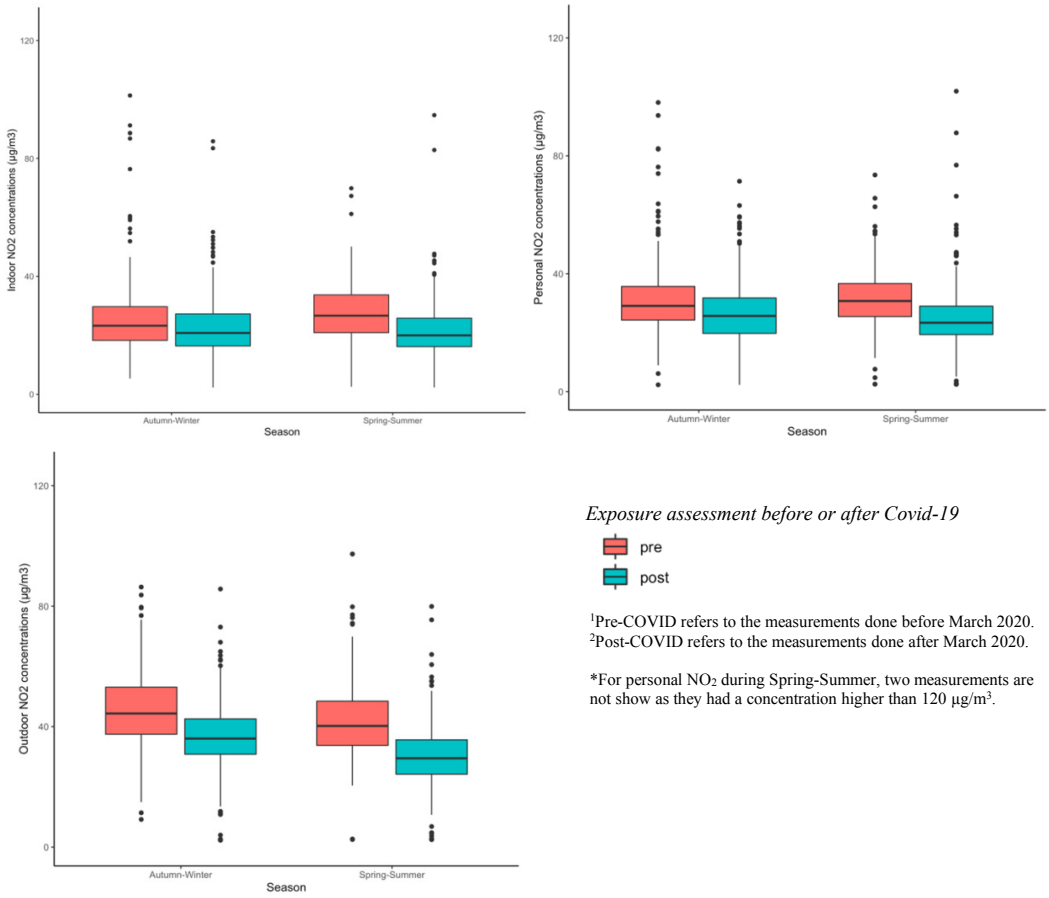

Figure 1. Median concentrations of indoor, personal, and outdoor NO_2 (pre- or post-COVID) by season of assessment with 25th and 75th percentiles in BiSC cohort.

Bivariate analyses

Mean indoor, personal, and outdoor NO_2 concentrations by selected socio-demographic baselines characteristics can be found in Supplementary material (Supplementary Excel, Table S2). Higher concentrations of NO_2 at 12w and 32w were found among pregnant women with a Latin American ethnic origin, moreover, indoor, and personal NO_2 concentrations at 12w were found to be larger in pregnant women with a primary or less educational level (Supplementary Excel, Table S2). Additionally, mean indoor and personal NO_2 concentrations by selected behaviour and home characteristics variables can be found in Table S3 (12w) and S4 (32w) of the Supplementary excel. The use of candles or incense at home seemed to be related to higher levels of indoor NO_2. Moreover, lower concentrations of indoor and personal NO_2 at 12w and 32w were observed when using the extractor while cooking, higher concentrations of indoor and personal NO_2 when using gas for cooking, and the presence of gaskets and windows frame made of wood were related to lower concentrations of indoor and personal NO_2. Finally, higher concentrations of indoor and personal NO_2 were observed in those groups with higher outdoor NO_2 concentrations (Supplementary excel, Table S2).

Linear Mixed-Effects Regression models

After adjustment for several potential predictors, outdoor NO_2 concentrations, the use of gas for cooking and Latin American ethnicity were the major statistically significant determinants of indoor NO_2 concentrations in BiSC cohort (Table 1). Levels of indoor NO_2 were slightly higher in Autumn-Winter (constant = 8.76 (95% confidence interval, CI = 5.4,12.12), p-value, $p < 0.001$) than in Spring-Summer (constant = 8.17 (CI = 5.49,10.84), $p < 0.001$). Homes with a gas cooker had an increase of 6.39 $\mu g/m^3$ (CI = 4.68,8.1), $p < 0.001$) in Autumn-Winter and of 4.84 $\mu g/m^3$ (CI = 3.31,6.36), $p < 0.001$) in Spring-Summer of indoor NO_2 compared to those with an electric cooker. Pregnant women who reported to have a Latin American ethnic origin had an increase of 3.85 $\mu g/m^3$ (CI = 1.54,6.15), $p = 0.0012$) in Autumn-Winter and of 2.10 $\mu g/m^3$ (CI =0.17,4.03), $p = 0.003$) of indoor NO_2 compared to women with a European ancestry. Finally, for each increase of 1 $\mu g/m^3$ of outdoor NO_2 we observed an increase of 0.22 $\mu g/m^3$ (CI = 0.15,0.29), $p < 0.001$) in Autumn-Winter and of 0.33 $\mu g/m^3$ (CI = 0.27,0.4), $p < 0.001$) in Spring-Summer of indoor NO_2.

For personal NO_2 models we considered that indoor NO_2 concentrations already included the beforementioned determinants, therefore the predictors of personal levels considered in the present study were indoor and outdoor NO_2 concentrations and period of exposure assessment regarding Covid-19. Results of the LMMs can be found in Table 2. Base levels of personal NO_2 were higher in Autumn-Winter (constant = 9.038 (CI = 7.28,10.79), $p < 0.001$) than in Spring-Summer (constant = 6.22 (CI = 3.86,8.58), $p < 0.001$). Moreover, for each increase of 1 $\mu g/m^3$ of indoor NO_2 there is an increase of 0.73 $\mu g/m^3$ (CI = 0.68,0.77), $p < 0.001$) in Autumn-Winter and of 0.79 $\mu g/m^3$ (CI = 0.7,0.87), $p < 0.001$)

in Spring-Summer of personal NO_2. Additionally, for each increase of 1 $\mu g/m^3$ of outdoor NO_2 an increase of 0.04 $\mu g/m^3$ (CI = 0,0.08), p = 0.0459) in Autumn-Winter and of 0.08 $\mu g/m^3$ (CI = 0,0.15), p = 0.0389) in Spring-Summer of personal NO_2 was observed.

The period of exposure assessment regarding Covid-19 pandemics was also considered as a predictor variable in indoor and personal NO_2 concentration models. However, statistically significant results were only obtained for indoor NO_2 concentrations in Spring-Summer season. Regarding those measurements done before Covid-19 an increase of 2.36 $\mu g/m^3$ (CI = 0.8,3.92), p = 0.0032) of indoor NO_2 was observed compared to those conducted after Covid-19. For personal NO_2 concentration in Autumn-Winter season, an increase of 1.75 $\mu g/m^3$ (CI = 0.71,2.8), p = 0.0010) of personal NO_2 was observed compared to those conducted after Covid-19.

Table 2. Results of linear mixed effects regression models of NO_2 indoor or personal concentrations stratified by season of assessment.

			Autumn- Winter		Spring-Summer	
	Socio-demographic or home characterization variables	**Categories or units**	**Beta (95% CI)**	**P-value**	**Beta (95% CI)**	**P-value**
Indoor NO_2 concentrations	Constant	$\mu g/m^3$	8.762 (5.4,12.12)	<0.001	8.167 (5.49,10.84)	<0.001
	Outdoor NO_2 concentrations	$\mu g/m^3$	0.22 (0.15,0.29)	<0.001	0.334 (0.27,0.4)	<0.001
	Period of exposure assessment regarding Covid-19	Post-COVID[1] Pre-COVID[2]	Ref. 1.615 (-0.23,3.46)	 0.0870	Ref. 2.362 (0.8,3.92)	 0.0032
	Location of the kitchen	Interior and open (American kitchen) Interior and separate (located inside the house; but separate from the other rooms) Others	Ref. 1.018 (-0.98,3.01) 1.826 (-3.68,7.33)	 0.3206 0.5157	Ref. 0.719 (-1.03,2.47) 6.079 (2.17,9.99)	 0.4219 0.0025
	Type of kitchen	Electric Gas (propane, butane or natural) Others	Ref. 6.388 (4.68,8.1) 3.436 (-10.14,17.01)	 <0.001 0.6201	Ref. 4.838 (3.31,6.36) 0.287 (-5.92,6.5)	 <0.001 0.9280
	Use of the extractor	Yes No	Ref. 1.54 (-1.21,4.29)	 0.2723	Ref. 1.294 (-0.95,3.54)	 0.2593
	Use of incense or candles	Yes No	Ref. -0.928 (-2.59,0.73)	 0.2737	Ref. -1.296 (-2.73,0.14)	 0.0774
	Self-reported ethnicity	European ancestry Latin American Other (including Arabian, Sub-Saharan Africa, Far East Asia, South Asia and Other)	Ref. 3.848 (1.54,6.15) 2.14 (-2.09,6.37)	 0.0012 0.3214	Ref. 2.101 (0.17,4.03) 1.839 (-2.09,5.77)	 0.0334 0.3598
	Number of observations Number of women		583 503		520 429	
Personal NO_2 concentrations	Constant		9.038 (7.28,10.79)	<0.001	6.22 (3.86,8.58)	<0.001
	Indoor NO_2 concentrations	$\mu g/m^3$	0.727 (0.68,0.77)	<0.001	0.787 (0.7,0.87)	<0.001
	Outdoor NO_2 concentrations	$\mu g/m^3$	0.042 (0,0.08)	0.0459	0.076 (0,0.15)	0.0389
	Period of exposure assessment regarding Covid-19	Post-COVID[1] Pre-COVID[2]	Ref. 1.752 (0.71,2.8)	 0.0010	Ref. 0.741 (-0.96,2.44)	 0.3926
	Number of observations Number of women		773 658		658 578	

