

*"The past grows gradually around one,
like a placenta for dying"*

-John Berger

Doctoral dissertation presented on Tuesday 21 June 2016 at Hasselt University

Members of the Jury

Prof. dr. M. Ameloot, Hasselt University, Diepenbeek, Belgium, Chairman

Prof. dr. T.S. Nawrot, Hasselt University, Diepenbeek, Belgium, Promoter

Prof. dr. A. Cuypers, Hasselt University, Diepenbeek, Belgium, Co-promoter

Prof. dr. K Smeets, Hasselt University, Diepenbeek, Belgium

Prof. dr. W. Gyselaers, Hasselt University, Diepenbeek, Belgium

Prof. dr. K. Michels, Harvard University, Boston, MA, USA

Dr. B. Izzi, Leuven University, Leuven, Belgium

SUMMARY

The '*Developmental Origins of Health and Disease*' (DOHaD) or Barker hypothesis suggests that small changes in the prenatal and early postnatal environment shape the future probability of the development of age-related diseases. Among the most hazardous early life environmental risk factors are fine particle air pollution and tobacco smoke, a personalized form of air pollution. While the specific mechanisms behind fetal programming are still poorly understood, evidence is mounting that the placenta plays a central role in an adaptive response to environmental perturbations resulting in altered fetal metabolism. During its nine month lifespan, the human placenta captures morphological, functional, genetic, and epigenetic information, representing thus a molecular 'footprint' that can reveal the impact of *in utero* exposures on the fetus.

In this dissertation, we investigated the effect of fine particle exposure on the most important organ in fetal development – the placenta. For this purpose, we focused on three important systems: epigenetics, mitochondria, and thyroid hormones, that could provide a biologically plausible link between *in utero* exposures and disease risks in adulthood. The specific objectives of this doctoral dissertation were:

1. To establish a population-based prospective birth cohort 'ENVIRONAGE'.
2. To evaluate the variation of gene-specific methylation in placenta tissue.
3. To replicate (molecular) effects of maternal tobacco smoke exposure during pregnancy.
4. To investigate the association between gestational PM exposure and placental mitochondria and to explore the role of thyroid hormones.
5. To investigate whether PM exposure during pregnancy is associated with genotoxic changes in cord blood.

A short description of each chapter in this thesis is summarized in **Table 1**. Findings of this thesis will contribute to the field of epigenetic epidemiology and provide a basis for future studies of early life exposures, epigenetic mechanisms, mitochondria, and the developmental origins of health and disease. In addition, it will promote preventive health care strategies and contribute to a healthier living environment for pregnant mothers and their children.

SUMMARY

Table 1. Summary of this doctoral dissertation

Chapter	What is known	What this study adds	Perspectives and conclusions
Chapter 2	<ul style="list-style-type: none">Studies on the health effects of air pollution exposure during the <i>in utero</i> period are scarceIn depth phenotyping is of crucial importance to understand the interaction between biological systems and environmental exposures	<ul style="list-style-type: none">Recommendations for the design to study human aging and its environmental influenceThe use of interesting molecular mechanisms to bridge the gap between exposure and disease	<ul style="list-style-type: none">Findings of the ENVIRONAGE birth cohort will promote:<ul style="list-style-type: none">Preventive health care strategiesA healthier living environment for pregnant mothers and their childrenCausal interference of environment with health
Chapter 3	<ul style="list-style-type: none">Gene methylation is tissue-specific and potentially even cell-specificLittle information is available about within-placental variation of DNA methylation patterns as well as concordance between cord blood and placenta	<ul style="list-style-type: none">In placental tissue, methylation patterns of most targets on the mitochondrial-telomere axis are not strongly influenced by sample locationThe methylation levels of the subtelomeric region and mitochondrial genome show concordance between cord blood and placenta	<ul style="list-style-type: none">Researchers interested in epigenetic or gene expression analysis should evaluate each gene of interest across placental sampling sites, as each gene may display site-specific differences
Chapter 4	<ul style="list-style-type: none">Maternal smoking during pregnancy increases the risk of low birth weight<i>CYP1A1</i> detoxifies constituents of tobacco smokeMitochondria contain mitochondrial DNA (mtDNA), which is extremely vulnerable and responsive to tobacco-induced oxidative stress	<ul style="list-style-type: none">Gestational tobacco smoke exposure is associated with:<ul style="list-style-type: none">Lower birth weightLower placental mtDNA contentHigher placental mtDNA methylationHigher CpG-specific methylation levels of <i>CYP1A1</i> in placenta	<ul style="list-style-type: none">Our findings will contribute to the understanding of molecular pathways underlying the association between gestational tobacco smoke exposure and low birth weightWhether these changes in molecular signatures relate to developmental defects in Belgian children will be investigated in a follow-up study of the ENVIRONAGE birth cohort
Chapter 5	<ul style="list-style-type: none">DNA methylation patterns are established during embryogenesisAlterations in DNA methylation is an important epigenetic mechanism in prenatal programmingEnvironmental factors influence DNA methylation patterns	<ul style="list-style-type: none">Prenatal fine particle ($PM_{2.5}$) exposure is associated with lower placental global DNA methylationExposure from fertilization to implantation showed a highly sensitive window for $PM_{2.5}$ exposure	<ul style="list-style-type: none">Our findings give mechanistic plausibility to the hypothesis that air pollution is linked to fetal programmingThere is need to further investigate how PM air pollution affects gene-specific DNA methylation patterns during fetal development

Table 1. (continued)

Chapter	What is known	What this study adds	Perspectives and conclusions
Chapter 6.1	<ul style="list-style-type: none"> Placental mitochondria play an important role in the proper formation and function of the placenta mtDNA content is a molecular marker of mitochondrial damage and inflammation Maternal smoking is associated with mtDNA depletion in placental tissue (chapter 4) 	<ul style="list-style-type: none"> Prenatal PM₁₀ exposure is associated with placental mtDNA content depletion, especially in the last period of pregnancy An increase in residential distance to major roads is associated with higher levels of placental mtDNA content No association of prenatal PM₁₀ exposure with mtDNA content in cord blood 	<ul style="list-style-type: none"> Alterations in mtDNA content may both reflect and intensify oxidative stress production Alterations in placental mtDNA may mediate the association between PM exposure and birth weight The health effects of decreased placental mtDNA content must be further elucidated
Chapter 6.2	<ul style="list-style-type: none"> Prenatal PM₁₀ exposure is associated with placental mtDNA content depletion (chapter 6.1) mtDNA can also be methylated Exposure to metal-rich PM₁ is associated with an increase in mtDNA methylation in adult blood 	<ul style="list-style-type: none"> Prenatal PM_{2.5} exposure is associated with higher placental mtDNA methylation levels mtDNA methylation correlates positively with mtDNA content mtDNA methylation substantially mediates the association between prenatal PM_{2.5} exposure and placental mtDNA content 	<ul style="list-style-type: none"> This study provides new insight into the mechanisms of altered mitochondrial function Future experiments should clarify whether mtDNA methylation in other regions of the mtDNA are sensitive to air pollutants mtDNA (methylation and copy number) can act as an environmental biosensor
Chapter 7.1	<ul style="list-style-type: none"> Thyroid hormones are essential for placental and fetal development Free thyroid hormones (FT₃ and FT₄) have effects on mitochondrial energetics and biogenesis <i>PPARGC1A</i> is a key regulator of mitochondrial genes and energy-linked metabolism 	<ul style="list-style-type: none"> Fetal FT₃ and FT₄ are positively associated with placental mtDNA content and negatively associated with mtDNA methylation mtDNA methylation substantially mediates the association between fetal FT₃ and FT₄ and placental mtDNA content Fetal FT₃ is negatively associated with placental <i>PPARGC1A</i> promoter methylation 	<ul style="list-style-type: none"> There exist coordinated events between mtDNA methylation, mtDNA content, and thyroid hormones Epigenetic modification of the mtDNA could intervene with thyroid-dependent regulation of mitochondrial biogenesis Findings should be confirmed in experimental studies

Table 1. (continued)

Chapter	What is known	What this study adds	Perspectives and conclusions
Chapter 7.2	<ul style="list-style-type: none"> Unbalanced thyroid function adversely affects the fetus (e.g. low birth weight and neurological development) Numerous chemicals interfere with thyroid hormone function in neonates and adults Fetal FT₄ levels associate negatively with birth weight 	<ul style="list-style-type: none"> Third trimester PM_{2.5} exposure is associated with a lower fetal FT₄/FT₃ ratio (as measured in cord blood), which is mainly attributed to a reduction in FT₄ Fetal FT₄ levels in cord blood substantially mediate the association between third trimester PM_{2.5} exposure and birth weight 	<ul style="list-style-type: none"> Whether PM_{2.5}-induced alterations in fetal thyroid function has bearing on pathological consequences later in life (especially as to cognitive development), requires further elucidation
Chapter 8	<ul style="list-style-type: none"> PM air pollution is classified as carcinogenic Micronuclei (MN) frequency is a valid biomarker for genotoxicity, with an important role played by the tumor suppressor protein P53 PM exposure is associated with MN frequency in cord blood 	<ul style="list-style-type: none"> Prenatal PM_{2.5} exposure is negatively associated with expression levels of the MN formation-related genes Specifically, PM_{2.5} exposure is associated with a down-regulation of <i>P53</i> gene expression P53 protein levels associate positively with PM_{2.5} exposure during pregnancy 	<ul style="list-style-type: none"> These alterations in expression of genes within the MN formation network can serve as potential biomarkers of PM_{2.5} exposure Our findings lend support to the development of a reliable reporter gene assay to screen chemically exposed human populations in a faster and less labor-intensive manner than using microarrays

SAMENVATTING

De hypothese van de foetale oorsprong van ziektes op volwassen leeftijd (DOHaD), of Barker hypothese, stelt dat blootstellingen aan risicofactoren in de prenatale en vroege postnatale omgeving de kans verhogen op de ontwikkeling van ouderdomsgerelateerde ziektes. Luchtverontreiniging en tabaksrook, een gepersonaliseerde vorm van luchtverontreiniging, behoren tot de meest schadelijke omgevingsblootstellingen. De specifieke mechanismen van foetale programmering zijn nog niet goed begrepen maar er wordt een belangrijke rol toegeschreven aan de placenta die zich aanpast aan omgevingsblootstellingen wat kan leiden tot permanente veranderingen in het foetale metabolisme. De placenta legt alle morfologische, functionele, genetische en epigenetische informatie vast waardoor dit orgaan gezien kan worden als een moleculaire "voetafdruk" waaraan de foetus is blootgesteld tijdens de zwangerschap.

In dit proefschrift onderzochten we het effect van luchtverontreiniging door fijn stof op de placenta, het belangrijkste orgaan tijdens de foetale ontwikkeling. Hiervoor hebben we ons gefocust op drie belangrijke systemen: epigenetica, mitochondriën en schildklierhormonen. De specifieke doelstellingen van dit proefschrift waren:

1. Het oprichten van een populatie-gebaseerd prospectief geboortecohort "ENVIRONAGE".
2. Het evalueren van de gen-specifieke methylation variatie in de placenta.
3. Het repliceren van de (moleculaire) effecten van blootstelling aan tabaksrook tijdens de zwangerschap.
4. Het onderzoeken van het verband tussen prenatale fijn stof blootstelling en mitochondriale (epi)genetica in de placenta en het exploreren welke rol schildklierhormonen in dit verband spelen.
5. Het onderzoeken of prenatale blootstelling aan fijn stof geassocieerd is met genotoxische veranderingen in navelstrengbloed.

Een korte beschrijving van elk hoofdstuk in dit proefschrift is samengevat in **Tabel 1**. De bevindingen van dit proefschrift zullen bijdragen leveren aan het onderzoeks domein epigenetische epidemiologie en vormen een basis voor toekomstig onderzoek naar de foetale oorsprong van ziektes. Dit werk zal ook preventieve gezondheidsstrategieën promoten en bijdragen tot een gezondere leefomgeving voor zwangere moeders en hun kinderen.