[1]Post-COVID refers to the measurements done after March 2020.
[2]Pre-COVID refers to the measurements done before March 2020.
Note: 95% CI = 95% confidence interval; NO_2 = dioxide of nitrogen.

Sensitivity analyses

As maternal education is widely considered an important variable to assess socioeconomic status, we added it as a predictor in the model of indoor NO_2, however results did not change (Supplementary Excel, Table S5). In the subset (number of observations: Autumn-Winter = 326 and Spring-Summer = 245) for which we had more detailed information on home characterization (i.e., type of water boiler and windows characteristics), we added as predictors time inside home during the week, the type of water boiler used, and the new self-created variable with information on windows characteristics (Supplementary Excel, Table S6). The base levels of indoor NO_2 are strongly lower in Spring-Summer season compared to the main model (constant = 2.841 (CI = -3.78,9.47), $p < 0.4016$), however similar results in relation to its determinants were obtained.

Finally, when performing the main model but separating the observations in measurements done before Covid-19 and after Covid-19, the estimates were similar, but in the post-Covid-19 group, ethnicity and outdoor NO_2 concentrations did not show a statistically significant effect on indoor and personal NO_2 concentrations, respectively (Supplementary Excel, Table S7).

DISCUSSION

Pregnancy is considered as one of the most sensitive windows of susceptibility to environmental threats. It is widely known that being exposed during this period to several environmental risk factors such as air pollution may lead to permanent changes in the human body and an increase risk of disease later in life (Barouki et al., 2012). For improving the exposure assessment among epidemiological studies aimed to study adverse health outcomes related to air pollution exposure is essential to characterize the relationship between home-indoor and personal exposure in pregnant woman. Furthermore, a better understanding of the different sources of home-indoor and personal air pollution could help promoting more effective preventive strategies to reduce their exposure.

In urban settings, NO_2 is widely known as a traffic-related pollutant as it is generated during combustion processes. Exposure to NO_2 can also occur in indoor environments not only because of the infiltration of outdoor NO_2, but also because the existence of combustion indoor sources of this pollutant such as cooking appliances (Vardoulakis et al., 2020). As people tend to spend most of their time in indoor environments, assessing indoor levels might better reflect individual's actual exposure than relying on outdoor concentrations. In addition, personal assessment of NO_2 could be considered the gold standard, as it considers all the possible microenvironments (including commuting) and the fraction of time spent on each. So far, most of the studies assessed the exposure to home-outdoor and home-indoor NO_2 concentrations in adult general population, and scarce evidence is available for pregnant woman and their personal levels of NO_2 (Nethery et al., 2008; Schembari et al., 2013; Valero et al., 2009). For

instance, previous studies assessing home-indoor or personal exposure concentrations included limited samples sizes which ranged from 50 to 108 pregnant women (Nethery et al., 2008; Schembari et al., 2013; Valero et al., 2009). To our knowledge our study comprises the largest monitoring campaign of personal and home-indoor and -outdoor exposure to NO_2 in pregnant women conducted in an urban area, in which measurements were done simultaneously. In our population, we observed higher correlations between personal and indoor than with ambient levels at home location, which is consistent with previous studies conducted in pregnant woman (Nethery et al., 2008; Schembari et al., 2013; Valero et al., 2009). To note, the personal, indoor, and outdoor weekly average levels observe in our study are higher than the guideline limits stablished by the WHO (24-hours average limit = 24 $\mu g/m^3$) (World Health Organization, 2021) and below the annual mean average set by the European Union legislation (annual average limit = 40 $\mu g/m^3$) (Directive 2008/50/EC).

The findings of our study show that the use of a gas cooker, ethnicity, home-outdoor NO_2 levels, and whether the assessment of the exposure was conducted before or after Covid-19 pandemics are the main determinants of indoor NO_2 in BiSC cohort. Previous studies have already observed higher concentrations of indoor NO_2 in homes with gas cookers in comparison with those with an electric cooker and an influence of outdoor NO_2 levels to indoor NO_2 concentrations. One of them, was a study conducted in Valencia and Sabadell (Spain) with 108 pregnant women, in which it was found that indoor NO_2 variability could be explained by the use of gas appliances (Valero et al., 2009). Similar results were found in another study developed in Barcelona with 54 pregnant women in which they observed that the type of the kitchen was the main determinant of indoor NO_2 levels (Schembari et al., 2013). These results support previous evidence in which other population groups such as children were evaluated (Esplugues et al., 2010). Another predictor of indoor NO_2 levels found in pregnant woman and general population was outdoor NO_2 concentrations (Valero et al., 2009; Vardoulakis et al., 2020). This can be explained because of the high outdoor NO_2 concentrations found in urban areas, which are characterised by a high combustion motor vehicles volume and population density (Salonen et al., 2019). Outdoor NO_2 may enter the indoor environment through open windows but also by infiltration through cracks and leaks in the building (Chen and Zhao, 2011). The latter will depend on several building conditions such as age of the building and type and features of the windows. However, in our study we were not able to evaluate age of the building due to the percentage of missing values on this variable was higher than 30%. Moreover, due to the Covid-19, those variables related to windows characteristics were only available for a subset of our population. In this subset we performed the main model adding new variables: time inside home during the week, type of water boiler and a self-created variable gathering information on windows characteristics. More isolating windows (Synthetic material instead of wood) could be better barriers for infiltration, for instance, a previous study found that an increase of indoor NO_2 for wood framed windows (Rivas et al., 2015), however we could not replicate this in our study population.

In relation to maternal self-reported ethnicity, higher levels were observed among non-European ancestry individuals. Previous studies have shown that socioeconomic status vary according to ethnic group. This leads to the emergence of social and economic inequalities which can be translated into poorer housing conditions (Braubach and Savelsberg, 2009). A recent study investigated systemic inequalities in indoor air pollution exposure in a city from a high-income country (London), and they discussed different reasons to explain why groups with a low SES are exposed to greater levels of indoor air pollution (Ferguson et al., 2021). They have hypothesized that 1) an inadequate housing might be linked to a lower capacity of dispersion of air pollutants at home and lower ventilation rates; 2) air infiltration rate differs and lower-income households experience higher levels of indoor air pollution from outdoor sources; 3) concentrations of indoor pollution from indoor sources are also higher among these group of population mainly because of their lifestyle (i.e., cooking practices and time of cooking) as well as they tend to spend more time at home. However, further studies are needed in other urban cities located in different geographical regions than London. Other factors that show significant associations in the bivariate analyses such as the use of the extractor, the location of the kitchen, the use of candles or incense, or maternal education, were not associated with indoor NO_2 levels in the multivariate analyses, which might indicate that the other variables included in the LMM had a stronger influence on indoor levels.

In the present study we also found that home-outdoor NO_2 concentrations, home-indoor NO_2 concentrations and whether the assessment of the exposure was carried out before or after Covid-19 pandemics are the main determinants of personal NO_2 concentrations. Furthermore, we observed that indoor NO_2 levels had a stronger influence than outdoor NO_2 levels on personal NO_2 levels, which goes in line with previous studies (Ramirez-Aguilar et al., 2011; Valero et al., 2009). This is consistent with the fact that people spend most of daily time indoors, and particularly pregnant women, however the addition of time inside home during the week was not related with personal NO_2 (data not shown). One explanation could be that, even people spend most of their time indoors, in our study we have only information on home-indoor levels, and other indoor microenvironments would need to be considered. Other variables that were associated in the bivariate analyses with personal levels were not considered as we hypothesized that indoor NO_2 levels were already capturing them, and we wanted to avoid over-adjusting the model.