Tabel 1. Samenvatting van dit proefschrift

Hoofdstuk	Wat is er geweten	Wat deze studie bijbrengt	Perspectieven en conclusies
Hoofdstuk 2	<ul style="list-style-type: none"> Studies naar de gezondheidseffecten van prenatale luchtverontreiniging zijn schaars Diepgaande fenotypering is van cruciaal belang om de interactie tussen biologische systemen en omgevingsblootstellingen te begrijpen 	<ul style="list-style-type: none"> Aanbevelingen voor studieontwerpen om menselijke veroudering en de invloed van de omgeving te bestuderen Het gebruik van interessante moleculaire merkers om blootstelling met ziekte te linken 	<ul style="list-style-type: none"> De bevindingen van ENVIRONAGE zullen volgende aspecten bevorderen: <ul style="list-style-type: none"> Preventieve strategieën in de gezondheidszorg Een gezondere leefomgeving voor zwangere moeders en hun kinderen Causale verbanden tussen leefomgeving en ziekten aantonen
Hoofdstuk 3	<ul style="list-style-type: none"> DNA methylatie is weefselspecifiek en waarschijnlijk zelfs celspecifiek Er is weinig informatie beschikbaar over de placentale variatie van DNA methylatie alsook in de overeenstemming tussen de placenta en navelstrengbloed 	<ul style="list-style-type: none"> DNA methylatiepatronen van de meeste genen in de mitochondriale-telomeer as zijn niet sterk beïnvloed door de locatie van staalname in de placenta De methylatie van de subtelomerische regio en het mtDNA tonen overeenstemming tussen navelstrengbloed en de placenta 	<ul style="list-style-type: none"> Onderzoekers die geïnteresseerd zijn in epigenetische of genexpressie metingen in de placenta zouden van elk gen de variatie binnen de placenta moeten bepalen om na te gaan of ze één of meerdere bioplen moet gebruiken om een representatief beeld van de placenta te krijgen
Hoofdstuk 4	<ul style="list-style-type: none"> Het risico op laag geboortegewicht verhoogt bij moeders die roken tijdens de zwangerschap <i>CYP1A1</i> speelt een rol in de detoxificatie van bepaalde bestanddelen van tabaksrook Mitochondriën bevatten mitochondriaal DNA (mtDNA) dat extreme kwetsbaar en adaptief is aan oxidatieve stress geïnduceerd door tabaksrook 	<ul style="list-style-type: none"> Actief roken tijdens de zwangerschap is geassocieerd met: <ul style="list-style-type: none"> Een laag geboortegewicht Een lagere placentale mtDNA inhoud Hogere mtDNA methylatie in de placenta Hogere CpG-specifieke methylatie van <i>CYP1A1</i> in de placenta 	<ul style="list-style-type: none"> Onze bevindingen zullen bijdragen tot het beter begrijpen van moleculaire mechanismen die onderliggend zijn in de associatie tussen een laag geboortegewicht en blootstelling aan tabaksrook tijdens de zwangerschap Of deze veranderingen in moleculaire merkers gerelateerd zijn aan ontwikkelingsstoornissen in Belgische kinderen zal onderzocht worden in een vervolgonderzoek van ENVIRONAGE
Hoofdstuk 5	<ul style="list-style-type: none"> DNA methylatiepatronen worden vastgelegd tijdens de embryogenese Veranderingen in DNA methylatie zijn belangrijke epigenetische mechanismen tijdens foetale programmering Omgevingsfactoren beïnvloeden DNA methylatie 	<ul style="list-style-type: none"> Prenatale PM_{2,5} blootstelling is geassocieerd met een lagere placentale DNA methylatie De periode vanaf de bevruchting tot de implantatie in de baarmoeder is een gevoelige periode voor veranderingen in DNA methylatie door PM_{2,5} blootstelling 	<ul style="list-style-type: none"> Onze bevindingen verklaren gedeeltelijk de hypothese dat luchtverontreiniging geassocieerd is met foetale epigenetische programmering Meer onderzoek is nodig om te verklaren hoe fijn stof gen-specifieke DNA methylatiepatronen tijdens de foetale ontwikkeling beïnvloedt

Tabel 1. (vervolg)

Hoofdstuk	Wat is er geweten	Wat deze studie bijbrengt	Perspectieven en conclusies
Hoofdstuk 6.1	<ul style="list-style-type: none"> • Placentale mitochondriën spelen een belangrijke rol in de ontwikkeling en functie van de placenta • mtDNA inhoud is een moleculaire merker van mitochondriale schade en ontsteking • Prenatale blootstelling aan tabaksrook is geassocieerd met daling van mtDNA in de placenta (hoofdstuk 4) 	<ul style="list-style-type: none"> • Prenatale PM₁₀ blootstelling, vooral in de laatste periode van de zwangerschap, is geassocieerd met een daling van de mtDNA inhoud in de placenta • Hoe groter de afstand tussen de woonplaats en een hoofdweg, hoe hoger de mtDNA inhoud in de placenta • Er is geen associatie tussen prenatale PM₁₀ blootstelling en mtDNA inhoud in navelstrengbloed 	<ul style="list-style-type: none"> • Veranderingen in mtDNA inhoud kan zowel oxidatieve stress productie weerspiegelen als versterken • Veranderingen in placentale mtDNA inhoud kan als mediator optreden tussen de associatie van fijn stof en geboortegewicht • De gezondheidseffecten van een verlaging in placentale mtDNA inhoud moet verder onderzocht worden
Hoofdstuk 6.2	<ul style="list-style-type: none"> • Prenatale PM₁₀ blootstelling is geassocieerd met een daling in placentale mtDNA inhoud (hoofdstuk 6.1) • mtDNA kan ook gemethyleerd zijn • Blootstelling aan metaalrijk PM₁ is geassocieerd met een verhoging in mtDNA methylatie in bloed van volwassenen 	<ul style="list-style-type: none"> • Prenatale PM_{2,5} blootstelling is geassocieerd met hogere placentale mtDNA methylatie • mtDNA methylatie correleert positief met mtDNA inhoud • mtDNA methylatie is een mediator in de associatie tussen prenatale PM_{2,5} blootstelling en placentale mtDNA inhoud 	<ul style="list-style-type: none"> • Deze studie geeft nieuw inzicht in mechanismen die leiden tot een verandering in mitochondriale functie • Toekomstig onderzoek moet uitwijzen of mtDNA methylatie in andere regio's van het mtDNA genoom gevoelig zijn aan luchtverontreiniging • mtDNA methylatie en mtDNA inhoud kan dienst doen als een omgevingsbiosensor
Hoofdstuk 7.1	<ul style="list-style-type: none"> • Schildklierhormonen zijn essentieel voor placentale en foetale ontwikkeling • Vrije schildklierhormonen (FT₃ en FT₄) spelen een rol bij mitochondriale energievoorziening en energieproductie • <i>PPARGC1A</i> is een belangrijke regulator van mitochondriale genen en het energiemetabolisme 	<ul style="list-style-type: none"> • Foetale FT₃ en FT₄ zijn positief geassocieerd met placentale mtDNA inhoud en negatief geassocieerd met mtDNA methylatie • mtDNA methylatie is een mediator in de associatie tussen foetale FT₃ of FT₄ en placentale mtDNA inhoud • Foetale FT₃ associeert negatief met placentale <i>PPARGC1A</i> promoter methylatie 	<ul style="list-style-type: none"> • Er is een gecoördineerd samenspel tussen mtDNA methylatie, mtDNA inhoud en schildklierhormonen • Epigenetische modificatie van het mtDNA kan tussenkomen in de schildklierhormoon-afhankelijke regulatie van mitochondriale biogenese • De bevindingen van deze studie moeten bevestigd worden in een experimentele studie

Tabel 1. (vervolg)

Hoofdstuk	Wat is er geweten	Wat deze studie bijbrengt	Perspectieven en conclusies
Hoofdstuk 7.2	<ul style="list-style-type: none"> Schildklierstoornissen kunnen negatieve gevolgen hebben voor de foetus (laag geboortegewicht en verstoerde neurologische ontwikkeling) Tal van chemicaliën interfereren met de schildklierfunctie in pasgeborenen en volwassenen Foetale FT₄ concentraties associëren negatief met geboortegewicht 	<ul style="list-style-type: none"> PM_{2.5} blootstelling tijdens het 3^{de} trimester is geassocieerd met een lager foetaal FT₄/FT₃ ratio (gemeten in navelstengbloed), wat vooraf te wijten is aan een verlaging in FT₄ Foetale FT₄ concentratie in navelstengbloed is een mediator in de associatie tussen PM_{2.5} blootstelling in het 3^{de} trimester en geboortegewicht 	<ul style="list-style-type: none"> Of PM_{2.5}-geïnduceerde veranderingen in foetale schildklierfunctie leidt tot pathologische gevolgen in het latere leven (vooral cognitieve ontwikkeling) moet verder uitgeklaard worden
Hoofdstuk 8	<ul style="list-style-type: none"> Fijn stof luchtverontreiniging is geclassificeerd als carcinogen Micronuclei (MN) frequentie is een gevalideerde biomarker voor genotoxiciteit waarbij een belangrijke rol is weggelegd voor het tumorsuppressor proteïne P53 Fijn stof blootstelling is geassocieerd met MN frequentie in navelstengbloed 	<ul style="list-style-type: none"> Prenatale PM_{2.5} blootstelling is negatief geassocieerd met de expressie van MN-gerelateerde genen die een rol spelen in de vorming van MN Meer specifiek, PM_{2.5} blootstelling is geassocieerd met een verlaging van de expressie van het P53 gen P53 proteïne waarden zijn positief geassocieerd met PM_{2.5} blootstelling tijdens de zwangerschap 	<ul style="list-style-type: none"> De veranderingen in expressie van genen binnen het MN netwerk kunnen dienst doen als potentiële biomarkers voor PM_{2.5} blootstelling Onze bevindingen ondersteunen de ontwikkeling van een betrouwbare screeningstest om individuen die blootgesteld zijn aan bepaalde stoffen te screenen, en dit op een snellere en minder labo-intensieve manier dan met het gebruik van microarrays

CONTENTS

Summary	i
Samenvatting.....	vii
Contents.....	xiii
List of abbreviations.....	xvii
Chapter 1. General introduction.....	1
1. The mysterious tree of a newborn's life	2
1.1 Placental development and the fetoplacental circulation	3
1.2 Role of thyroid hormones in placental and fetal development.....	6
2. Ambient air pollution.....	6
2.1 Origin and characteristics of air pollution	7
2.2 Particulate matter legislation	9
2.3 Health impact of particulate matter	9
2.4 Health impact of particulate matter in susceptible groups	10
3. Biomarkers: Epigenetics and mitochondria	11
3.1 Epigenetics	11
3.2 Mitochondria	13
4. Aims of the study	17
Chapter 2. The ENVIRONAGE birth cohort: A cohort profile	19
Why was the cohort set up?	20
Who is in the cohort?.....	21
How often are cohort members being followed-up?.....	25
What has been measured?	28
What has it found? Key findings and publications	36
What are the main strengths and weaknesses?	40
Can I get hold of the data? Where can I find out more?	41
Profile in a nutshell.....	41

Chapter 3. Variation of DNA methylation in candidate age-related targets on the mitochondrial-telomere axis in cord blood and placenta.....	43
Abstract.....	44
Introduction	45
Materials and methods.....	48
Results	52
Discussion.....	56
Conclusion	59
Supplemental material.....	61
Chapter 4. Placental mitochondrial DNA and CYP1A1 methylation as potential biomarkers for low birth weight in smoking pregnant women.....	65
Abstract.....	66
Introduction	67
Materials and methods.....	68
Results	71
Discussion.....	77
Conclusion	80
Supplemental Material	82
Chapter 5. Placental DNA hypomethylation in association with particulate air pollution in early life.....	85
Abstract.....	86
Introduction	87
Materials and methods.....	88
Results	92
Discussion.....	99
Conclusion	104
Supplemental material.....	106
Chapter 6.1. Placental mitochondrial DNA content and particulate air pollution during <i>in utero</i> life	107
Abstract.....	108
Introduction	109

Materials and methods.....	110
Results	115
Discussion.....	121
Conclusion	125
Supplemental material.....	126
Chapter 6.2. Placental mitochondrial methylation and exposure to airborne particulate matter in the early life environment: An ENVIRONAGE birth cohort study.....	129
Abstract.....	130
Introduction	131
Materials and methods.....	133
Results	138
Discussion.....	144
Conclusion	148
Supplemental material.....	149
Chapter 7.1. Epidemiological evidence for fetal thyroid-dependent regulation at the (epi)genomic level in placental mitochondria	155
Abstract.....	156
Introduction	157
Materials and methods.....	158
Results	162
Discussion.....	170
Conclusion	172
Supplemental material.....	175
Chapter 7.2. Fetal thyroid function, birth weight and <i>in utero</i> exposure to fine particle air pollution: a birth cohort study	181
Abstract.....	182
Introduction	183
Materials and methods.....	184
Results	188
Discussion.....	195
Conclusion	199

Supplemental material	200
Chapter 8. Expression of micronuclei-related genes in cord blood in association with <i>in utero</i> particulate matter exposure: P53 as a central hub	209
Abstract	210
Introduction	211
Materials and methods.....	211
Results	217
Discussion.....	222
Conclusion	226
Supplemental material.....	227
Chapter 9. General discussion.....	229
Establishment of the population-based prospective birth cohort 'ENVIRONAGE'	231
Methodological considerations for studying placental epigenetics.....	232
An explorative analysis on the effects of tobacco smoke.....	234
Fine particle exposure and epigenetics	235
Fine particle exposure and mitochondria	238
An integrated network of thyroid hormones, mitochondria, and epigenetics.....	240
Fine particle exposure and effects on cord blood	243
Outlook.....	243
Reference list.....	245
Curriculum Vitae.....	269
Bibliography	271
International peer-reviewed publications	272
Books	273
Reports.....	273
Abstracts	273
Awards	274
Grants	274
Dankwoord	275

LIST OF ABBREVIATIONS

3-NTp	3-nitrotyrosine
5-mdC	5'-methyl-deoxycytidine
8-OHdG	8-hydroxy-2'-deoxyguanosine
ACTB	beta actin
Ahr	aryl hydrocarbon
ARNT	Ahr nuclear translocator protein
ATP	adenosine-5'-triphosphate
BAX	BCL2-associated X protein
BC	black carbon
BMI	body mass index
BP	blood pressure
BTEX	benzene, toluene, ethylbenzene, and xylene
CDKN1A	cyclin-dependent kinase inhibitor 1A (<i>p21</i>)
CI	confidence interval
CV	coefficient of variation
CYC1	cytochrome c-1
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1
D-loop	displacement-loop
D4Z4	non-satellite subtelomeric tandem repeat D4Z4
dC	deoxycytidine
DNMTs	DNA methyltransferases
DOHaD	developmental origins of health and disease
ENVIRONAGE	ENVIRonmental influence <i>ON</i> early AGEing
EU	European Union
FFQ	food frequency questionnaire
GIS	geographical information system
ICC	intra-class correlation coefficient
ICP-MS	inductively coupled plasma mass spectrometry
IPO8	importin 8
IQR	interquartile range
IRC	inter-run calibrator
miRNA	microRNA
MN	micronuclei

mtDNA	mitochondrial DNA
MTF3212/R3319	mitochondrial forward primer from nucleotide 3212 and reverse primer from nucleotide 3319
MT-ND1	mitochondrial encoded NADH dehydrogenase 1
MT-RNR1	mtDNA-encoded 12S rRNA
NBAS	neonatal behavior assessment scale
nDNA	nuclear DNA
NIH	National Institute of Health
NO _x	nitrogen oxides
NTC	non-template control
O ₃	ozone
p43	43 kDa c-Erb A alpha 1 protein
PAH	polycyclic aromatic hydrocarbons
PCNA	proliferating cell nuclear antigen
PM	particulate matter
PM _{2.5}	particles with an aerodynamic diameter < 2.5 µm
PM ₁₀	particles with an aerodynamic diameter < 10 µm
PPARG	peroxisome proliferator-activated receptor γ-
PPARGC1A	peroxisome proliferator-activated receptor γ-coactivator1α
POLGA	DNA polymerase γ A
PSS	perceived stress scale
qPCR	quantitative polymerase chain reaction
ROS	reactive oxygen species
RPLP0	acidic ribosomal phosphoprotein P0
SIRT1	sirtuin 1
SNP	single nucleotide polymorphism
SO _x	sulfur oxides
SDQ	strength and difficulties questionnaire
TBP	TATA box binding protein
TFAM	mitochondrial transcription factor A
TP53	tumor suppressor protein 53
TSH	thyroid stimulating hormone
(F)T ₄	(free)thyroxine

(F)T ₃	(free)triiodothyronine
UFP	ultra fine particles (particles with an aerodynamic diameter < 0.1 µm)
UPLC	ultra-pressure liquid chromatography
WHO	World Health Organization

CHAPTER 1

GENERAL INTRODUCTION

As our knowledge of developmental biology expands, there is greater awareness that health complications in adults can have their roots in risk factors operative in early life. The Barker hypothesis or '*Developmental Origins of Health and Disease*' (DOHaD) evolved from a study focusing on deprived prenatal environment associated with low birth weight, and a greater subsequent risk of type-2 diabetes and cardiovascular disease in adult life.¹ While the specific mechanisms behind fetal programming are still poorly understood, a large body of evidence suggests the human placenta as a main target organ since it serves as the gatekeeper between mother and fetus. In addition, evidence is mounting that sub-optimal fetal development has life-long and intergenerational consequences and is more likely to be the result of an interaction of multiple external and internal factors rather than a single cause.²

A report from the World Health Organization (WHO) in 2006 stated that approximately one-quarter of the global disease burden, and more than one-third of the burden among children, is due to modifiable environmental factors.³ Some 2.5 million people die every year from non-communicable diseases such as cardiovascular diseases and cancers that are attributable to environmental factors, including work-related stress, as well as chemicals, secondhand smoke exposures, and air pollution. In the last decade, air pollution has been pinpointed as one of the major global health problems affecting people worldwide.⁴ In addition to the prominent health effects in the adult population such as cardiovascular events,^{5,6} and lung cancer incidence,⁷ growing evidence shows that exposure to ambient air pollution during the most vulnerable stage in life, the *in utero* period, is of critical importance.^{8,9} Since the placenta captures all information it has been exposed to during its nine months' lifespan, it might play a key role in unraveling the complex interplay between external and biological factors which may increase our understanding of DOHaD.

1. THE MYSTERIOUS TREE OF A NEWBORN'S LIFE

"*The [umbilical] vessels join on the uterus like the roots of plants and through them the embryo receives its nourishment*"-Aristotle, *On the Generation of Animals*, ca. 340 B.C. Since ancient history, the placenta has been recognized as being of vital importance for fetal development, and at the same time quite

mysterious - even somewhat mystical. In some cultures, the organ has spiritual meaning with rituals including preservation after birth (lotus birth), burial, or placentophagy.¹⁰ During pregnancy, the placenta plays the role of many organs of the fetus such as respiration, endocrine system, liver, and kidney. It is a temporary organ by which oxygen and nutrients are carried from the mother to the fetus while at the same time removes potentially toxic substances like carbon dioxide.¹¹ Until the 1960s, it was common to believe that one could consume alcohol, smoke, and use medication without affecting the child's health. The 1961 thalidomide tragedy definitely refuted the hypothesis that the placenta acts as a protective barrier between mother and child.¹² Chemical compounds can cross the placenta through passive diffusion or transporters between the maternal and fetal side of the placental membrane. Although the placenta can metabolize some foreign chemicals during pregnancy, others accumulate and affect placental function and morphology.¹³ Since the placenta is in contact with all nutritional, hormonal, and other environmental stress factors throughout pregnancy, it contains essential information and is often referred to as the 'footprint' of intrauterine life. Still, given its vital role, shockingly little is known about the placenta. The National Institute of Health (NIH-USA) called the placenta: "*the least understood human organ and arguably one of the most important, not only for the health of a woman and her fetus during pregnancy but also for the lifelong health of both.*" The NIH recently funded the 'Human Placental Project', an initiative to understand and monitor the development of the human placenta during pregnancy.¹⁴

1.1 Placental development and the fetoplacental circulation

The human placenta is attached to the baby by the umbilical cord and is composed of tissues derived from the fetal embryo, as well as maternal tissues of the endometrium. Five days after fertilization in the fallopian tube, the blastocyst (approximately 150 cells) reaches the uterine wall and is ready for implantation into the maternal endometrium (**Figure 1**). The blastocyst is a sphere made up of an outer layer of trophoblasts, which forms the fetal portion of the placenta, and an inner cell mass, which is to become the embryo. During implantation (6-12 days), the trophoblast differentiates into an inner cytotrophoblast layer and an overlying invasive syncytiotrophoblast layer which

are multinucleated cells that digest endometrial cells to firmly secure the blastocyst to the uterine wall.¹⁵ At this stage, the implanted blastocyst is supplied by oxygen and metabolic substrates from the maternal circulation, but during prenatal weeks 4-12, the developing placenta gradually takes over the role of nourishment. As the syncytiotrophoblasts reach deep into the uterine wall, a network of finger-like structures, called chorionic villi, are formed which project into the endometrium like tree roots making up the fetal portion of the placenta. Invasive extravillous trophoblasts migrate from the villous tips to the maternal endometrium to anchor the placenta. In addition, these cells remodel maternal spiral arteries to augment maternal blood flow surrounding the villi. Placentation is complete by the end of the first trimester of pregnancy.¹¹

While oxygen, carbon dioxide and lipid-soluble substances cross the walls of the villi by simple diffusion, other substances like amino acids and iron are moved across the placenta by active transport, without commingling of fetal and maternal blood because blood cells cannot move across the placenta. However, throughout gestation, the exchange increases extensively and villi become thinner and increasingly branched. The placenta is permeable to lipid-soluble fetotoxic substances such as alcohol, nicotine, barbiturates, antibiotics, certain pathogens, and many other substances that can be dangerous or fatal to the developing embryo or fetus.¹¹

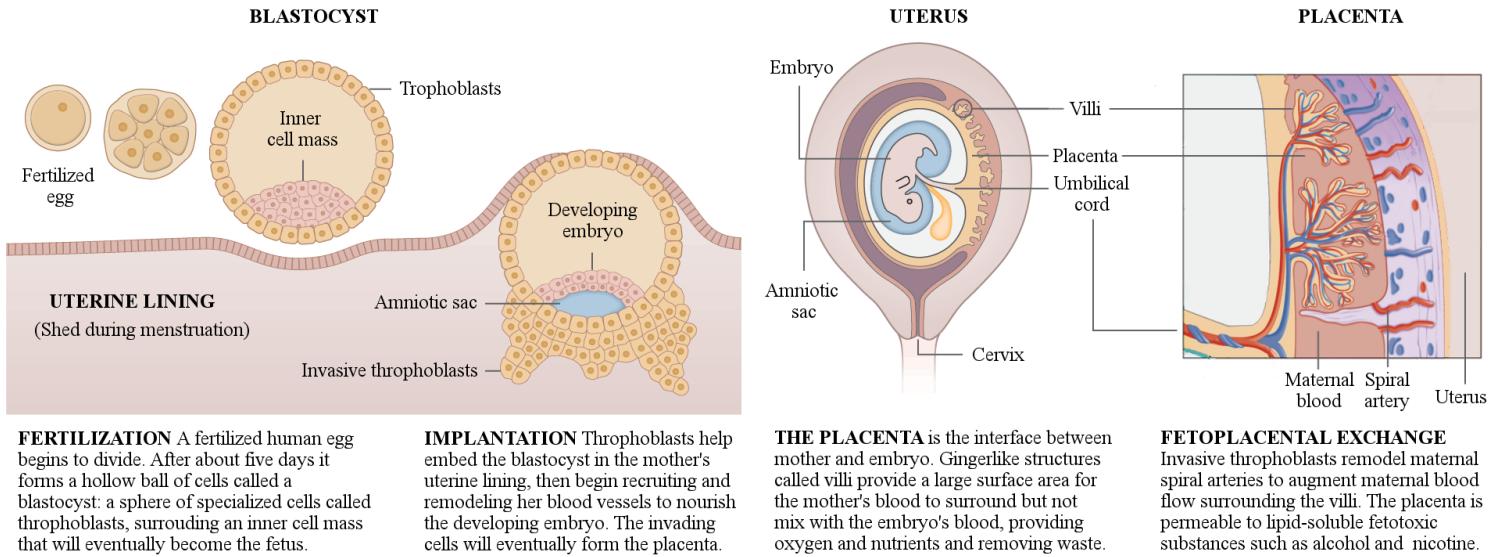


Figure 1. Development of the human placenta from fertilization onwards and a detailed magnification of the chorionic villous tree.

1.2 Role of thyroid hormones in placental and fetal development

During prenatal life, thyroid hormones are critical for placental¹⁶ and fetal development.¹⁷ Thyroxine (T₄), the major form of thyroid hormone, and triiodothyronine (T₃), the active form, are controlled by thyroid-stimulating hormone (TSH) and released by the thyroid gland. Bound to plasma proteins, these thyroid hormones are transported throughout the body and diffuse from maternal blood across the placenta to reach the fetus.¹⁸ However, it is the unbound, free fraction of these hormones (FT₄ and FT₃) that are taken up by different cell types to regulate their function.¹⁹ The human placenta regulates the passage of thyroid hormones from the maternal to the fetal circulation, ensuring that the required levels are present in the fetus at each stage of development.¹⁶ From the second trimester of gestation onwards, the fetal thyroid gland becomes functional and the fetus is able to produce its own supply of thyroid hormones in addition to the maternal supply.²⁰ Two types of iodothyronine deiodinases (D2 and D3) are expressed in the placenta that are capable of metabolizing FT₄ to FT₃.²¹ Fetal FT₃ stimulates the production of factors that control trophoblast growth and development.²² Therefore, thyroid hormones play an important role in normal placentation and development of the fetus. Unbalanced thyroid function influences pregnancy outcomes and adversely affects the fetus. In particular, both maternal hypo- and hyperthyroidism are associated with increased risk of low birth weight,^{23,24} while other studies also suggest an important role of fetal thyroid function in regulating fetal growth.^{25,26} Besides placental and fetal development, thyroid hormones are known to have profound effects on mitochondrial energetics and biogenesis.^{27,28}

2. AMBIENT AIR POLLUTION

Industrial activities and mining increased since the beginning of the industrial revolution in the late 19th and early 20th century. Together with this fast growing industry, the problem of (traffic-related) air pollution raised extensively. For the earliest episodes of human health effects due to extreme levels of air pollution, one often refers to the increased rates of mortality during the Meuse valley fog of 1930²⁹ or the London smog of 1952.³⁰ Although air quality has improved

considerably during the last decades in most European countries,⁴ the effects of ambient air pollution remain to be of great concern in terms of human health.^{6,31,32}

2.1 Origin and characteristics of air pollution

Air pollutants originate from natural (geological dust, forest fires, volcanoes, methane) or anthropogenic sources (fossil fuel burning, refineries/power plants, agriculture, industry, transport). Primary pollutants are emitted directly in the air e.g. ash from volcano eruptions, volatile organic compounds, nitrogen oxides (NO_x), sulfur oxides (SO_x) from factories and toxic metals from metal producing and using factories. Secondary pollutants are formed due to chemical reactions involving UV-light, ozone (O_3), gaseous pollutants [e.g. SO_2 , NO_x , ammonia (NH_3)], and organic compounds. Taken together, air pollution consists of both gaseous and particulate matter (PM) pollutants.^{4,33}

PM is a heterogenic mixture of particles suspended in a gas or in liquid droplets that may vary in size, mass, and chemical composition. The majority of PM in ambient air are respirable particles with an aerodynamic diameter lower than 10 μm . The particle fraction between 2.5 and 10 μm are defined as the 'coarse fraction' ($\text{PM}_{10-2.5}$), less than 2.5 μm as 'fine particles' ($\text{PM}_{2.5}$), and less than 0.1 μm as 'ultrafine particles' ($\text{PM}_{0.1}$ or UFPs) (**Figure 2**). Black carbon (BC) is one of the constituents of $\text{PM}_{2.5}$ and is a product of incomplete combustion of fossil fuels, biofuel, and biomass. Concentrations of BC increase sharply in the vicinity of traffic sources and when inhaled, can cause human morbidity and premature mortality.³⁴ Indoor activities such as cooking, tobacco smoke, candles, and stoves contribute significantly to indoor concentrations of $\text{PM}_{2.5}$.³⁵ The PM composition of ambient air is complex and naturally controlled by meteorological conditions.