The ventilation rate is known to be higher during the warm season than in the cold season, due to people usually having their windows opened for longer times during that period. This tendency is reported in our study, in which the mean of hours that the window from parent's room or from the kitchen is opened for ventilation is higher at summer compared to winter. Consequently, the air exchange is higher in the warm season compared to the cold season which might cause a higher contribution of outdoor NO_2 concentrations to indoor and personal NO_2. Furthermore, a seasonal variability can be specifically

observed for outdoor NO_2. Therefore, because of the infiltration process, the influence of outdoor NO_2 to indoor and personal NO_2 levels directly depends on the outdoor concentrations which can also differ across seasons (Dėdelė and Miškinytė, 2016). Hence, all our models were stratified by season. For indoor NO_2 concentrations, a stronger influence by outdoor NO_2 is observed in the warm season compared to the cold season which goes in line with the hypothesis that during this season ventilation rate is higher compared to autumn and winter. The influence of using a gas cooker is higher during the cold season which might be explained by the lower ventilation rates and the less dispersion of the pollutants generated indoor or might indicate that people tend to cook less in the warm season compared to the cold one. Nonetheless, similar levels of indoor NO_2 are observed within both seasons, but we find higher personal NO_2 levels in the warm season, which might indicate that less time is spend inside home during that period. This is also linked to the finding of the influence of indoor NO_2 levels to personal concentrations not changing across the year, while the influence of home-outdoor NO_2 levels to personal concentrations is stronger when evaluating the spring-summer months.

Our study was conducted from September 2018 to February 2022; hence, half of the study was carried out under Covid-19 pandemics. In relation to this, we find that an increase of indoor and personal NO_2 levels occurred when evaluating measurements assessed before March 2020 compared to those measures obtained after March 2020. Therefore, we stratified our analyses both for season and pre- or post-Covid-19 situation to investigate whether the influence on indoor or personal levels of the previous commented determinants varied. Interestingly, our analyses show that for indoor NO_2 levels the variability caused by outdoor levels, type of kitchen and ethnicity did not change when comparing pre- and post-Covid-19. However, the influence of outdoor NO_2 levels during spring-summer on personal levels is lower in post-Covid-19 than in pre-Covid-19, contrary to indoor levels that have a higher influence on personal levels in post-Covid-19 than in pre-Covid-19. This could be explained because, levels of outdoor NO_2 post-Covid-19 are lower, and people during post-Covid-19 tended to be more time inside home. Nonetheless, further studies are required to confirm our results.

One of the main strengths of our study is the much larger number of NO_2 measurements assessed in pregnant women compared to previous studies. Another strength is the fact that we measured simultaneously home-outdoor, home-indoor, and personal concentrations of NO_2. Most of the published studies assessed home-outdoor exposure, however as the number of studies evaluating the adverse health outcomes related to environmental risk factors during pregnancy has exponentially increased, a more sensitive and accurate assessment of the exposure levels, including home-indoor and personal exposure, is needed. In addition, 1-week measurements were conducted at two different points during gestation (12w and 32w), which allowed us to have data on different seasons for the same individual. Finally, we studied indoor and personal NO_2 determinants, including 1st and 3rd trimester, as we were

able to gather information on several factors related different dimensions such as socioeconomic status, maternal behaviour, and home characteristics.

However, our results should be interpreted in the context of its limitations. First, the Covid-19 pandemics have clearly influenced the study. We were forced to change the NO_2 assessment protocols and the way in which we collected further information regarding socioeconomic, behaviour and home characterization variables through questionnaires. Consequently, we had a loss of information on several variables, specially from home characterization questionnaires as the fieldworkers were not able to enter the houses and a reduced version to collect essential data was handled to the volunteers. Moreover, during the complete lockdown from March 2020 to June 2020 we were not able to collect any NO_2 measurement. Second, in the present study we assessed in the bivariate analyses the number of hours the volunteer reported spending indoors over a week and the total time spent going to and from work, as well as how she got there (i.e., walking or by car, among others), non-conclusive results were found. Hence, it would also be interesting to investigate the influence on personal NO_2 concentrations of the total time spent outside home (i.e., at work or other outdoor environment) as well as the time spent cooking and the actual time and type spent in commuting (i.e., from specific time-activity diaries for the days of measurements instead of using the average commuting time reported in questionnaires for the full trimester of pregnancy). Third, we observed that the use of a gas cooker was a determinant of indoor NO_2 levels, however determining which type of cooker is (i.e., propane, methane, or butane) could also be of interest. Finally, most of the data was collected in our study through questionnaires which is an easy and simple way to gather information. However, some of them were self-reported which can be related to different types of biases such as self-reported bias (i.e., social desirability bias or recall bias), measurement error bias or confirmation bias.

CONCLUSIONS

Overall, we conducted the largest monitoring campaign of personal and home-indoor exposure to NO_2 during pregnancy in an urban area. Our results provide a better understanding of the relationship between indoor and personal NO_2 exposure levels, which is still scarce. For instance, the use of gas cooker, outdoor NO_2 levels, not being of European ancestry and exposure before Covid-19 pandemics were related to home-indoor NO_2 concentrations. Moreover, indoor, and outdoor NO_2 levels, and exposure before Covid-19 pandemics were suggested to be predictors of personal NO_2. These findings reinforce the importance of assessing indoor and personal air pollution exposure and their determinants among vulnerable groups as being exposed to this harmful pollutant can come from other sources besides traffic emissions. Future research evaluating the chemical composition of other pollutants and their toxicity, as well as the influence of Covid-19 pandemics to air pollution levels and lifestyle among pregnant women are needed.

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6. DISCUSSION

In this Thesis, we investigated the effect of several environmental factors on child molecular traits using data from longitudinal birth cohorts. We have presented and discussed the results of the different studies in the Chapter 5 of this Thesis. The Chapter 6 aims to provide a summary of the main findings and the contribution to the current evidence in the field, a general discussion of the methodological considerations of the study, including the exposure assessment, the biological functions, the study design, and some statistical considerations, and it also comprises different suggestions for future research, and the implications for public health.

6.1 Main findings and contribution to evidence

In concordance to the different objectives proposed in the present Thesis, the studies carried out have contributed to the understanding of: 1) the influence of early life exposome on epigenetic age acceleration in children, 2) the influence of ambient NO_2 and $PM_{2.5}$ exposure and active maternal tobacco smoking during pregnancy on placental epigenetic age acceleration, 3) the effects of ambient NO_2, PM_{10} and $PM_{2.5}$ exposure during childhood on blood plasmatic proteins and blood pressure, and 4) the indoor and personal NO_2 determinants during pregnancy in the new BiSC cohort.

6.1.1 Influence of environmental exposures on biological aging

The study presented in paper I, is one of the first to evaluate the link between a wide range of pregnancy and childhood environmental exposures and epigenetic age acceleration in children. We observed a positive association between maternal tobacco smoking during pregnancy and exposure to parental smoke in childhood and epigenetic age acceleration. In addition, a positive association was found between childhood indoor PM_{abs} and epigenetic age acceleration. Finally, a relationship was observed between higher DMDTP and PCB exposure and decreased age acceleration.

Our results of the effect of maternal smoking during pregnancy and parental tobacco smoking during childhood on increased accelerated epigenetic age were in line with previous evidence in children (Javed et al., 2016; Simpkin et al., 2016; Wu et al., 2019b; Yang et al., 2019). Equivalently, active smoking in adult and elderly populations was also linked to an increased epigenetic age acceleration (Yang et al., 2019). In our study we also observed that SHS was associated with increased age acceleration in children. However, this association may have been partly confounded by the exposure during the pregnancy period, as when adjusting the model for maternal smoking during pregnancy the association was attenuated. Additional support to this is the fact that pregnancy active smoking implies a higher dose than childhood passive smoking and that smoking effects on blood DNAm are known to be persistent (Vives-Usano et al., 2020). We also

studied the dose dependent effect of tobacco smoke during pregnancy and childhood, and we observed that a longer duration or a higher dose of the exposure increased the estimates, which is consistent with previous evidence linked to other health outcomes (Vives-Usano et al., 2020; Zhuge et al., 2020).

As far as we know there are no studies of the indoor PM_{abs} effects on epigenetic age in children, thus we were not able to compare the positive association observed between epigenetic age acceleration and childhood indoor PM_{abs} as a proxy of elemental/black carbon (EC or BC). However, two longitudinal studies conducted in adults, found that outdoor BC and ambient $PM_{2.5}$ were associated to increased age acceleration (Nwanaji-Enwerem et al., 2016; Ward-Caviness et al., 2016). We knew that tobacco smoking can be one of the sources of indoor PM, however, the association observed in our study did not change after adjusting the models for childhood SHS based on parental smoking, which implies that other sources of PM were involved.

Besides the associations observed with air pollution and tobacco smoke, we also found that a higher exposure to DMDTP and PCB-138 was associated with decreased age acceleration, despite both exposures are considered as risk factors for human health. On one side, we hypothesized that DMDTP exposure was related to fruit/vegetable intake, which could be a protective exposure. We tested the association between levels of a urinary metabolite called hippurate, which is a marker of fruits and vegetables consumption,

and epigenetic age acceleration to determine if the association found between DMDTP and epigenetic age acceleration was explained by a higher intake of fruit and vegetables. However, no significant results were obtained although the hippurate levels where higher in those children with DMDTP over the limit of detection. On the other side, we hypothesized that the association observed for PCBs was capturing the relationship between epigenetic age acceleration and BMI. PCBs are highly lipophilic and are stored in fat tissues (Domazet et al., 2020). When we additionally adjusted the models for child BMI the association was largely attenuated. Based on beforementioned, further investigation is needed to elucidate the role of DMDTP as a proxy of fruit and vegetables intake, and future studies should consider separately BMI and adipose tissue in the association models.

Due to the findings of the first paper, we decided to further evaluate the association between active maternal tobacco smoking and ambient air pollution exposure during pregnancy and placental age acceleration, as indoor exposure was not available in INMA cohort. However, we did not obtain any statistically significant associations. Placental epigenetic age was estimated using the CPC developed by Lee et al., 2019. To our knowledge this study was the first one to evaluate this association, and due to the lack of studies we were not able to compare our null results with others. Only one study evaluated residential NO_x exposure during 1st trimester of pregnancy and placental aging. They observed that early exposure to high levels of

NO_x in women with preeclampsia was associated with placental epigenetic aging (Domazet et al., 2020).

Overall, we conducted a screening of the association of the early life environmental exposures with biological aging in two tissues (blood and placenta) calculated using epigenetic clocks in two highly vulnerable periods throughout life: *in utero* and childhood.

6.1.2 Influence of environmental exposures on inflammation

In paper III, we undertook one of the first studies that simultaneously evaluated the associations between several time windows (including 1-day, 1-week, and 1-year) of different air pollutants and levels of various cytokines, apolipoproteins, adipokines and growth factors in children. Moreover, we studied their relationship with blood pressure (BP). In our study we found that higher levels of 1-week exposure to NO_2, $PM_{2.5}$ and PM_{10} both at school and at home were associated with higher levels of hepatocyte growth factor (HGF). Another positive relationship was observed between 1-week PM_{10} exposure and levels of interleukin 8 (IL8). Levels of 1-week NO_2 exposure were also linked to higher SBP, and the statistical mediation analyses suggested that HGF might be implicated within this relationship.

On one side, IL8 is a pro-inflammatory factor that can be produced by a wide range of cells such as macrophages, epithelial, endothelial or lymphocytes in response to inflammation (Benakanakere et al.,

2016). It is involved in mitogenesis, inhibition of angiogenesis, chemotaxis, neutrophil degranulation, calcium homeostasis and leukocyte activation (Brennan and Zheng, 2007). In line with our study, previous evidence has found a positive association between IL8 levels and PM. Two studies in adults observed increased levels of IL8 among other proteins (MCP1, TNF-α and IL6) in relation to short-term exposure to $PM_{2.5}$ (Pope et al., 2016; Zhang et al., 2020). Another study found that children living in a region with higher air pollution levels had higher levels of salivary IL8 (Mehrbani et al., 2016). Furthermore, increased IL8 concentrations in primary human bronchial epithelial cells were detected after 24h of exposure to PM_{10} (Mehrbani et al., 2016). Moreover, IL8 gene expression is increased in macrophages located in the pulmonary alveoli in response to air pollution exposure (Drumm et al., 1999). Finally, an elevated expression of this cytokine has been previously related to hypertension (Martynowicz et al., 2014), and chronic obstructive pulmonary disease (Gilowska, 2014).

On the other side, levels of HGF were associated with short-term exposure to NO_2, PM_{10} and $PM_{2.5}$. When adjusting the models for other covariates results did not change and were consistent across cohorts, which suggests a robust association. Scarce evidence has been published on the influence of air pollution on HGF levels. One study found a positive association between short-term exposure to NO_2 and HGF in adult population (Dadvand et al., 2014), and another one did not find any association with $PM_{2.5}$ (Riggs et al., 2020). HGF, is not considered a canonical inflammatory marker, however,

recently it has been seen that is involved in different functions such as anti-inflammation or anti-apoptosis in vascular endothelial cells, myocardial cells, and other types of cells (Naito et al., 2018). Moreover, it is known to be involved in the HGF/c-Met signalling axis that regulates proliferation, differentiation, survival and mitogenesis of endothelial cells which are linked to the reparation of tissues in different organs such as heart (Neuss et al., 2004; Oliveira et al., 2018). Previous evidence has shown a possible relationship between HGF and BP. The mediation analyses found that 19% of the effect of air pollution on SBP could be explained through HGF levels. Nonetheless our results need to be interpreted cautiously as the direction for the association is still uncertain. In vitro models suggest that HGF could be a downstream product of increased blood pressure to counteract the endothelial damage caused by hypertension (Nakamura et al., 1996; Shimizu et al., 2016) Consequently, further studies are needed to elucidate the causal connection between HGF and BP within air pollution exposure.

Finally, only two out of the thirty-six proteins showed statistically significant associations. However, previous literature relates short- and medium-term exposure to air pollution to increased levels of CRP, plasminogen activator inhibitor-1 (PAI1), TNF-α, IL6 and IL10. A meta-analysis of 40 studies carried out in adults confirmed higher levels of circulating CRP and, long- and short-term exposure to $PM_{2.5}$ and PM_{10} (Liu et al., 2019). Moreover, long-term exposure to traffic related pollutants during first year of life was related to high levels of IL6 and IL10 measured at 8 years old (Gruzieva et al.,

2017a). The lack of replication of these associations in our study could have different explanations, which are extensively developed in section 6.2 and 6.3, where we discuss the limitations of the present Thesis.

Overall, our study provides further evidence that especially short-term exposure to air pollution could increase levels of SBP and of the circulating proteins, IL8 and HGF. The direct causation between HGF and higher BP in the context of air pollution needs further investigation, but our findings reinforce the adverse cardiovascular effects of air pollution in children. Importantly, higher BP during first years of life can impact on health across the lifespan.

6.1.3 Determinants of home-indoor and personal NO_2 concentrations during pregnancy

To the best of our knowledge, paper IV comprises the largest monitoring campaign conducted in an urban area of personal, home-indoor, and home-outdoor NO_2 levels in pregnant women within the framework of the BiSC cohort. Previous studies included limited samples sizes which ranged from 50 to 108 pregnant women (Nethery et al., 2008; Schembari et al., 2013; Valero et al., 2009), in comparison to our study population that included 919 individuals and 4646 samples of NO_2 (including personal, home-indoor and home-outdoor).

Although not directly comparable due to different averaging periods, in our study, personal, indoor and outdoor weekly average levels were higher than the guideline limits established by the WHO (24-hours average limit = 24 $\mu g/m^3$) (World Health Organization, 2021b) and below the annual mean average which is set by the European Union legislation (annual average limit = 40 $\mu g/m^3$) (Directive 2008/50/EC). In consistence with previous studies, higher correlations were observed between personal and indoor concentrations than with ambient levels at home, which goes in line with the fact that people tend to spend most of their daily time at home and other indoor environments. We evaluated the determinants of home-indoor and personal NO_2 levels during pregnancy in BiSC cohort. The former was linked to the use of gas cooker, ethnicity, home-outdoor NO_2 and whether the assessment of the exposure was conducted before or after Covid-19 pandemics, and the latter was mainly related to home-outdoor and -indoor NO_2 concentrations, and consequently to all their determinants.

In regard to indoor NO_2 levels, our results are in line with those reported in previous studies including pregnant women in which the authors have observed higher indoor NO_2 concentrations in those homes with gas cookers compared to those with an electric cooker (Valero et al., 2009). This is also consistent with other evidence published considering pregnant women or other population groups such as children (Esplugues et al., 2010; Schembari et al., 2013). Outdoor NO_2 concentrations were also considered as a predictor variable of indoor NO_2 levels, which is consistent with other studies

(Valero et al., 2009; Vardoulakis et al., 2020). This could be explained because outdoor NO_2 can enter the indoor environment through ventilation and infiltration, which are related to the number of hours that windows are open and housing characteristics such as the type or features of windows. However, in our study we did not find any significant association between the number of hours that participant's windows were open or windows characteristics. Maternal self-reported ethnicity was also linked to indoor levels, we found that individuals with a non-European ancestry were exposed to higher concentrations of NO_2. SES can vary among ethnic groups, causing social and economic inequality, which can be translated to poorer housing conditions. Several reasons have been proposed for explaining why low SES groups are exposed to greater levels of indoor air pollution (i.e., inadequate housing implies less dispersion and ventilation of air pollutants, a higher infiltration rate may occur and more outdoor NO_2 can enter or higher emissions from indoor sources mainly due to their lifestyle) (Ferguson et al., 2021). Finally, other variables such as the use of the extractor, location of the kitchen or maternal education seemed to have less influence on indoor levels.

In relation to personal levels, only few studies have evaluated this exposure and investigated its determinants (Ramirez-Aguilar et al., 2011; Valero et al., 2009). In our study, we found that indoor NO_2 levels had a stronger influence on personal NO_2 concentrations compared to outdoor NO_2. This is consistent with other studies, and with the fact that people tend to spend most of their daily time in indoor environments. However, we did not find any association

between time spend inside home during the week and personal NO_2. In future studies, it would be of interest to also consider data of other indoor microenvironments besides home. Apart from home-indoor and -outdoor NO_2 levels, in the bivariate analyses other variables showed an association with personal NO_2 levels, however we hypothesized that indoor NO_2 was already capturing them, and we wanted to avoid over-adjusting our models.