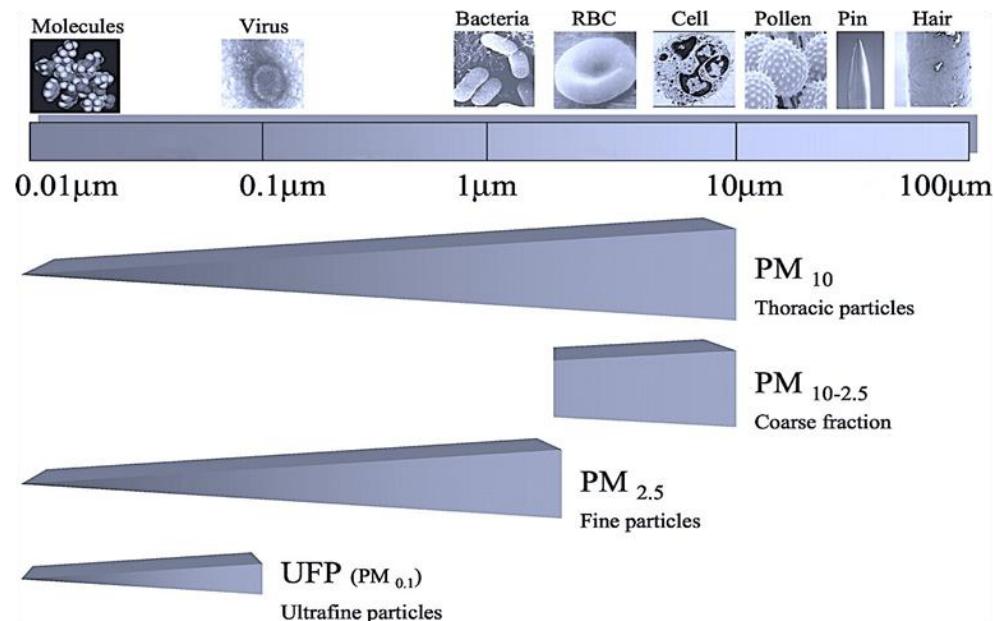


Figure 2. Different fractions of particulate matter. PM₁₀ consist mainly of fugitive/geological dust from roads and industries, biologic material (sea salt, endotoxins, pollen, spores) but encloses also smaller components. PM_{2.5} arises mainly from combustion of fossil fuels used in power generation, industry, and diesel engines and can consists of toxic metals, elemental carbon, black carbon, and reaction products with inorganic compounds (NH₃, NO₂, and SO₂). Moreover, particles are constantly changing and interacting with semi-volatile/volatile compounds such as polycyclic aromatic hydrocarbons (PAHs) that can absorb reactive metals. UFPs are formed during gas-to-particle conversion or during incomplete fuel combustion of diesel engines and contain condensates with toxic metals. Although the composition of UFPs is generally compatible with PM_{2.5}, the classification is based on their small size, large surface area, and high alveolar deposition. *Reprinted from Robert D. Brook et al. Circulation. 2004;109:2655-2671*³⁶

Deposition of inhaled PM in the airways is determined by the particle size, the anatomy of the respiratory tract, and respiration rate. Coarse particles are deposited in thoracic airways and mostly eliminated by mucociliairy clearance ending up in the gastrointestinal tract. PM_{2.5} goes deeper in the bronchial/bronchiolar parts of the lung, while UFPs penetrate into the deep lung (alveoli) with the potential to translocate to the systemic circulation.³⁷ At the epithelium, particles can be cleared by dissolution or phagocytosis by alveolar macrophages.³⁸ Systemic translocation of UFPs has been demonstrated in animal experiments³⁹ and humans.⁴⁰ Taken together, the deposited fraction of particles increases with decreasing size and deeper respiration, e.g. physical exercise.

2.2 Particulate matter legislation

In 2005, the WHO updated the Air Quality Guidelines for PM, NO₂, SO₂, and O₃. The current guidelines, which are not legally imposed, are based on a review of evidence on the relationship between air quality and adverse human health effects. The average annual limit value for PM₁₀ and PM_{2.5} is set respectively at 20 µg/m³ and 10 µg/m³ for the WHO and at 40 µg/m³ and 25 µg/m³ by the European Union (EU) (**Table 1**). The EU has set less strict air quality standards since economic feasibility is taken into account next to health reasons. The last years, successful efforts have been made in the EU to reach the WHO guidelines. For Belgium, in contrast to the year 2012 and 2013, the annual averages of PM₁₀ and PM_{2.5} in 2014 were lower or similar compared with the WHO guidelines.⁴¹ However, the number of days on which the daily average PM₁₀ concentration was higher than 50 µg/m³ is still above three days as tolerated by the WHO recommendation (**Table 1**).

Table 1. Guidelines concerning particulate matter set by the EU and the WHO

Pollutant	2014†			European guidelines	WHO guidelines
	Min.	Mean	Max.		
PM ₁₀					
Annual average (µg/m ³)	5	15	26	40	20
Number of days >50 µg/m ³	0	5	21	Max. 35 days	Max. 3 days
PM _{2.5}					
Annual average (µg/m ³)	6	10	19	25	10

† Values from the annual report air quality in Belgium 2014 provided by IRCEL.⁴¹

2.3 Health impact of particulate matter

Due to substantial spatial and temporal variation, the effects of environmental air pollution are difficult to describe on the individual level, but have a great impact on the population level. Exposure to pollutants such as PM, NO₂, and O₃ has been associated with increases in mortality and hospital admissions due to respiratory and cardiovascular diseases. These effects have been found in short-term studies, which relate day-to-day variations in air pollution and health, and long-term studies, which have followed cohorts of exposed individuals over time.⁴² It is estimated that PM_{2.5} exposure in 2010 reduced life expectancy by 6.3 months on average in the EU countries and by 9.9 months in the Belgian

population.⁴³ In the EU, a total of 403,000 premature deaths were attributable to PM_{2.5} exposure in 2012, of which around 9,300 in Belgium (total population in 2012 approximately 11 million civilians).⁴⁴ Air pollution as a whole, as well as PM as a separate component of air pollution aerosols, have been classified as carcinogenic to humans (IARC Group 1).⁴⁵

2.4 Health impact of particulate matter in susceptible groups

PM-related health effects are more prominent in susceptible groups including elderly, infants, and people with pre-existing heart and lung disease. Inhalation of PM causes irritation or damage of lung tissue. Exposure to PM may lead to pulmonary inflammation and trigger respiratory diseases including asthma, chronic bronchitis, obstruction of airways, and reduced lung function.^{46,47} Atheromatous plaques, increased viscosity of blood, and thrombosis are cardiovascular effects associated with long- and short-term exposure to PM_{2.5}.⁴⁸

Fetuses and newborns are particularly susceptible to PM due to their physiologic immaturity and exposure during critical developmental periods (i.e. higher rates of cell proliferation or changing metabolic capabilities).⁹ Short-term exposure to PM₁₀ is associated with increased risk of infant mortality.⁴⁹ Exposure to ambient PM_{2.5} pollution during pregnancy has been found to be significantly associated with increased risk of low birth weight at term in mother-child cohorts of 12 European countries³¹ and preterm birth (20-36 weeks of gestation) in a very large cohort of singleton pregnancies from three states in the USA.⁵⁰ Air pollution may affect maternal respiration or general health and, in turn, impair the fetoplacental circulation, and nutrient and oxygen transport to the fetus. Fetuses adapt their energy metabolism to cope with alterations in the maternal environment. In doing so, this 'metabolic reprogramming' may be at the origin of adverse pregnancy outcomes including low birth weight and increased susceptibility for the risk of developing coronary heart disease,⁵¹ metabolic syndrome,⁵² chronic lung disease,⁵³ and osteoporosis⁵⁴ in later life (Barker hypothesis).

3. BIOMARKERS: EPIGENETICS AND MITOCHONDRIA

Biomarkers, or biological markers, generally refer to quantifiable characteristics or indicators of some biological state or condition in a certain tissue in the human body. In the field of health risk assessment, biomarkers of effect are very useful to indicate if exposure to an environmental compound leads to quantifiable changes that may result in a health effect. The fast-growing 'omics' technologies (epigenomics, transcriptomics, proteomics, metabolomics) opened opportunities to discover molecular changes and markers of environmental exposure beyond the genome (genomics). Recently, there is a new area of research that stretches beyond the nuclear genome called 'mitochondriomics'.⁵⁵ It is well recognized that the (patho)physiological role of mitochondria widely exceeds that of solely being the biochemical power plant of the cells. Mitochondriomics is dedicated to discover whether mitochondria are novel biosensors or mediators of environmental effects.

3.1 Epigenetics

Although most of the cells in the human body contain the same DNA, gene expression, function, and phenotype vary dramatically among different tissues. This implies that, apart from genetic programming, the phenotype is regulated by another phenomenon. The developmental biologist Conrad Waddington was the first to define 'epigenetics' in the 1940s as "...*the interactions between genes and their products which bring phenotype into being*".⁵⁶ Epigenetics, literally meaning 'above genetics', is defined as the study of changes in phenotype or gene expression caused by mechanisms other than changes in the underlying DNA sequence, either by histone modification, DNA methylation, or non-coding RNA. One often compares epigenetics with an orchestral score, in which DNAs are the notes on the score, and the marks added to the score are crucial to interpret and control the dynamics of a symphonic performance. The score is not modified, only the way the score is executed. Changes in the epigenetic landscape can occur throughout an individual's lifetime in response to environmental factors, stress, disease, nutrition, hormones, and aging.⁵⁷

The most commonly characterized epigenetic mark is DNA methylation which involves the addition of a methyl group to the carbon-5 position of cytosine residues of the dinucleotide CpG. Changes in DNA methylation patterns can be observed at the global level (entire genome), which usually is associated with genomic instability, or at specific genes, which is more associated with gene regulation. Methylation of gene promoters is commonly linked to silencing whereas methylation in the gene body does not block transcription or silence alternative transcripts in a tissue-specific manner.⁵⁸ It is assumed that chemical modification of cytosines in CpG islands, which are small regions of DNA in the promoter region of genes, remodels the DNA making it inaccessible to the transcriptional and regulatory machinery. Epigenetic marks including DNA methylation undergo critical modification during the early *in utero* life.⁵⁹ After fertilization and prior to implantation, DNA methylation patterns are largely erased but are re-established by *de novo* DNA methyltransferases (DNMTs) in the blastocyst stage (**Figure 3**).⁶⁰

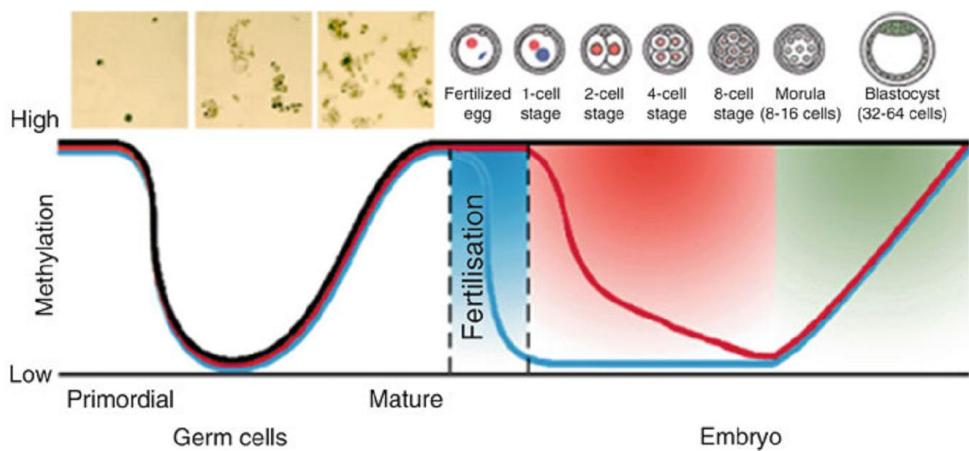


Figure 3. Methylation reprogramming during mouse development. Mammalian development is characterized by bimodal DNA methylation reprogramming that occurs during germ cell development and pre-implantation. The parental epigenome is largely erased during gametogenesis, followed by epigenetic reprogramming as primordial germ cells differentiate into oocyte and sperm. After fertilization, the non-imprinted gene regions in the parental genomes are triggered to undergo an asymmetrical round of epigenetic reprogramming that restores totipotency. Black line: Methylated imprinted genes; Red: Maternal genome; Blue: Paternal genome. Reprinted from Santos and Dean, Reproduction 2004;127:643-51.⁶¹

The dramatic waves of epigenetic reprogramming likely make early embryonic development a critical period during which nutritional, environmental, and metabolic factors affect the developmental establishment of epigenetic regulation.⁶² Animal studies showed that certain transient environmental influences during *in utero* life could produce persistent changes in epigenetic marks that have life-long consequences.⁶³⁻⁶⁵ For example, when F0 mice were conditioned to an odor fear for acetaminophen before conception, they subsequently conceived F1 and F2 generations that had an increased behavioral sensitivity to the F0-conditioned odor, but not to other odors. The transgenerational epigenetic effects at the DNA methylation level were inherited via parental gametes.⁶⁴ In humans, data from the Dutch Hunger Winter (1944-45), when food rations dropped below 1,000 kcal/day for six months, indicate that prenatal environmental conditions can cause epigenetic changes that persist throughout life, increasing the risk of obesity among offspring of mothers exposed to the famine during the first and second trimester.⁶⁶ Evidence from animal and human studies in adults indicate that PM air pollution affects global and gene-specific DNA methylation.⁶⁷⁻⁷⁰ A number of studies described DNA methylation patterns in placental tissue⁷¹⁻⁷³ providing an attractive mechanism linking PM air pollution with altered fetal development that may lead to health consequences in adulthood.⁶²

3.2 Mitochondria

The inter-genomic crosstalk between the nucleus and mitochondria is complex. Growing evidence suggests that the epigenetic landscape of the nuclear genome is tightly related to mitochondrial function.^{74,75} While it is intuitive that mitochondria play an important role in disease processes,⁷⁶ given that every cell of the body is dependent on energy metabolism, it is less clear how environmental exposures impact mitochondrial mechanisms that may lead to increased risk of disease.⁵⁵ Mitochondria are intracellular organelles that are essential for cellular energy provision through the production of adenosine-5'-triphosphate (ATP) via oxidative phosphorylation. They also play a critical role in calcium homeostasis, oxidant signaling, apoptosis, regulation of cell proliferation, and metabolism. Each cell contains approximately 200 to 2,000 mitochondria, each carrying 2-10 copies of mitochondrial DNA (mtDNA) that are

bound to protein structures.⁷⁷ The human mtDNA is a double stranded, circular molecule of 16.6 kb and contains 37 genes, encoding 13 proteins that are essential for oxidative phosphorylation and ATP production. Intra-mitochondrial synthesis of these proteins requires mtDNA-encoded 12S (*MT-RNR1*) and 16S rRNAs and 22 tRNAs for their translation.^{78,79} (**Figure 4**).