On one hand, the ventilation rate differs between the warm and the cold season, and the levels of outdoor NO_2 are subjected to a seasonal variability. Consequently, all our models were stratified by season. Similar levels of indoor NO_2 were observed within both seasons, but slightly higher levels of personal NO_2 were found in the warm season, indicating that participants could be spending less time inside home during that period. In addition, the influence of indoor NO_2 to personal concentrations did not differ across the year, however the influence of home-outdoor NO_2 levels was stronger when considering the warm season. On the other hand, we found higher levels of home-indoor and personal NO_2 levels when the measurement was obtained before March 2020 compared to those values obtained after March 2020, which is the beginning of the Covid-19 pandemics. Therefore, as a sensitivity analyses, models were also stratified by pre- or post-Covid-19. Our findings showed that for indoor NO_2 levels the influence of outdoor NO_2, type of kitchen and ethnicity did not change between pre- and post-Covid-19. However, when evaluating personal NO_2, the influence of outdoor levels on personal concentrations was lower in post-Covid-

19 than in pre-Covid-19 during the warm season, which was contrary to indoor levels that had a stronger influence during post-Covid-19 than in pre-Covid-19. Further studies are required to confirm our results; however, we have hypothesized that one explanation could be that outdoor NO_2 concentrations during post-Covid-19 are lower, and that people during this period also tends to spend more time inside home.

Overall, our findings highlight the importance of assessing home-indoor and personal levels of air pollution among vulnerable groups such as pregnant women in urban areas. Moreover, studying its determinants provides a better understanding on those indoor sources which are less known. Future studies would need to focus on the chemical composition of other pollutants, and study in more depth the influence of Covid-19 pandemics.

6.2 Exposure assessment

6.2.1 Early-life exposome

In paper I, a detailed and comprehensive assessment of the early-life exposome in six cohorts across Europe was evaluated. We were able to assess a wide range of environmental exposures in a relatively large sample size in two different periods of time considered as vulnerability windows for children's development, which are pregnancy and childhood. Different methods were used to assess each environmental exposure, with their specific strengths and

limitations. Consequently, we cannot directly contrast the effect sizes and significance levels, and thus results should be interpreted cautiously. Furthermore, following standardized protocols should be a priority in future research to be able to compare the results within cohorts and previous published evidence. Moreover, in those epidemiological studies investigating the exposome, an improvement of the exposure assessment is required. To achieve that, we suggest using more sensitive and precise GIS tools complemented with data from personal sensors and behaviour information. Additionally, considering more high-throughput metabolomics would improve the measurement of toxic chemicals.

6.2.2 Air pollution exposure during pregnancy and childhood

In this Thesis, ambient, indoor, and personal air pollution exposure were assessed during pregnancy or childhood.

Regards to ambient air pollution, different outdoor atmospheric pollutants were estimated based on LUR models developed within the framework of the ESCAPE project. All these models were temporally adjusted to measurements made in local background monitoring stations, which limited measurement error. The final estimates of each air pollutant were assigned to each individual of the cohort based on residential (Paper I, II and III) and school geocoded addresses (Paper III). The exposure estimated through the whole period of pregnancy and childhood was used as the main exposure in

paper I and II. However, in paper III different time windows of exposure during childhood were also obtained for the evaluated air pollutants by averaging them over 1-day, 1-week, and 1-year before the clinical and molecular assessment. Nonetheless, results when evaluating these windows of exposure did not change from the main analyses. For effective interventions in public health, calculating different time windows is essential to evaluate whether there are any differences on the effect depending on the length of exposure and on the specific time in which the individual was exposed. Nevertheless, due to the low within-subject variability observed in our study we were not able to clearly identify which were the most sensitive windows of exposure because high correlations were found between 1-day and 1-week exposures.

Despite the strengths mentioned above, there also some limitations. First, most of the exposures were assessed as the mean levels of exposure during the whole pregnancy and childhood period which was estimated at the residential address of the mothers and the offspring. However, the exposure to other microenvironments such as workplace, school, indoor environments, or during commuting was not considered, hence we might not capture the real exposure of the mother and their offspring during the period of interest. The individual exposure in urban areas is the result of a dynamic process between the individual's behaviour and urban air, therefore the quantifying and assessment process is complex. Consequently, conducting large-scale population-based studies collecting information through sensors related to personal monitoring exposure,

indoor exposure at home or school, exposure in other microenvironments or during commuting routes as well as time spent indoors are needed to provide a more accurate and precise exposure assessment. Furthermore, gathering data on physical activity behaviour could help to better understand the impact of air pollution in health in urban environments. Previous studies have found that in highly polluted locations, air pollution could negate the beneficial effects of physical activity (Li et al., 2015; Si and Cardinal, 2017), and that active travel increases the intake of air pollution which can trigger to negative health consequences (Tainio et al., 2021). Second, we need to consider that the sources of the different air pollutants are unknown in our studies, and they might be different across cohorts. Third, we were not able to identify the chemical compounds of PM, that might be more important to evaluate rather than the overall concentration of PM to which the individuals are exposed. Finally, in our study we found stronger associations within one-week exposure which might imply acute effects of air pollution on the evaluated traits. However, our exposure assessment of 1-year average exposures to air pollution did not collect information on peaks of air pollution during that year, and those peaks could be the main contributors to the increased levels of proteins or BP measurements. Determining whether air pollution peaks are more harmful than annual levels may be important for public health, as it can help to target prevention campaigns directly when such peaks exist.

In HELIX subcohort, indoor PM_{abs} exposure during childhood was also evaluated. It was estimated through a prediction model trained

in a subgroup (n=157) of children using home personal measurements and questionnaire data about indoor sources such as cooking, heating, cleaning, and ventilation. A more comprehensive assessment of home- outdoor, home-indoor, and personal exposure to NO_2 during pregnancy was carried out within the framework of the BiSC cohort in paper IV. Measurements were done during one week at first trimester and third trimester with an environmental passive dosimeter. The dosimeter was placed in the participant's room (indoor), in the most exposed façade (outdoor) and it was worn by the participant in a necklace or attached to the backpack (personal). All measurements were done simultaneously and at two different points during gestation, which allowed us to have data on different seasons from the same individual. Moreover, throughout questionnaires several data on different dimensions such as SES, maternal behaviour and home characteristics was collected, and we could investigate if they were determinants of indoor and personal NO_2 levels. Our study is one of the largest assessing home-indoor and personal NO_2 concentrations in pregnant women. However, some limitations need to be mentioned. First, because of the Covid-19 pandemic, the assessment protocol was modified as fieldworkers could not enter to the participants' home and the tubes had to be installed by the participants. Furthermore, during that period a short questionnaire to collect essential data was handled to the participants, and we loss information on several variables mainly related to home characterization. Second, we did not collect information on the type of gas cooker (i.e., propane, methane, or butane), the number of hours that they spent cooking weekly, the total time spent outside home as

well as the actual time and type spent in commuting, which could be of interest for future studies. Finally, we assessed only NO_2, which is one of the pollutants found in urban areas, however, other pollutants with a higher toxicity need to be considered.

Overall, future studies, would need to measure concentrations of the different chemical compounds found in PM, develop techniques to clarify the possible sources of each pollutant, investigate the determinants of indoor and personal exposure in different settings and across cohorts, and evaluate peaks of air pollution.

6.2.3 Exposure to tobacco smoke

Active maternal smoking during pregnancy and childhood SHS was assessed based on self-reported questionnaire data during one or several times in pregnancy and childhood. This is a relatively simple, easy, and less expensive way to collect data from a large population sample. However, it is accompanied by different types of biases such as self-reported bias (i.e., social desirability bias or recall bias), measurement error bias or confirmation bias. To reduce exposure misclassification, future studies might also evaluate maternal smoking based on biomarkers of exposure such as cotinine, which is a metabolite of nicotine that can be measured in urine, saliva, and plasma samples. However, this metabolite only accounts for short term exposure to smoking as its average half-life ranges from 16 to 19 hours and it is mainly used to quantify recent smoking exposure

(Fernandes et al., 2020). Thus, repeated measurements during pregnancy, at least at 1st and 3rd trimester, should be taken for an accurate assessment. Another possibility would be to predict pregnancy exposure to tobacco smoke through an epigenetic score based on DNAm data (Rauschert et al., 2020).

6.3 Biological functions

The present Thesis mainly focuses on two biological pathways common to several environmental exposures, including smoke and air pollution, which are aging and inflammation. In the following section we comment on methodological considerations and future directions within this field.

6.3.1 Blood and placental epigenetic clocks as markers of aging

In paper I and II, biological aging was calculated through different epigenetic clocks using DNAm data measured in blood or placental samples with the *methlyclock* R package.

On one side, blood epigenetic age was estimated in HELIX children (mean age = 8.1 years) using four different clocks: Horvath's All Tissue clock, Horvath's skin and Blood clock, the Paediatric-Buccal-epigenetics' (PedBE) clock and Wu's methylation-based age prediction model (Horvath, 2013; Horvath et al., 2018; McEwen et

al., 2020; Wu et al., 2019a), but the main analyses was conducted with the Horvath's Skin and Blood clock as showed the strongest correlation with chronological age. The Horvath's Skin and Blood epigenetic clock assessed was trained using samples with a wide range of ages, thus, we need to be aware that it was not specific for children. The only epigenetic clock covering children's age range (0-20 years) was trained on buccal epithelial cells, and its correlation with chronological age in our study was lower compared to the other clocks. Hence, it seems that clocks are less portable across tissues than across age ranges. It could be of interest the development of an improved epigenetic clock for children based on blood cells to decrease measurement error.