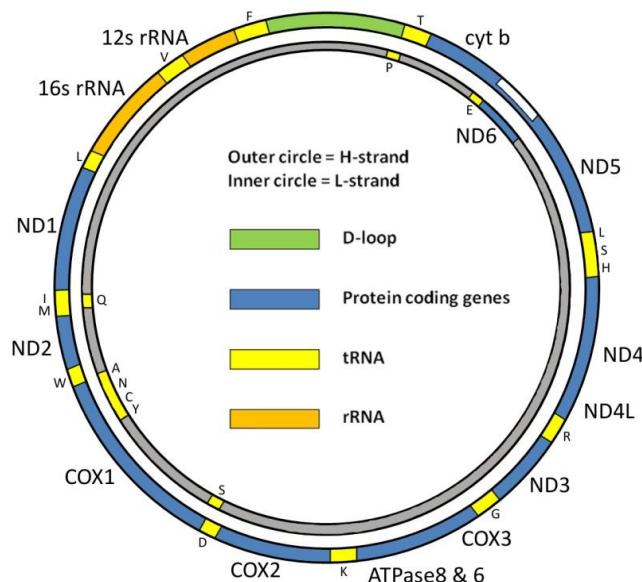


Figure 4. Structure of the human mitochondrial genome (16,569 bp). The mitochondrial genome consists of the Heavy-strand (outer circle) and the Leading-strand (inner circle). The majority of genes lie on the H-strand. Yellow boxes represent the genes coding for 22 tRNAs and the associated single letter codon refers to the amino acid. The two rRNAs (12S and 16S) and 13 polypeptides (ND, COX, ATPase and Cyt b) are depicted by orange and blue boxes respectively. The *D-loop* (green box) is a DNA triple helix with two copies overlapping the H-strand. It contains the origin of replication and place where promoters initiate transcription of the H-strand. Abbreviations: *NADH dehydrogenase (ND)*; *Cytochrome c oxidase (COX)*; *F₁F₀-ATP synthase (ATPase)*; *Cytochrome b (Cyt b)*; *Displacement-loop (D-loop)*.

Not all proteins present in the mitochondria are encoded by the mtDNA. Mitochondria are genetically semiautonomous in that they rely heavily on the tight interplay of nuclear and mitochondrial gene products to maintain mitochondrial function. The vast majority of proteins and enzymes that reside in the mitochondria are nuclear gene products that are synthesized on cytosolic

ribosomes and imported into the mitochondria by multiple complex mechanisms.⁸⁰ Nuclear genes specify most of the structural catalytic components of the five inner membrane complexes of the oxidative phosphorylation system.⁸¹ However, mtDNA encodes a small, but essential, subset of the oxidative phosphorylation machinery as well as the RNA components of the mitochondrial translation system required for its expression in the mitochondrial matrix.

The estimated mutation rate of mtDNA is 5-10 times higher compared to nuclear DNA.⁸² Partly, the mutation rate depends on oxidative stress generated by intra- and extracellular factors. mtDNA is particularly susceptible to reactive oxygen species (ROS) generated by the respiratory chain due to its proximity. The major difference between human nuclear DNA (nDNA) and mtDNA is that the latter lacks protective histones, chromatin structure, and introns. Additionally, the mtDNA repair mechanisms work less efficiently than that of nDNA.⁸³ Mutations in the mtDNA occur more frequently in NADH dehydrogenase subunit-4 gene (*ND-4*) and in the *D-loop* region (**Figure 4**). The *D-loop* is a non-coding area that controls replication and transcription of mtDNA. It contains the site of origin of replication and the place where promoters initiate transcription. Consequently, mutations in this region are one of the causes that the rate of mtDNA replication is affected.⁷⁹ On the other hand, defects in mtDNA replication due to mutations in nuclear genes such as the DNA polymerase γ A gene (*POLGA*), which is a mtDNA polymerase responsible for mtDNA replication, repair, and copy number are linked with mitochondrial diseases.⁸⁴ This gene is regulated by epigenetic mechanisms through DNA methylation of CpG islands within exon 2 leading to reduction of its expression. Additionally, steady-state levels of *POLGA* expression and mtDNA copy number are linearly correlated.⁸⁵

Alterations in mtDNA content (total amount of mtDNA copies) is an established marker of mitochondrial damage and function⁸⁶ and has been identified as an etiological determinant in a variety of human diseases.⁸⁷ Decreased mtDNA content of white blood cells has been shown in type-2 diabetes,⁸⁸⁻⁹⁰ breast cancer,^{91,92} multiple sclerosis,⁹³ renal cell carcinoma,⁹⁴ and low birth weight.⁹⁰ Alternatively, increases in mtDNA content have been associated with diseases such as lung cancer,⁹⁵ pancreatic cancer,⁹⁶ and intrauterine growth restriction in human placenta.^{97,98} In addition, Lin and Beal⁹⁹ summarized the role of

mitochondrial function in the pathogenesis of neurodegenerative diseases. Some mitochondrial disorders can be passed on from mother to child since mtDNA is only transmitted through the female germ line (maternally inherited).

In the last years, it has been shown that the mitochondrial genome can undergo epigenetic modifications. The rationale of studying mtDNA methylation is underscored by its role in the etiology of a variety of human diseases¹⁰⁰⁻¹⁰³ and aging.¹⁰⁴ Growing evidence suggests mitochondrial epigenetics as a novel mechanism to understand the pathophysiology of diseases with a mitochondrial dysfunction involvement.^{102,105} The biological mechanisms how mitochondrial function is linked to disease outcome is not completely understood, but mutations and epigenetic modifications in nuclear genes and mitochondria may play an important role. Mitochondria are particularly sensitive to environmental stressors including PM air pollution highlighting the role of mtDNA as an important environmental biosensor.¹⁰⁶

4. AIMS OF THE STUDY

Studies on health effects of air pollution exposure during the most vulnerable stages in life, the *in utero* period, are still scarce. While the specific mechanisms behind fetal programming are still poorly understood, a large body of evidence suggests the human placenta as a main target organ, since it serves as the gatekeeper between mother and fetus. During its nine month lifespan, the human placenta captures morphological, functional, genetic, and epigenetic information, representing thus a molecular 'footprint' that can reveal the impact of *in utero* exposures on the fetus. Unraveling the complex interplay between environmental exposures and different biological factors will increase our understanding of DOHaD and promote preventive health care strategies that contribute to a healthier living environment for pregnant mothers and their children.

The specific objectives of this doctoral dissertation were:

1. To establish a population-based prospective birth cohort 'ENVIRONAGE'. We designed the ENVIRONAGE birth cohort (ENVIronmental influence ON early AGEing) to explore new dimensions in the present understanding of human aging and its environmental influence. In **chapter 2**, we summarized the characteristics of the cohort and explained the rationale of the strong focus on the molecular mechanisms to bridge the gap between exposure and health effects occurring in early and later life.
2. To evaluate the variation of gene-specific methylation in placenta tissue. In this methodological chapter, we examined whether specific age- and mitochondrial-related genes show considerable variation within placental tissue (**chapter 3**).
3. To replicate (molecular) effects of maternal tobacco smoke exposure during pregnancy. Since tobacco smoke exposure is a personalized form of air pollution, we investigated in **chapter 4** whether the effects of maternal tobacco smoke exposure, that are already described in literature, are observable in our cohort.

4. To investigate the association between gestational PM exposure and placental mitochondria and to explore the role of fetal thyroid hormones. For this purpose, we first investigated the association between gestational PM exposure and placental global DNA methylation (**chapter 3**), mtDNA content (**chapter 6.1**), and mtDNA methylation (**chapter 6.2**). Afterwards, the role of fetal thyroid hormones on mtDNA content and mtDNA methylation was examined in **chapter 7.1**, and in **chapter 7.2** whether thyroid hormones were associated with gestational PM exposure.
5. To investigate whether PM exposure during pregnancy is associated with genotoxic changes in cord blood. We investigated the carcinogenic properties of PM by examining the association between gestational PM exposure and expression of genes involved in micronuclei formation, a valid biomarker for genotoxicity (**chapter 8**).

We performed measurements on samples that were available at a certain time point, and therefore, we used different sample sizes in each chapter of this doctoral dissertation. The sequence of the chapters does not follow the chronological sequence in which these measurements were performed (**Figure 5**).

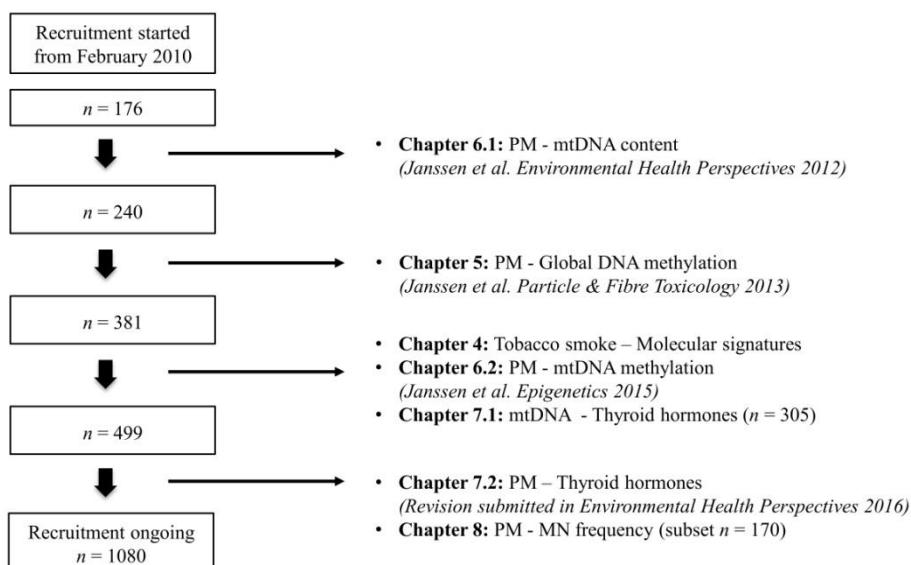


Figure 5. Flowchart of the sample sizes for each chapter.

CHAPTER 2

THE ENVIRONAGE BIRTH COHORT: A COHORT PROFILE

Bram G. Janssen,¹ Narjes Madlhoum,¹ Wilfried Gyselaers,^{2,3} Esmée Bijnens,¹ Diana B. Clemente,¹ Bianca Cox,¹ Janneke Hogervorst,¹ Leen Luyten,¹ Dries S. Martens,¹ Martien Peusens,¹ Michelle Plusquin,¹ Eline Provost,¹ Harry A. Roels,^{1,4} Nelly D. Saenen,¹ Maria Tsamou,¹ Annette Vriens,¹ Ellen Winckelmans,¹ Karen Vrijens,¹ Tim S. Nawrot^{1,5}

¹ Centre for Environmental Sciences, Hasselt University, Diepenbeek, Belgium

² Department of Obstetrics, East-Limburg Hospital, Genk, Belgium

³ Biomedical Research Institute, Hasselt University, Diepenbeek, Belgium

⁴ Louvain Centre for Toxicology and Applied Pharmacology (LTAP), Université catholique de Louvain, Brussels, Belgium

⁵ Department of Public Health & Primary Care, Leuven University, Leuven, Belgium

WHY WAS THE COHORT SET UP?

Although universal and unavoidable, aging does not occur in a uniform way. Aging is a complex phenotype responsive to a plethora of environmental stressors from early life onwards. Within the ongoing population-based prospective birth cohort study 'ENVIRONAGE' (ENVIROnmental influence ON early AGEing), we explore new dimensions in the current understanding of human aging and its interaction with the environment.

Age-related diseases often find their origin in risk factors operative in early life.^{107,108} The potential risk of developing cardiovascular,⁵¹ metabolic,⁵² respiratory,⁵³ and even neurological pathologies^{109,110} later in life increases in neonates with adverse gestational outcomes such as low birth weight and preterm birth. From conception onwards (and even preconceptionally), humans are exposed to a variety of environmental hazards that could lead to physiological and metabolic adaptations.⁵⁹ Therefore, identifying the effects of environmental exposures, even subtle changes, as from conception to later in childhood and adolescence, is a growing area of research interest with major public health implications. Relevant early life exposures include maternal nutrition, medication, alcohol consumption, tobacco smoke, physical activity, noise, pesticides, radiation, ambient air pollution as well as hormonal and genetic determinants. One of the main public health concerns in the ENVIRONAGE birth cohort study is the life-long health effect of ambient air pollution. In large areas of the world, fine particle air pollution is an omnipresent environmental risk factor causing major health problems in adult populations.^{5,42} Studies on the health effects of air pollution exposure during the most vulnerable stages in life, the *in utero* period, are still scarce.³¹ The ENVIRONAGE birth cohort study is designed to carry out prospective epidemiological follow-up as from the newborn stage to obtain evidence on the interactions of environmental exposures with processes of aging including mitochondrial function, telomere length, epigenetic mechanisms, and DNA repair as the core axis of aging.

Specific objectives include†:

- 1/ to study at birth the variability of telomere length and mitochondrial DNA content (placental tissue and cord blood) in association with environmental exposures *in utero* and DNA repair capacity;
- 2/ to explore the associations between mitochondrial function, epigenetics (total methylation and DNA gene-specific methylation), and telomere length in cord blood and placental tissue of newborns with health outcomes such as birth weight and cardiovascular phenotypes including arterial stiffness;
- 3/ to identify early life gene expression patterns (including neurobehavioral genes) involved in the aging processes (aging phenotypes in infants including cardiovascular phenotypes) and its interaction with environmental exposures.

Mother-child pairs are being recruited from February 2010 onwards according to the principles outlined in the Helsinki Declaration for investigation of human subjects and following procedures approved by the Ethical Committee of Hasselt University and the East-Limburg Hospital. The birth cohort is supported by the European Research Council (ERC-2012-StG.310898), and by funds of the Flemish Scientific Research council (FWO, N1516112 / G.0.873.11.N.10).

WHO IS IN THE COHORT?