On the other side, placental epigenetic age and age acceleration were calculated in the INMA study using four different clocks: Mayne's clock, RPC, CPC and refRPC (Lee et al., 2019; Mayne et al., 2017). However, we conducted the main analyses with CPC developed by Lee et al., 2019 as it showed the strongest correlation with gestational age in our sample. This clock was trained on placental samples that were designated as "control" and without placental pathologies such as gestational diabetes or preeclampsia. While blood clocks have already been used in epidemiological studies and there is existing literature on them, there are no studies on placental epigenetic age for comparison.

While the tobacco smoke and indoor air pollution was related to epigenetic age in blood, we did not find any effect in placenta. This

discrepancy has different potential explanations. First, the study in placenta had lower statistical power than the study in blood (379 placentas vs. 1,173 samples for blood). Second, the accuracy of the clocks might be different due to the training sets used to develop both. Thus, development of new and more accurate clocks for placenta based on larger samples might be necessary. Third, aging is a multifactorial and complex process that is related to different biological mechanisms which might differ across organs and life stages (López-Otín et al., 2013). Hence, each clock in each tissue might be capturing one of these specific aging processes. Indeed, we found that genes annotated to the CpGs included in the placental epigenetic clock were related to processes such as development and neuronal process, whereas CpGs of the epigenetic clock in blood were linked to genes enriched for cell cycle and apoptosis, detoxification, and immune response. In this line, a recent study observed that epigenetic gestational age of the same individual differed depending on the tissue that was used to estimate it: Lee's clock for placenta and another clock known as Bohlin's for cord blood (Bohlin et al., 2016). Hence epigenetic age might be specific-tissue characteristic more than a general characteristic of the individual (Dieckmann et al., 2021). Finally, although both placenta and blood are heterogenous in terms of their cell types, blood can be homogenized before obtaining DNAm thus getting a good representation of it, while for placenta obtaining identical biopsies across samples is quite difficult. Therefore, the disparity between placenta training samples and the testing samples in terms of cell composition might explain the lower performance of this clock.

Moreover, placenta shows a higher variation in cell composition during gestation in comparison to blood. We think that the location of the placental biopsy collection is critical and difficult to harmonize, thus, we are a bit reluctant that placental clocks will be as powerful as blood clocks. In our study the correlation between Horvath's Skin and Blood clock and chronological age was 0.85, and between CPC and gestational age was 0.57. Similarly, estimation of chronological gestational age, even if assessed through last menstrual period and ultrasounds, is less accurate than postnatal life chronological age.

Overall, an improvement of future studies using epigenetic clocks might address the following key points. First, the actual epigenetic clocks are mainly based on DNAm data which reflect the average in different populations of cells within the tissue. In our study we have evaluated epigenetic age acceleration adjusted and non-adjusted for cell type proportions and results did not differ, however, further investigation is required in relation to the known cell-to-cell heterogeneity. Second, it has been observed that the tissues within an individual can age at different relative rates, therefore the development of tissue specific epigenetic clocks might provide more accurate information. However, in the epidemiological field the access to tissues other than blood or placenta is quite limited. Therefore, when evaluating aging through epigenetic clocks, we might need to consider multi-tissue epigenetic clocks or if using tissue-specific clocks we need to clarify that the aging process is investigated in that tissue. Third, the epigenetic clocks are trained on

chronological age, and they show a high accuracy at predicting age, thus they might not be ideal for measuring biological age acceleration. In relation to this, a new era of biological clocks based on DNAm has appeared. One of them is PhenoAge, that to predict chronological age uses blood DNAm plus information on nine markers related to tissue function and immune function (Levine et al., 2018). Forth, more studies focusing on a better understanding of the biological mechanisms captured by epigenetic clocks are essential (Field et al., 2018). Finally, it would be advisable in future studies to investigate in the same population several markers of aging (i.e., epigenetic age and telomere length) as they capture different aspects of the aging process.

6.3.2 Plasmatic protein levels as markers of inflammation

In paper III, we assessed plasmatic levels of a total of 36 proteins of the 1,170 individuals of the HELIX subcohort. During the clinical examination of the HELIX children follow-up, blood samples were collected. Afterwards, plasma samples were analysed to quantify levels of various cytokines, apolipoproteins, adipokines and other proteins such as growth factors. Previous epidemiological studies have focused on a few specific proteins such as interleukins, CRP, or adipokines. Therefore, one of the strengths of our study was the evaluation of multiple proteins.

The collection of blood samples and the analyses of protein levels was conducted following harmonized protocols across the six

cohorts, which implies less measurement error. To quantify protein levels, we used the Luminex technique. This technique is a bead-based immunoassay that allows to detect up to 100 analytes simultaneously and is based on the detection of antibodies through fluorescence. However, we are aware that in the end only 36 proteins could be evaluated in our study, which is still limited if you consider all the circulating proteins that can be found in the human body. At the beginning of our study, a set of 43 proteins were chosen based on the literature and the Luminex kits commercially available. A total of three kits were selected which assessed a total of 50 measurements that represented 43 unique proteins, as 7 proteins were repeated between two kits. The protein measurements obtained were subjected to a quality control process. However, protein quantification methods with less measurement error and higher throughput are needed such as *Olink,* which is based on a multiplex immunoassay-PCR that increases the number of proteins per assay and its sensitivity.

Additionally, the proteome is known to be complex and dynamic, therefore we need to interpret our results cautiously as the measurement of the protein levels at one particular time point can result in poor replicability of the results (Guo et al., 2021). Longitudinal studies could be the best approach to determine if the association between air pollution exposure and inflammation biomarkers is causal. Measuring plasmatic protein levels in different follow-ups could provide more accurate information regarding the influence of the short-, medium- and long- term exposure to environmental risk factors and would help to elucidate if the effects

on health are acute or chronic. Besides inflammatory proteins, studies could be complemented with other biomarkers of the chronic inflammation such as mitochondrial DNA content (Knez et al., 2017) Finally, most of the previous evidence was carried out in adults while we studied children, in which the inflammatory response to the environmental factors might differ and be lower compared to adults. However, evaluating children's response might be relevant to prevent early life consequences of systemic inflammation.

To conclude with, future studies should extend their investigations to other biological processes, besides inflammation and aging, that may also be sensitive to environmental exposures. Those studies should also assess the mediating role of these biological process within the association between the exposure and the occurrence of adverse health outcomes.

6.4 Study design and statistical considerations

6.4.1 Study design and causality

The four studies presented in this Thesis were based on prospective population-based birth cohorts. Longitudinal cohorts are considered as one of the most powerful observational designs because of their prospective nature. This design helps to establish causal relationships between potential risk factors during pregnancy or early life, and adverse health outcomes in childhood or later life. However, this design has some limitations. Firstly, creating and establishing a birth

cohort is costly and time-consuming, which implies that the sample size of the cohorts is usually not very large compared to cross-sectional designs. Future research within this field should be directed, in the first instance, to encourage collaboration between cohorts to pool information and resources to achieve studies with larger population samples from which even small effects can be identified. Secondly, some of the information related to covariates, outcomes and in some cases to exposure variables is collected via questionnaires, which might imply self-reported bias (i.e., social desirability bias or recall bias), measurement error bias or confirmation bias. In consequence, new techniques should be addressed to reduce biases in the collection of self-reported data. Finally, birth cohorts are exposed to the risk of the loss of subjects to follow-up leading to possible selection bias and a reduced statistical power of the study over time. Therefore, the promotion and care of individuals involved in the cohort is essential to ensure that losses through follow-ups are kept to a minimum.

In the present Thesis, we must consider that although our studies were based on birth cohorts, papers I (childhood exposome), II and III were cross-sectional epidemiological studies. Therefore, we studied the exposure and the outcome of the individuals at one specific point of time, very closely to the molecular and clinical assessment. Thus, further longitudinal studies are required assessing the associations observed through this Thesis to elucidate the possible causal link between the exposure and the outcome.

6.4.2 ExWAS approach, confounding, and multiple testing

In paper I, to assess the association between the environmental exposures and health outcome an ExWas was conducted. The ExWAS is a single exposure approach which allows to evaluate the association between different outcomes and many environmental exposures successively and independently. In paper I we did this through the *rexposome* package in the R software. Although, the ExWAS enables to present results for many different exposures at once, this approach is limited in terms of confounding and multiple testing correction.

First, when conducting an ExWAS all the models are adjusted for the same set of confounders, which is not ideal, as depending on the exposure we might consider different variables as confounders. In epidemiological studies, the existence of other factors that can be associated with the exposure and with the outcome can alter the observed association. They are known as confounder variables and its effect can be limited to some extent adjusting the statistical models for these potential confounders. In our analyses, we have adjusted our models for a set of variables related to socio-economic factors besides other covariates such as sex, cohort, age, or ethnicity. However, we acknowledge that residual confounding might still be present.

Second, given the large number of exposures embedded in the exposome we need to correct for multiple comparison to limit false positive results. Hence, an adaptation of the Bonferroni procedure was applied. This adaptation was done because the Bonferroni procedure assumes that the exposures are independent, and this assumption is questionable in the exposome context (Santos et al., 2020).

Third, the correlation structure of the exposome makes difficult to identify the causal relationship. For instance, in our study as we found that maternal tobacco smoke during pregnancy and childhood parental smoking were both related to the outcome response, we run mutually adjusted models to disentangle the effect between both exposures. The effect estimates in both cases were slightly smaller. Furthermore, in relation to the other significant association with indoor PM_{abs}, to ensure that the association was not confounded by parental smoking behaviour or maternal smoking during pregnancy, we further adjusted the model for each of these variables, and no differences in the estimate coefficients were found, which suggested independent effects. Within the context of the exposome, other statistical approaches could be interesting to apply in future studies to select a subset of exposure variables such as the deletion-substitution-addition (DSA) algorithm, the elastic net (ENET), the graphical unit evolutionary stochastic search (GUESS) algorithm (Santos et al., 2020) or the Bayesian kernel machine regression (BKMR) (Bobb et al., 2018). However, it is still under debate which

is the more accurate strategy to study the estimated effects of the exposome in relation to health.

6.4.3 Mediation

Mediation models are constructed to identify whether a variable known as a mediator can explain partially or totally an observed association between an exposure and an outcome. In paper III, a mediation study was conducted to determine if protein levels of HGF, that were related to higher exposure to air pollution, were somehow mediating the association between air pollution and SBP. Although we found the potential effect by HGF, however, due to the cross-sectional nature of our study we were not able to prove the causal directionality of the relationship. Future research using in vitro or in vivo models, interventions or Mendelian randomization should be applied to disentangle this, and indeed environmental epidemiologists should work closely with molecular biologists and toxicologists to address causality.

6.4.4 Heterogeneity and representativeness

This Thesis has used data from different cohorts. Studying distinct populations that are living in different geographical zones, belonging to different cultures and settings, allowed us to capture how the exposure levels, the outcomes, and the background population characteristics diverge across regions. To identify common effects

across population we conducted a pooled analyses adjusted by cohort. Then, we checked heterogeneity across cohorts by carrying out cohort-by-cohort analyses for the statistically significant associations found in paper I and III. In general, for the main findings no significant differences were found among cohorts.

Another point to consider is representativeness. Although the cohorts are population-based, we must be aware that they do not represent the wider population, as usually vulnerable groups (i.e., ethnic minorities or lower SES) are underrepresented. Consequently, depending on how representative our sample is of the general population, the results may or may not be generalized. Further investigations considering other settings, involving rural and urban populations, promoting the participation of vulnerable groups, and stimulating research on low- and middle-income countries are needed.

6.4.5 Publication bias and replication studies

To avoid selective reporting bias, we have reported all the estimates without selecting them based on their nature or direction, even null results were shown. Publishing them supplies all the information obtained through the study, and this can be useful for other researchers when planning and conducting other investigations as it can avoid a waste of resources and time.

Finally, the replication of the different studies conducted within this Thesis in other birth population-based cohorts is necessary to provide robust knowledge. Especially the study conducted in placenta as the outcomes assessed have not yet been widely used and our sample size was smaller.

6.5 Implications for public health

Living in safe, healthy, and sustainable environments is recognized as a human right since 2021 by the United Nations Human Rights Council. Consequently, ensuring the existence of such environments for the entire population should be a top public health priority. Public health is based on the promotion and protection of health, as well as the prevention of diseases or adverse health effects. Therefore, one of the prevention strategies that need to be implemented and defended is the reduction of the levels of exposure to certain environmental factors which are already known to be risk factors. Consequently, increasing evidence on the influence of these risk factors on human health is also enforced.

In the present Thesis we have focused on two environmental exposures which are already widely accepted as global public health problems, air pollution and tobacco smoke exposure mainly during pregnancy. On one side, 99% of the population is breathing polluted air. On the other side, maternal smoking during pregnancy is still high: the estimated prevalence of smoking during pregnancy was found to be 8.1% in Europe (Lange et al., 2018). Besides these two

exposures, we also analysed the early life exposome, which includes many factors related with urban life, such as green spaces or air pollution. Data obtained through this study it is an important step to be aware of the day-to-day exposure levels in an urban setting regards to a vulnerable group of the population. In addition, the latest study has allowed us to unravel some of the sources of indoor and personal levels of air pollution in one of the most polluted cities worldwide, as we have been able to investigate the determinants of the observed air pollution concentrations. The results of the study highlighted the importance of assessing the personal and home-indoor exposure among pregnant women following a more accurate methodology.

Beforementioned exposures are of global concern; however, a national and local approach is needed. In most of the cases, it is local legislation that has the greatest impact on people's lives. In addition, these legislations must be linked to long-term changes, as long-lasting actions may be the only way to bring about real changes and healthier effects. Finally, the fact that the entire population is exposed to some of the environmental factors implies that any action in favour of their elimination not only promotes health among vulnerable groups, such as pregnant women or children, but also among the general population.

Additionally, this Thesis contributes not only to the existing evidence in relation to the clinical health outcomes associated with environmental exposures, but also provides evidence on the underlying biological mechanisms, which reinforces epidemiological

studies with biological plausibility. Promoting studies that include the molecular process can contribute to the existing evidence and provide further information to keep completing the complex picture of how the environment affects health as commented below. First, the process of aging is considered as a public health issue worldwide, thus new evidence on how this is related to environment might drive new policies to reduce environmental exposures and promote "healthy aging" from early stages of life, which afterwards would have a direct impact in society. Further investigation is required to clearly understand which aging biological processes captured by the epigenetic clocks. Second, we investigated inflammation and its possible relationship with BP. Hypertension is known to be one of the major contributors to cardiovascular diseases, which are considered as one of the leading causes of mortality and disability worldwide. In addition, it is known that children with higher blood pressure are more likely to develop cardiovascular diseases during childhood. Moreover, we found that that short-term exposure to air pollution increases SBP. Therefore, our findings might promote further strategies and public policies to reduce exposure to air pollution as an important prevention strategy to decrease the incidence of cardiovascular diseases.

Furthermore, one of the main implications in public health of this Thesis is the increased awareness not just in the academic world but also in general population. Some of the results presented in this Thesis were divulgated through media communication, the obtained results reached the general population and somehow, we managed to

attract their attention. However, there is still a long way to go in the field of scientific communication. In relation to this, the involvement of the general population is fundamental for the promotion and creation of new policies driven to reduce environmental exposures. Therefore, without their involvement it will be more complicated to make a definitive change. One of the priority objectives for the scientific world would be to build bridges with society and achieve an organised, inclusive, and accessible dissemination that might allow the citizens to actively participate in science, facilitate critical thinking, and understand the world we live in.

To conclude with, population health is the result of a complex network of connections and interrelationships between a wide range of factors related to environment, society, culture, and economics, among others. In most of the cases they cannot be considered simultaneously. However, when doing research within the field of public health we must be aware of this complexity and consider each study as a small part, but strongly essential, of the whole picture, since as Rachel Carson stated, "In nature nothing exists alone".

7. CONCLUSIONS

Main conclusions of the analyses of the effects of the environmental factors on epigenetic aging:

- In the context of the early life exposome, pregnancy and childhood exposure to tobacco smoke and childhood exposure to indoor $PM_{2.5}$ were associated with accelerated epigenetic aging in blood of children from 6 European birth cohorts.
- In contrast, DMDTP and PCB-138 exposure during childhood were associated with decreased epigenetic aging in children's blood, likely due to the link between DMDTP exposure and fruit or vegetable intake, and the lipophilic character of PCBs that might partially capture the relationship between body mass index and epigenetic age.
- Regarding placenta, neither ambient air pollution nor active maternal tobacco smoking during pregnancy were related to epigenetic gestational aging of new-borns from three Spanish birth cohorts.
- CpG sites of the blood epigenetic clocks were functionally annotated to genes involved in immune response, cell cycle and apoptosis, and detoxification; while CpG sites of placental epigenetic clocks were annotated by proximity to genes involved in development and neuronal processes.

Main conclusions of the analyses of the effects of short- and medium-term exposure to air pollution on blood pressure and plasmatic proteins:

- Short-term exposure to NO_2, defined as 1 week before outcome measurement, was associated to increased systolic blood pressure in children from 6 European birth cohorts. No effects were observed on diastolic blood pressure.
- Moreover, short-term exposure to NO_2, PM_{10} or $PM_{2.5}$ was related to increased levels of hepatocyte growth factor (HGF), a growth factor involved in tissue reparation; and PM_{10} exposure to increased levels of the pro-inflammatory protein interleukin 8 (IL8).
- Statistical analyses suggested that hepatocyte growth factor (HGF) levels could be mediating the effect of air pollution on blood pressure, however further studies are needed to prove the direction of the causal relationship.

Finally, in relation to the analyses of the determinants of indoor and personal NO_2 levels in BiSC:

- The use of gas cooker, outdoor NO_2 levels, not being of European ancestry and exposure before Covid-19 pandemics were related to home-indoor NO_2 concentrations.
- Moreover, indoor, and outdoor NO_2 levels, and exposure before Covid-19 pandemics were suggested to be predictors of personal NO_2.

- Our findings reinforce the importance of assessing indoor and personal air pollution exposure among vulnerable groups as indoor and personal levels come from different sources besides traffic emissions.

8. REFERENCES

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9. APPENDIX

About the Author

Paula de Prado Bert is graduated in Human Biology at the Pompeu Fabra University (UPF) (2012-2016) and did her Master of Public Health at the Pompeu Fabra University (UPF) (2016-2018). She is currently undertaken the Master of Cooperation, Development and Globalization at the Barcelona University (UB) (2021-Present). She conducted her final degree thesis (2016) and her final master thesis (2018) at ISGlobal. She finally joined ISGlobal in 2018 as a PhD researcher under the supervision of Mariona Bustamante and Jordi Sunyer. A summary of the research activity of the author during the thesis is provided below.