Mother-newborn pairs are recruited when they arrive for delivery at the East-Limburg Hospital in Genk (Belgium) and provide written informed consent to collect biological specimens as well as medical and lifestyle data. In the post-delivery ward, participating mothers complete an extensive study questionnaire. The catchment area of the hospital includes the province of Limburg (Flanders, Belgium) and combines urban, suburban, and rural areas with population densities of the municipalities ranging from 82 to 743 inhabitants/km² (**Figure 1**). Each year, there are around 2,000 live singleton births and 50 live twin births in the hospital. While recruitment is still going on, we enrolled during weekends between February 2010 and November 2015 a study population of 1,080 singleton and 40 twin births (as a subset), making this the largest prospective birth cohort in Belgium (**Figure 2**). Mothers without planned caesarian section and able to fill out a Dutch language questionnaire are eligible

† These are the overall objectives of the ENVIRO/NAGE birth cohort. The specific objectives of this doctoral dissertation are given at page 17-18.

for the cohort. Currently, the participation rate of eligible mothers is 61% and enrollment is spread equally over all seasons of the year. Midwives record the reason of nonparticipation, which are in descending importance: omission to ask for participation, communication problems, or complications during labor. At the time of recruitment, we ask whether mothers are willing to participate in a follow-up study at the age of four to six of their newborn, which resulted in a preliminary response rate of 75%. The first stage of the follow-up started in January 2015 with an actual response rate of 70%.

Demographic and lifestyle characteristics of the ENVIRONAGE birth cohort participants (**Table 1**) are similar to those of the Flemish birth register of all births between 2002-2011 in the Northern part of Belgium as to maternal age, education, parity, child's sex, ethnicity, and birth weight (**Table 1**).¹¹¹ Therefore, our mother-child cohort is representative for the gestational segment of the population in Flanders.

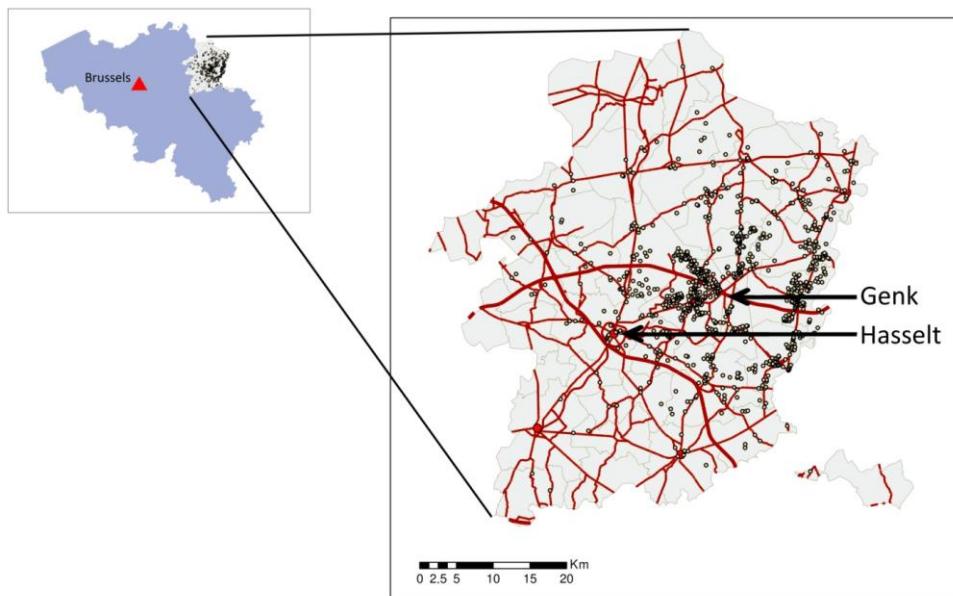


Figure 1. The catchment area of the current 1,080 participants in the ENVIRONAGE birth cohort study is located in the northeast of Belgium (the province of Limburg, Flanders). Main roads are depicted in red and dots represent the residences of the mothers. Although the catchment area is spread throughout the entire province, a great number of mothers are living close to the East-Limburg hospital in Genk.

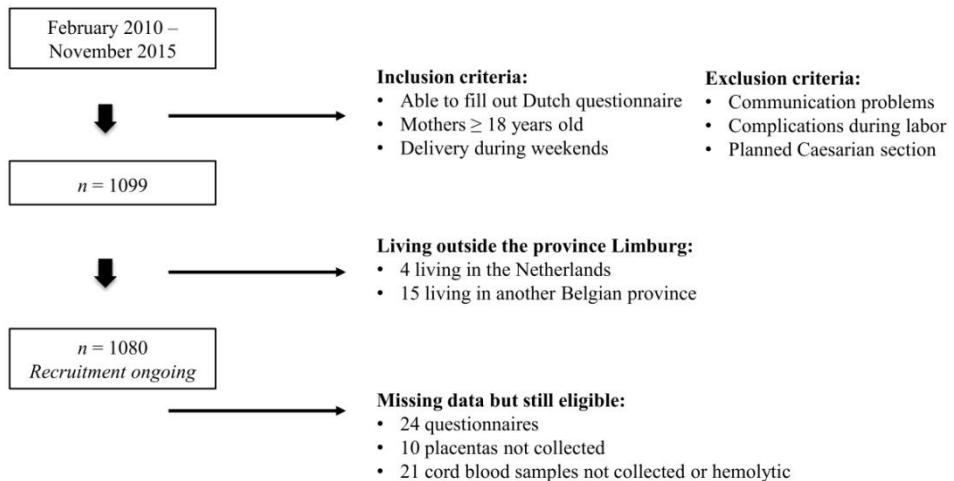


Figure 2. Flowchart of the ENVIRO/NAGE birth cohort.

Table 1. Characteristics of the ENVIRONAGE birth cohort participants compared to the characteristics of all births in Flanders (Northern part of Belgium) from 2002-2011

Characteristic	ENVIRONAGE (<i>n</i> = 1,080)			Flanders (<i>n</i> = 606,877) [§]	
	Missing	Values	Values		
Mothers					
Age, y		29.4 [23-35]	29.5 [23.5-35.8]		
< 25		160 (14.8)	98,419 (16.2)		
25-35		817 (75.6)	428,781 (70.7)		
> 35		103 (9.6)	79,677 (13.1)		
Pre-pregnancy BMI, kg/m ²	2	24.3 [19.5-30.7]	N/A		
Maternal education [†]	26				
Low		140 (13.3)	58,743 (13.1)		
Middle		385 (36.5)	183,410 (40.8)		
High		529 (50.2)	5,968 (46.1)		
Self-reported smoking status	10		N/A		
Never-smoker		685 (64.0)			
Cessation before pregnancy		244 (22.8)			
Smoker during pregnancy		141 (13.2)			
Indoor passive smoking	30	92 (8.8)	N/A		
Alcohol consumption	30		N/A		
None		890 (84.7)			
Occasionally		160 (15.3)			
Parity					
1		583 (54.0)	284,770 (46.9)		
2		367 (34.0)	108,134 (34.7)		
≥ 3		130 (12.0)	114,347 (18.4)		
Caesarean section		44 (4.1)	N/A		
Epidural		861 (79.7)	N/A		
Newborns					
Sex					
Female		528 (48.9)	295,257 (48.6)		
Ethnicity [‡]	5				
European-Caucasian		929 (86.4)	384,522 (87.7)		
Gestational age, w		39.1 [37-41]			
Season of delivery					
Winter (Dec - Mar)		249 (23.1)	147,471 (24.3)		
Spring (Mar - Jun)		283 (26.2)	152,326 (25.1)		
Summer (Jun - Sep)		251 (23.2)	157,788 (26.0)		
Autumn (Sep - Dec)		297 (27.5)	149,292 (24.6)		
Apgar score 5 min after birth					
7 or 8		595 (55.1)			
9		398 (36.8)			
10		87 (8.1)			
Birth weight, g		3,384 [2,795-4,000]	3,360 [2,740-3,965]		
Birth length, cm	10	50.2 [47.5-53.0]			
Head circumference, cm		34.1 [32.0-36.0]			

Values are numbers (percentages) or means [10th-90th percentiles].[†] Maternal education was categorized as 'low' (no diploma or primary school), 'middle' (high school) or 'high' (college or university degree).[‡] Ethnicity was defined as European-Caucasian when two or more grandparents of the newborn were European, or non-European when at least three grandparents were of non-European origin.[§] Cox et al. 2013.¹¹¹

HOW OFTEN ARE COHORT MEMBERS BEING FOLLOWED-UP?

The ENVIRO/NAGE birth cohort is a longitudinal study, starting with recruitment at birth and follow-up at the age of four to six and at preadolescent age (**Figure 3**).

Pregnancy period

We have access to all medical records during and after pregnancy including anthropometric and fetal ultrasound data in addition to lifestyle factors derived from questionnaires. Furthermore, daily concentrations of air pollutants are calculated to which the mothers have been exposed before and during the course of their pregnancy (*see details below*).

Birth

Midwives record all anthropometric data in the medical files and these are double checked through a questionnaire the mothers receive after birth. During a postpartum exam three days after delivery, maternal and neonatal blood pressure is measured and a cognitive development assessment is obtained, using the Neonatal Behavior Assessment Scale (NBAS).

Follow-up 1 (at age four to six)

Mother-child pairs are re-invited by phone (or email if phone number is missing) to participate in the ongoing follow-up examination of the ENVIRO/NAGE study at age four to six. The first follow-up wave commenced in January 2015 and is yet to be finalized. The examination includes a Strengths and Difficulties Questionnaire (SDQ) and another questionnaire to retrieve general information and details on nutrition, life-style habits and medical history since birth. We also record anthropometric and clinical measurements (detailed in **Table 2**) and collect biological specimens. This is conducted in a well-equipped, child-friendly research room located at Hasselt University.

Follow-up 2 (at age nine to ten)

A second follow-up examination will be scheduled at the age of 9-10 years. At that time, we will again assess the cardiovascular and neurological characteristics similar to the exams performed at the first follow-up.

Furthermore, both mother and child will be asked for the collection of biological specimens (blood, saliva) to perform molecular analyses. This second follow-up will allow us to study gradual changes in cardiovascular and molecular parameters.

In an attempt to increase general awareness of the importance of the local birth cohort with an extensive follow-up, we have enlisted social media to increase knowledge on the ENVIRONAGE birth cohort in the population. As such, we have created a website [www.environage.be] and a Facebook page on which we provide details of our study as well as highlights of relevant recent literature in layman's terms. We also invite radio and television on a regular basis for communicating updates on the progress of our study, and hand-outs about our birth cohort are distributed as well.

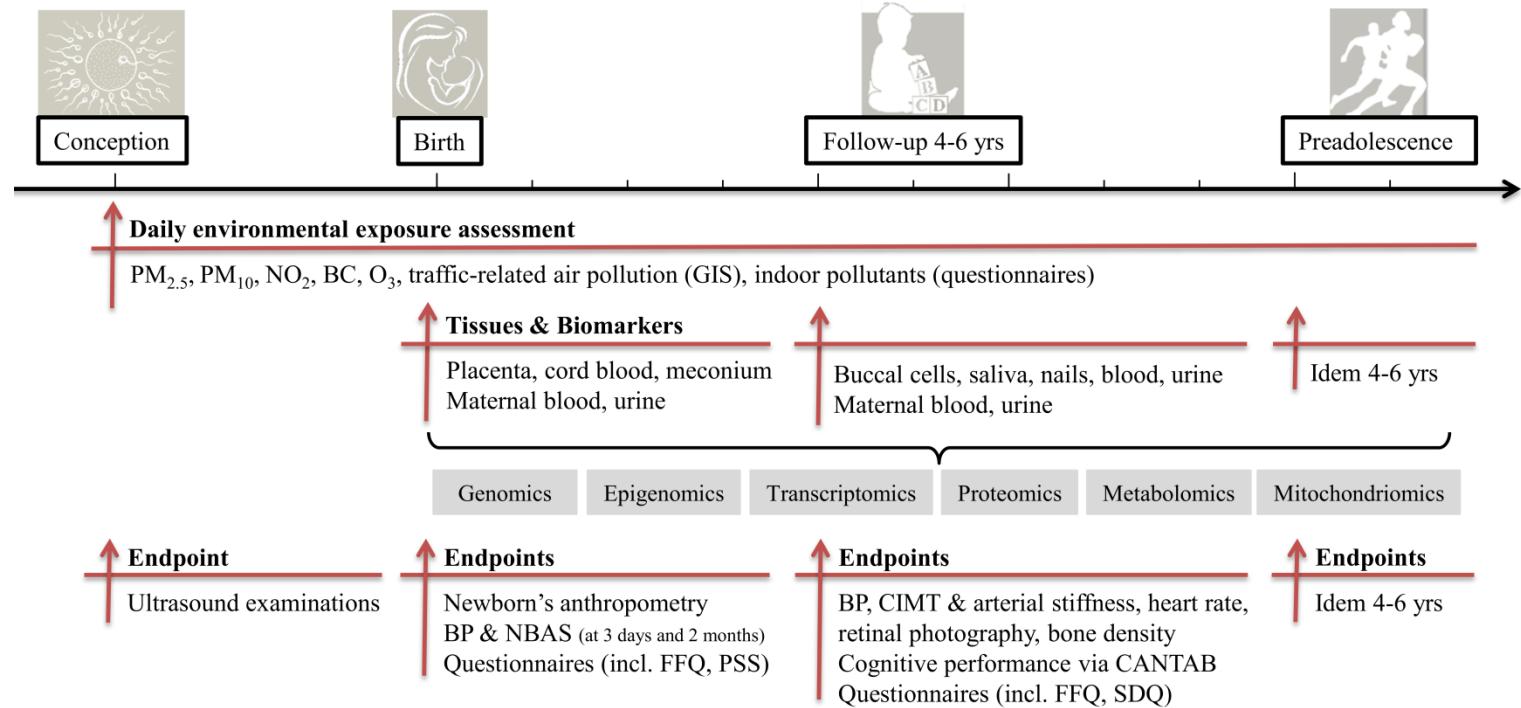


Figure 3. Study scheme of the ENVIRONAGE birth cohort. Recruitment of the mother-child pairs started in February 2010 and is still ongoing. ENVIRONAGE is a longitudinal study, starting with recruitment at birth and follow-up at the age of four to six, and the preadolescent age. Abbreviations: PM: Particulate Matter; NO₂: Nitrogen dioxide; BC: Black Carbon; O₃: Ozone; GIS: Geographical Information System; BP: Blood Pressure; NBAS: Neonatal Behavior Assessment Scale; FFQ: Food Frequency Questionnaire; PSS: Perceived Stress Scale; CIMT: Carotid Intima-Media Thickness; CANTAB: Cambridge Neuropsychological Test Automated Battery; SDQ: Strengths and Difficulties Questionnaire.