A. Other co-authored papers

Mohammad Miri*, **Paula de Prado Bert***, Ahmad Alahabadi, Moslem Lari Najafi, Abolfazl Rad, Alireza Moslem, Hamideh Ebrahmi Aval, Mohammad Hassan Ehrampoush, Mariona Bustamante, Mohammad Javad Zare Skhvidi, Tim Nawrto Jordi Sunyer, Payam Dadvand. **Association of greenspace exposure with telomere length in preschool children**. *Environmental Pollution.* 2020; 266. DOI: 10.1016/j.envpol.2020.115228

Alireza Moslem, Abolfazl Rad, **Paula de Prado Bert**, Ahmad Alahabadi, Hamideh Ebrahimi Aval, Masoumeh Miri, Abdolmajid

Gholizadeh, Mohammad Hassan Ehrampoush, Jordi Sunyer, Tim S. Nawrot, Mohammad Miri, Payam Dadvand. **Association of exposure to air pollution and telomere length in preschool children**. *Science of The Total Environment*. 2020; 722. DOI: 10.1016/j.scitotenv.2020.137933.

Edwina H. Yeung, Weihua Guan, Xuehuo Zeng, Lucas A. Salas, Sunni L. Mumford, **Paula de Prado Bert**, Evelien R. van Meel, Anni Malmberg, Jordi Sunyer, Liesbeth Duijts, Janine F. Felix, Darina Czamara, Esa Hämäläinen, Elisabeth B. Binder, Katri Räikkönen, Jari Lahti, Stephanie J. London, Robert M. Silver and Enrique F. Schisterman. **Cord blood DNA methylation reflects cord blood C-reactive protein levels but not maternal levels: a longitudinal study and meta-analysis**. *Clinical Epigenet*ics. 2020; 12**:** 60. DOI: 10.1186/s13148-020-00852-2.

Dolors Pelegí-Sisó, **Paula de Prado**, Justiina Ronkainen, Mariona Bustamante, Juan R González. **methylclock: a Bioconductor package to estimate DNA methylation age.** *Bioinformatics*. 2021 Jul 19;37(12):1759-1760. DOI: 10.1093/bioinformatics/btaa825.

M.J. Taeubert, **P. de Prado-Bert**, M.L. Geurtsen, G. Mancano, M.J. Vermeulen, I.K.M. Reiss, J. Sunyer Deu, G.C. Sharp, J. Julvez, M.U. Muckenthaler, J.F. Felix. **Maternal iron status in early pregnancy and DNA methylation in offspring: an epigenome-wide meta-analysis.** *Clinical Epigenetics.* 2022 May 03; 15:59. DOI: 10.1186/s13148-022-01276-w.

GWAS air pollution and lung function with PACE consortium (Under construction)

Maternal and paternal determinants of offspring DNA methylation age acceleration (Under construction)

B. Grants and awards

Involved in the research team as a PhD for the project "*Air pollution during pregnancy and early-life, miRNAs and child health*" funded by "Beca de Recerca Clínica de l'Acadèmia 2019". *Acadèmia de Ciències Mèdiques i de la Salut de Catalunya i de Balears*

2nd call for Research Proposals of the Planetary Wellbeing Initiative 2020. *University Pompeu Fabra*

Award for the 3rd best oral presentation. *ISGlobal PhD Symposium 2021.*

C. Presentations and attendance at scientific conferences

The PhD Candidate has participated in different National and International scientific conferences and meetings, presenting the work conducted during her PhD.

Attendance:

Attendance to the "15a Jornadas Científicas INMA 2018", held in Donostia-San Sebastian, Spain during the 14th and 15th of November 2018.

Attendance to the European Society of Human Genetics 2020.

Poster and attendance:

Poster presentation to the fifth **ISGlobal PhD Symposium 2018**. *"Air pollution and green spaces exposure and telomere length in primary schoolchildren"*.

Poster and video presentation to the **Epigenomics of common Diseases 2020**. *"The early-life exposome and epigenetic age acceleration in children"*. Video available at: https://www.youtube.com/watch?v=MvPsqlxbJS4

Chalk talk at the sixth **ISGlobal PhD symposium 2019**. *"The early-life exposome and epigenetic age acceleration in children"*.

Oral presentation and attendance:

Oral presentation to the 31st annual conference of the International Society for Environmental Epidemiology (**ISEE 2019**)", in Utrecht. *"Green Spaces and telomere length in preschool children".*

Oral presentation to the "**16a Jornadas Científicas INMA 2020**". *"Early Life exposome and epigenetic age in HELIX subcohort".*

Oral presentation to the Young International Society for Environmental Epidemiology (**ISEE young 2021**). *"Early Life exposome and epigenetic age in HELIX subcohort".*

Oral presentation to the seventh **ISGlobal PhD Symposium 2021**. *"Short- and medium-term air pollution exposure, plasmatic protein levels and blood pressure in children".*

Oral presentation to the "**17a Jornadas Científicas INMA 2021**". *"Short- and medium-term air pollution exposure, plasmatic protein levels and blood pressure in children".*

D. Workshops and training activities

Attendance to the course of "How to write a scientific article" of the PRBB Intervals - CÍCLIKS, held at the PRBB (November 2018).

Attendance to the online course "COVID-19: Tackling the novel coronavirus" from the London School of Hygiene & Tropical Medicine and UK Public Health Rapid support team, for 3 weeks, 4 hours per week.

Attendance to the online course organized by the Bristol University regarding Basic Epigenetic Epidemiology and Advanced Epigenetic Epidemiology (a 3-day and a 1-day online course, respectively).

Attendance to the PRBB intervals course related with Sharpen your Reasoning skills (October 2020).

Attendance to the Exposome Boot Camp: Measuring exposures on an omics scale by Columbia University (July 2021).

E. Reviews for Peer-Reviewed Scientific journals

A. Environmental Health (2x)
B. Epigenetics Communications (1x)

F. Supervision of students

October 2021 – June 2022: Mariona Isern. “Association between noise perception and cortisol levels in pregnant women”. Degree of Biomedical Sciences, Barcelona University (UB), Barcelona, Spain.

G. Media attentions

- Wrting posts at ISGlobal blog:

Paula De Prado , Investigadora predoctoral

SALUD AMBIENTAL, CORONAVIRUS, VIRUS EMERGENTES

El Proyecto BiSC y la COVID-19: Así medimos el impacto de la contaminación atmosférica en el embarazo durante la pandemia

13.5.2020

Foto: **Laura Guerrero/Ajuntament de Barcelona** - Control de entrada en la avenida Meridiana (Barcelona) por las medidas de contención de la pandemia de la COVID-19

Paula de Prado , Predoctoral Researcher

ENVIRONMENTAL HEALTH

What Is the Relationship Between Air Pollution and Blood Pressure in Children?

25.5.2022

Photo: **Clara Soler Chopo / Barcelona City Council**

- Media dissemination based on published manuscripts:

https://s.kmni.eu/t/tpnqrNiT-DqokKoNFwEa5sIdGogBHmg-pdf-zC/Ara_20210825100000

https://www.ccma.cat/324/lexposicio-al-fum-de-tabac-durant-lembaras-i-la-infantesa-accelera-lenvelliment/noticia/3115445/

https://medicalxpress.com/news/2021-08-exposure-tobacco-early-life-biological.html

INVESTIGACIÓN

La exposición al humo de tabaco en las primeras etapas de la vida se asocia con una aceleración del envejecimiento biológico

Un nuevo estudio analiza la asociación entre más de 100 exposiciones ambientales y el 'reloj epigenético' de más de 1.000 niños y niñas en seis países europeos

26.08.2021

Foto: Carlo Navarro / Pascal Meier / Unsplash

SALUD

Exponer a humo del tabaco a bebés y niños les acelera su envejecimiento biológico

• Una conclusión a la que llega un estudio liderado por el Instituto de Salud Global de Barcelona (ISGlobal), que ha analizado datos biológicos de 1.173 niños de entre 6 y 11 años de seis países europeos, incluida España

Salut

SOCIETAT | 25/08/2021

El fum del tabac accelera l'envelliment biològic de nadons i infants

Un estudi suggereix que l'exposició a contaminants pot augmentar el risc de patir malalties metabòliques i neurodegeneratives

H. Other activities

Within the PhD Programme, the PhD candidate has been in charge of: (i) the organization of internal weekly ISGlobal seminars, from September 2020 to April 2021, (ii) the creation of the video "The magical clock" for the Open PRBB 2020 (available here: http://y2u.be/PvP5iN2I2Yg), and (iii) the organization of the 7th ISGlobal PhD Symposium (September 2021) with the creation of the opening video regarding women in science (available here: http://y2u.be/0S0ig554vrE). She also participated as a volunteer in the "The Global Forum on Childhood Pneumonia" held at Cosmocaixa, Barcelona, between the 29-31 of January 2020.

Finally, she was involved on the last edition of "100tífiques" programme carried out on February 11th (International Day of Women and Girls in Science) conducting a talk at *Nou Patufet* School in Barcelona.