WHAT HAS BEEN MEASURED?

The outline of the ENVIRONAGE birth cohort study is summarized in **Table 2** (and **Table 3**). It includes questionnaires, bio-banked samples for molecular measurements, whole blood samples for biochemical and metal analyses, and maternal history of exposure to air pollution. The first follow-up includes history and lifestyle updates of the past years, micro- and macro-vascular phenotypes, and exposure to environmental pollutants. Because this is an ongoing cohort, we indicated in the table the different items as 'already performed, '; 'planned to be performed, ()', and 'measured in a subgroup, S'.

Personal characteristic questionnaires

Extensive questionnaires are administered at birth and at each follow-up examination to collect information on anthropometry of the mother and father, socio-demographic status, occupation, smoking status, body mass index (BMI), diet, physical activity, medication, health status, use of assisted reproductive technologies, and various lifestyle factors. We asked the mothers whether they consumed alcohol during pregnancy. Former-smokers were defined as those who quit smoking at the beginning of pregnancy, while smokers continued during pregnancy. Based on the native country of the newborn's grandparents, we classified the neonate's ethnicity as European-Caucasian when two or more grandparents were European, or non-European when at least three grandparents were of non-European origin. Maternal education was coded as 'low' (no diploma or primary school), 'middle' (high school) or 'high' (college or university degree). Maternal stress is assessed using the Perceived Stress Scale (PSS), the most widely adopted psychological instrument for measuring the perception of stress.¹¹² At follow-up, the child's behavior is assessed through a Strengths and Difficulties Questionnaire (SDQ) filled out by the mother. Environmental household exposures include indoor smoking, the way of heating, ventilation, usage of cleaning products, and number of (house) pets.

Dietary acrylamide intake

Maternal dietary exposure to acrylamide-containing foods is assessed by questions on several potato-based (crisps, chips, baked potatoes) and cereal-based (cookies, breads, pastry, etc.) products, and coffee. The amounts of these

foods consumed are multiplied by acrylamide levels found in Flemish and Dutch foodstuffs^{113,114} and summed to obtain total dietary acrylamide intake.

Dietary antioxidant intake

Intake of antioxidants is registered through detailed semi-quantitative food frequency questionnaires (FFQ), asking the mothers about their food pattern during gestation and that of their child during the last three months before the first follow-up examination. Antioxidant intake is estimated on the basis of a modified validated FFQ¹¹⁵ comprising 54 items that contribute mostly to the antioxidant intake (fruits, vegetables, cereals, etc). Antioxidant content of the food items is calculated using the USDA Database for the Oxygen Radical Absorbance Capacity (ORAC) of Selected Foods, release 2. Furthermore, since adherence to a Mediterranean diet has been described to protect against obesity^{116,117} and has been shown to reduce overall mortality in a European cohort,¹¹⁸ we also included a validated 14-item Mediterranean diet assessment tool¹¹⁹ including questions on the consumption of olive oil, fish, preference for red or white meat, fruit and vegetable intake.

Exposures

Ambient air pollution

For the exposure assessment at the mother's residential address, we use an advanced spatial temporal interpolation method that uses pollution data collected from a governmental stationary monitoring network ($n = 34$) and land cover data obtained from satellite images (CORINE land cover data set)¹²⁰ in combination with a dispersion model.^{121,122} This model chain provides daily air pollution concentrations using data from the Belgian telemetric air quality network, and point and line sources which is then interpolated in a high resolution receptor grid (25 x 25 m). For Flanders, the validation statistics of the interpolation tool gave a spatiotemporal explained variance of more than 0.8.¹²³ With this method, we calculate the daily concentrations of air pollutants during the entire pregnancy, specific exposure windows of interest during gestation (e.g. separate trimesters, last month, etc.), and even the preconception period. In addition, we calculate long-term exposure to air pollutants to which the child and parents have been exposed before the first follow-up study. We take into

account address changes and calculate exposure concentrations at places the child and parents spent most of their time per day such as school or workplace. The air pollutants concentrations that can be interpolated using this model are particulate matter ($PM_{2.5}$ and PM_{10} : particles with an aerodynamic diameter $\leq 2.5 \mu m$ and $\leq 10 \mu m$ respectively), nitrogen dioxide (NO_2), ozone (O_3) and black carbon (BC).

Indoor air pollution

In a subgroup of the ENVIRONAGE birth cohort, we measure indoor air pollution. We apply a hanging air exposure system in each participant's living room for a period of two weeks to evaluate concentrations of NO_2 , BTEX (benzene, toluene, ethylbenzene, and xylene) and formaldehyde.

Traffic exposure

We also determine traffic exposure using Geographic Information System (GIS) functions with ArcGIS 10 software. We collect information on two traffic indicators at the mother's residence i.e. distance to major road and traffic density. In addition, we are able to estimate greenness, residential, and industrial areas in a 5,000 m radius from the residential address based on CORINE land cover 2006 (European Environment Agency).

Biomarkers of internal acrylamide exposure

As a biomarker of integrated internal acrylamide exposure during approximately the previous four months, acrylamide and glycidamide to hemoglobin adducts are assessed in cord blood using modified Edman degradation and high-performance liquid chromatography-tandem mass spectrometry (HPLC/MS/MS)¹²⁴.

Toxic and essential metals

We determine levels of toxic (arsenic, cadmium, lead, mercury) and essential elements (manganese, selenium) in placental tissue and mother and child blood using inductively coupled plasma mass spectrometry (ICP-MS).

Biological specimens

At birth, we collect approximately 42 ml of venous umbilical cord blood in suitable tubes to retrieve DNA, RNA, blood/plasma biochemistry, and metals. Placental biopsies are collected for DNA and RNA extraction according to a fixed sampling scheme (**Chapter 3, Figure 2**).¹²⁵ Briefly, we take biopsy samples of approximately 1-2 cm³ at each quadrant of the fetal side across the middle region of the placenta approximately four cm away from the umbilical cord and 1-1.5 cm below the chorio-amniotic membrane. An extra biopsy is taken from the maternal side of the placenta. Three regions between the quadrants are collected for placental metal measurements. One day after delivery, we collect maternal blood (20 ml) and urine, as well as newborn's meconium samples. At the follow-up exams, we collect buccal cells from the children using SK-2 Isohelix buccal swabs (Cell Projects Ltd, UK), as well as saliva, and fingernail clippings. If both the participating child and the accompanying adult consent, a blood sample is collected from child and mother in addition to a urine sample collected in metal-free containers.

All the biological material (original specimens and extracted material) are stored at -80°C except urine, blood, and placenta tissue for metal analysis, and placenta tissue stabilized in RNAlater RNA Stabilizing Reagent (Qiagen, Inc., Venlo, the Netherlands) which can be stored at -20°C, according to the manufacturer's instructions.

Blood biochemistry

Complete blood cell counts and differential leukocyte counts are determined using an automated cell counter with flow differential (Cell Dyn 3500, Abbott Diagnostics, Abott Park, IL, USA). Plasma levels of ferritin, cholesterol, LDL and HDL cholesterol, cystatin C, thyroid hormones [free triiodothyronine (T₃), free thyroxine (T₄) and thyroid-stimulating hormone (TSH)], folic acid, homocysteine, insulin, vitamin D, estradiol, interleukin-6, immunoglobulin E, luteinizing hormone, follicle stimulating hormone, and sex hormone binding globulin are measured with an electro-chemiluminescence immunoassay using the Modular E170 automatic analyzer (Roche, Basel, Switzerland).

Molecular 'omics' measures

The ENVIRONAGE birth cohort is designed with a strong focus on early molecular mechanisms to bridge the gap between exposure and disease. The study concept is to perform measurements at the different 'omics' levels in relevant biological tissues (**Table 3**).

Genomics

At the level of the genome, we identify whether environmental exposures lead to the formation of DNA adducts and whether certain genetic determinants such as single nucleotide polymorphisms (SNPs) make newborns more susceptible to develop diseases in adulthood. We have special interest in exploring the role of early environmental exposures on telomeres, the distal hexameric repeats at the end of the chromosomes, which provide stability and protection to the coding DNA. Telomere length declines with each cell division and is considered as a marker of biological aging.¹²⁶ Telomere length is measured as telomere repeat copy number relative to a single gene copy number (T/S ratio) by a modified version of the previously described PCR-based telomere assay by Cawthon.¹²⁷

Epigenomics

Epigenomics comprises the complete set of epigenetic marks that modify chromatin structure and gene expression without changing the underlying DNA sequence. DNA methylation is the most commonly characterized epigenetic modification which involves the addition of a methyl group to the carbon-5 position of cytosine residues of the dinucleotide CpG. Changes in DNA methylation patterns can be observed at the global level (entire genome), which usually is associated with genomic instability, or at specific genes, which is more associated with gene regulation. MicroRNAs (miRNA), small non-coding RNA molecules, and histone modifications are two other epigenetic marks that can regulate gene expression.

Transcriptomics

At the transcriptome level we perform candidate gene expression measurements in combination with DNA-microarray technology. The combination of these two technologies provides a better understanding of varying expression profiling due to environmental stimuli.

Proteomics and metabolomics

We measure candidate protein levels in newborn tissues but in the near future we will characterize the total information flow within the cell and the organism, through protein pathways and networks, with the eventual aim of understanding the functional relevance of proteins. In addition, we measure blood plasma metabolites, the final downstream product of genes that are closest to the phenotype of the biological system.

Mitochondriomics

There is a new area of research that stretches beyond the nuclear genome called 'mitochondriomics'.⁵⁵ It is well recognized that the (patho)physiological role of mitochondria largely exceeds that of solely being the biochemical power plant of the cells. Mitochondriomics is dedicated to clarify whether mitochondria are novel biosensors or mediators of environmental effects by exploring mitochondrial DNA abundance, mutations and deletions, epigenetics, and mtDNA-encoded proteins.

Phenotypic measures

Pregnancy and birth

On three prenatal visits at fixed time points during gestation, the gynecologist performs fetal ultrasound examinations to establish gestational age and fetal growth patterns such as estimated weight, nuchal translucency (first echo), crown-rump length, biparietal diameter, occipital frontal diameter, head circumference, transcerebellar diameter, abdominal contour, and femur length. Maternal length and weight is also recorded during these examinations. At birth, the newborn's anthropometry assessment includes weight, length, and head circumference. Other relevant data include arterial and venous cord blood pH, epidural or Caesarean delivery, hour of delivery and delivery of the placenta, parity, gestational age, gender, Apgar score, and pregnancy complications.

At the postpartum exam (hospital) as well as at the age of two months (home visit), a well-trained academic midwife/researcher performs the NBAS test. The NBAS is a neurobehavioral scale that is used to examine in a highly individualized way the effects of a wide range of perinatal variables on newborn

behavior and developmental behavior. The NBAS assesses the neonate's behavioral repertoire on 28 items, each scored on a 9-point scale covering four domains of neurobehavioral functioning, i.e. autonomic regulation, motor organization, state regulation, and social interaction. The scale also includes an assessment of the infant's neurological status on 18 reflex items, each scored on a 4-point scale. Scoring the neonatal behavior provides a comprehensive profile of a full range of neurobehavioral functioning as well as identifying areas of difficulty or deviation.¹²⁸ In addition, neonatal and maternal blood pressure is recorded up to five times with a 1-minute interval using the Welch Allyn Vital Signs Monitor 6000 Series and Omron 705IT (Omron Corporation) respectively.

Follow-up 1

Birth cohort children at the age of four to six years are invited to participate and undergo an extensive clinical examination. A physical examination of the children includes the measurements of height, weight, and waist circumference. Child and mother receive a clinical examination that consists of five blood pressure readings (Omron HBP 1300 automated monitor with special sized cuff for children), retinal photography (Canon 45 6.3-megapixel digital nonmydriatic camera, AGE Reader), and measurements of carotid intima-media thickness and arterial stiffness (MyLabOne, Esoate Benelux). The bone density (BeamMed Sunlight MiniOmni) and the heart rate (portable wireless Zephyr Biopatches) of children is measured. Furthermore, cognitive performance of the study participants is assessed using the Cambridge Neuropsychological Test Automated Battery (CANTAB) Research Suite, investigating measures for the neuropsychological domains of attention (sustained), reaction time, visual memory and executive function.

Table 2. Overview of questionnaires, analyses, and measures within the ENVIRO/NAGE birth cohort

	Pregnancy period	Birth	Infancy 2 months	Follow-up 1 (age 4-6)
Questionnaires				
Lifestyle factors (nutrition, smoking, etc.)	☒	☒		☒
Medication	☒	☒		☒
Physical activity	☒	☒		☒
Anthropometric data	☒	☒		☒
Allergy questions	☒	☒		☒
Perceived Stress Scale (PSS)	☒	☒		☒
Strengths & Difficulties Questionnaire (SDQ)				☒
Environmental exposures				
PM ₁₀ & PM _{2.5}	☒	☒	☒	☒
Black carbon	☒	☒	☒	☒
NO ₂	☒	☒	☒	☒
O ₃ & UV	☒	☒	☒	☒
Temperature & humidity	☒	☒	☒	☒
Indoor air pollution (BTEX & formaldehyde)			S	
GIS (traffic density & distance to roads)	☒	☒	☒	☒
Metals		☒		☒
Biological specimens				
Placenta & cord blood		☒		
Newborn meconium		☒		
Maternal blood & urine		☒		☒
Child blood & urine				☒
Child buccal cells				☒
Child saliva				☒
Child nails				☒
Blood analysis				
Hematology		☒		☒
Blood biochemistry		☒		☒
Toxic and essential metals		●		●
Molecular measures (see details at birth in Table 3)				
Mitochondrial DNA content		☒		☒
Mitochondrial DNA methylation		S		●
Global DNA methylation [†]		S		●
Gene-specific DNA methylation		S		●
Telomere length		☒		☒
Gene expression candidates		S		●
MicroRNA expression candidates		S		●
Microarrays		S		●
8-OHdG		☒		●
SNPs		☒		●
Candidate protein levels		S		●
Metabolomics		☒		
Phenotypic measures				
Ultrasound data		☒		
Retinal photography				☒
Carotid ultrasound				☒
Blood pressure		☒	S	☒
Heart rate				☒
Bone density				☒
Neurological battery (Stroop & NES tests)		☒		☒
NBAS		☒	☒	

☒ have been performed and ● are planned. S = Assessment only in a subgroup.

[†] Global DNA methylation obtained by quantifying 5-methyl-2'-deoxycytidine and 2'-deoxycytidine using ultra-pressure liquid chromatography (UPLC), in combination with tandem mass spectrometry (MS-MS).

Abbreviations: 8-OHdG: 8-hydroxy-2'-deoxyguanosine; SNPs: Single Nucleotide Polymorphisms; PM: Particulate Matter; NO₂: Nitrogen dioxide; O₃: Ozone; BTEX: benzene, toluene, ethylbenzene and xylene; NES: Neurobehavioral Evaluation System; NBAS: Neonatal Behavior Assessment Scale.

Table 3. Details of molecular measurements at birth in the ENVIRONAGE birth cohort

Molecular measure	Placenta tissue	Cord blood	Maternal blood
mtDNA content	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
mtDNA methylation	S	S	•
Global DNA methylation†	S	•	
Gene-specific DNA methylation	S	S	•
Telomere length	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
Gene expression candidates	S	S	•
miRNA expression candidates	S	•	•
Microarrays	S	S	•
8-OHdG	•	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
SNPs		<input checked="" type="checkbox"/>	
Candidate protein levels	S	S	
Metabolomics		<input checked="" type="checkbox"/>	

have been performed and • are planned. S = Assessment only in a subgroup.

† Global DNA methylation obtained by quantifying 5-methyl-2'-deoxycytidine and 2'-deoxycytidine using ultra-pressure liquid chromatography (UPLC), in combination with tandem mass spectrometry (MS-MS).

Abbreviations: 8-OHdG: 8-hydroxy-2'-deoxyguanosine; SNPs: Single Nucleotide Polymorphisms.

WHAT HAS IT FOUND? KEY FINDINGS AND PUBLICATIONS

Findings from the ENVIRONAGE birth cohort are currently limited to the first stage of the study (birth), since the follow-up, which started in January 2015, is still ongoing (**Table 4**). We have shown that air pollution exposure during pregnancy induces molecular, cellular, and hormonal changes in placental tissue and cord blood, which are most representative for the fetus/neonate. The placenta is considered the main target organ since it serves as the gatekeeper between mother and fetus for all nutritional, hormonal, and environmental stress factors throughout pregnancy. A summary of published articles within the framework of the ENVIRONAGE birth cohort study can be found in **Table 4**, or in the section 'For Researchers' on the website [www.environage.be], or using the term 'ENVIRONAGE' in Pubmed [<http://www.ncbi.nlm.nih.gov/pubmed>].

Oxidative stress markers

Oxidative and nitrosative stress are two putative main mechanisms by which air pollutants may exert their toxic effects. We observed a positive association between placental 3-nitrotyrosine (3-NTp) levels, a biomarker of oxidative stress and inflammation, and entire pregnancy exposure to PM_{2.5} and BC. These findings highlight the relevance of placental 3-NTp as a marker of

cumulative airborne particulate matter-induced prenatal oxidative stress (*Saenen et al. Accepted article in American Journal of Epidemiology 2016*).

Epigenetics

With the exception of imprinted genes, all DNA methylation patterns are established during embryogenesis, and play an important role in gene regulation which may comprise a biologically plausible link between *in utero* exposures and disease risks in adulthood.¹²⁹ We observed a lower degree of global DNA methylation in placental tissue in association with exposure to fine particulate air pollution (PM_{2.5}) during early pregnancy. More specifically, the period from fertilization up to and including implantation, a critical period for methylation reprogramming, was a highly sensitive window for the effect of PM_{2.5} exposure on placental DNA methylation at birth.¹³⁰

We analyzed miRNA expression by qRT-PCR in a subset ($n = 210$) of placenta samples. Findings up to now show negative associations between placental expression of miRNAs (miR-21, miR-146a, and miR-222) and 2nd trimester PM_{2.5} exposure. In addition, placental expression of Tumor suppressor Phosphatase and Tensin homolog (*PTEN*), a common target of the miRNAs, is significantly associated with third trimester PM_{2.5} exposure in our study population (*Tsamou et al. Accepted article in Epigenetics 2016*).

Transcriptomics

Early life exposure to PM_{2.5} negatively influenced placental transcription of brain-derived neurotrophic factor (*BDNF*) and Synapsin 1 (*SYN1*), two genes implicated in neural development. Furthermore, the effects of PM_{2.5} exposure are potentially transmitted through the phospholipase gamma and son of sevenless signaling cascades of the *BDNF* pathway.¹³¹

Mitochondriomics

Given that every cell of our body is dependent on energy metabolism, it is intuitive knowledge that mitochondria play an important role in disease processes. However, it is less clear to what extent environmental exposures impact mitochondrial mechanisms that may lead to enhanced risk of disease.⁵⁵ Mitochondria have a unique sensitivity to oxidative stress induced through

environmental toxicants, such as tobacco smoke for which alterations in placental mtDNA copy number have been showed (each mitochondria carries 2-10 copies of mtDNA).¹³² We found a lower mtDNA copy number in placental tissue in association with *in utero* exposure to PM₁₀, which reflects signs of mitophagy and mitochondrial death.¹³³ In a subsequent study, which combines two independent European cohorts, INMA (INFancia y Medio Ambiente), Spain and ENVIRONAGE, we demonstrated that mtDNA copy number is one of the potential mediators in the association between prenatal air pollution exposure and birth weight.¹³⁴ Furthermore, we found that epigenetic modifications in the mitochondrial genome substantially mediate the association between gestational PM_{2.5} exposure and placental mtDNA content, which indicates that mtDNA methylation might be a method or pathway by which mitochondrial biogenesis and function is regulated.¹³⁵ Lastly, we showed that prenatal PM air pollution exposure was positively associated with mitochondrial 8-OHdG in maternal and cord blood, a marker of oxidative DNA damage.¹³⁶ Hence, these findings show that exposure to PM air pollution in early life plays a role in increasing systemic oxidative stress at the level of the mitochondria, both in mother and fetus.

Table 4. Summary table of published articles of the ENVIRONAGE birth cohort (2012-2016)

Reference	n	Target tissue	Effect measure	Exposure	Effect [95% CI]	Conclusion
Janssen et al. (2012) ¹³³	174	Placenta & cord blood	mtDNA content	A 10 µg/m ³ increment in PM ₁₀ during the last trimester of pregnancy	Associated with a placental mtDNA depletion (relative -17.4% [-31.8, -0.1]) No association in cord blood	Alterations in mtDNA content may reflect and intensify oxidative stress production
Janssen et al. (2013) ¹³⁰	240	Placenta	Global DNA methylation	A 5 µg/m ³ increment in PM _{2.5} during pregnancy	Associated with lower global DNA methylation (relative -2.19% [-3.65, -0.73]), especially in the 1st trimester (implantation)	Our findings give mechanistic plausibility to the hypothesis that air pollution is linked to fetal programming
Janssen et al. (2015) ¹³⁵	381	Placenta	mtDNA methylation	An IQR (7.8 µg/m ³) increment in PM _{2.5} during the 1 st trimester of pregnancy	Associated with higher mtDNA methylation (absolute +1.27% [0.23, 2.32])	mtDNA methylation substantially mediates the association between gestational PM _{2.5} exposure and placental mtDNA content
Saenen et al. (2015) ¹³¹	90	Placenta	Gene expression in BDNF pathway	A 5 µg/m ³ increment in PM _{2.5} during the 1 st trimester of pregnancy	Associated with a relative decrease of 15.9% [-28.7, -3.2] in BDNF and 24.3% [-42.8, -5.8] in SYN1	Placental expression of BDNF and SYN1, two genes implicated in normal trajectories neurodevelopment, decreased with exposure to PM _{2.5}
Clemente et al. (2016) ¹³⁴	726†	Placenta	mtDNA content & birth weight	A 10 µg/m ³ increment in NO ₂ during pregnancy	Associated with a relative decrease of 4.9% [-9.3, -0.3] in mtDNA content and a decrease in birth weight of 48 g [-87, -9]	mtDNA content can be a potential mediator of the association between prenatal air pollution exposure and birth weight
Grevendong et al. (2016) ¹³⁶	224	Maternal blood	mtDNA 8-OHdG	An IQR (3.0 µg/m ³) increment in PM ₁₀ during pregnancy	Associated with a relative increase in mtDNA 8-OHdG of 18.3% [5.6, 32.4]	Particulate air pollution exposure in early life plays a role in increasing systemic oxidative stress, at the level of the mitochondria, both in mother and fetus
	293	Cord blood	mtDNA 8-OHdG	An IQR increment in PM ₁₀ during trimester 1 (7.2 µg/m ³) and 2 (6.4 µg/m ³)	Associated with a relative increase in mtDNA 8-OHdG of 23.0% [5.9, 42.8] and 16.6% [1.8, 33.5] respectively	
Saenen et al. (in press, 2016)	336	Placenta	3-nitrotyrosine	An IQR (3.5 µg/m ³) increment in PM _{2.5} during pregnancy	Associated with a relative increase of 33.5% [13.8, 56.5] in 3-NTP, especially in the 1 st and 2 nd trimester	Our findings suggest a pivotal relation between PM exposure and placental 3-nitrotyrosine
Tsamou et al. (in press, 2016)	210	Placenta	miR-21, miR-146a, miR-222	A 5 µg/m ³ increment in 2 nd trimester PM _{2.5}	Associated with a relative decrease in miR-21 (-33.7% [-53.2, -6.2]), miR-146a (-30.9% [-48.0, -8.1]), miR-222 (-25.4% [-43.0, -2.4])	These candidate placental miRNAs that relate to PM exposure may be involved in PM-induced effects in fetal programming.
	181	Placenta	Gene expression (PTEN)	A 5 µg/m ³ increment in 3 rd trimester PM _{2.5}	Associated with a relative increase in PTEN (+59.6% [26.9, 100.7])	

† Total sample size 726 of which 550 from ENVIRONAGE birth cohort and 376 from the INMA cohort.

Abbreviations: BDNF: brain-derived neurotrophic factor; SYN1: Synapsin 1; 8-OHdG: 8-hydroxy-2'-deoxyguanosine; PTEN: Tumor suppressor Phosphatase and Tensin homolog.

WHAT ARE THE MAIN STRENGTHS AND WEAKNESSES?

The major strength of the ongoing ENVIRONAGE birth cohort study is that detailed repeated physiological and clinical measures allow the integration of molecular and cellular mechanisms in order to bridge the gap between environmental exposure in early life and subsequent onset of diseases. The future of biomolecular epidemiology depends on innovative strategies and new technologies, both in the measurement of environmental agents, and their pathophysiological mode of action and outcomes.¹³⁷ Deep phenotyping of participants is of crucial importance to understand the dynamic interrelationships between biological systems and environmental exposures. We set up multi-disciplinary collaborations with diverse laboratories for highly quantitative bisulfite pyrosequencing, mass spectrometry, proteomics, etc... to integrate all these in-depth omics technologies in our laboratory. Another strength is biobanking of an extensive collection of target biospecimens which are very representative for the fetal exposure and which not only include cord blood and multiple samples of placental tissue, but also maternal samples. Besides, we recruit twins to study gene-environment interactions in this subgroup. Despite very good validation statistics,¹²³ our interpolation method to assess exposure at the mother's residence does not represent perfect air pollution levels at the individual level, but the main advantage is that we have daily concentrations from pre-conception onwards in a high resolution scale which enables us to explore the health effects of long-term exposure to air pollution.

The study design of ENVIRONAGE has some limitations. We restrict our mother-newborn recruitment to the weekends and to one hospital. This means that we lack power to study some rare exposures or outcomes. In addition, the majority of our study population is White Caucasian which reduces confounding but leads to an under-representation of other ethnic groups, limiting the extrapolation of our study findings to other ethnic population groups. Nevertheless, our study findings are generalizable to the gestational segment of the neonates in Flanders (**Table 1**). Due to the fact that we are only able to recruit at time of birth, some recall bias may be present with regard to nutrition intake and smoking behavior recorded with questionnaires. As in all observational studies, estimates or conclusions should be interpreted with caution because the underlying assumptions of causality cannot be verified. However, with our repeated

measure design across frequent time points, from early life to preadolescence, we can obtain a better understanding of disease onset. Currently, attempts are made to describe the totality of human environmental (i.e. non-genetic) exposures from conception onwards, referring to the 'exposome'.¹³⁸ Although this holistic approach is the most complete way to study environmental effects on human health, study designs focusing on detailed and well-defined exposures (i.e. air pollution) definitely add knowledge in unraveling the onset of exposure-related human diseases. Findings from the ENVIRONAGE birth cohort study will promote preventive public health care strategies and contribute to a healthier living environment for pregnant mothers and their children.

CAN I GET HOLD OF THE DATA? WHERE CAN I FIND OUT MORE?

Please contact the principal investigator Prof. dr. Tim Nawrot at his email address [tim.nawrot@uhasselt.be] to request access to the data or to set up a collaboration. Since this is an ongoing cohort, we are able to collect specific biological specimens of interest and explore innovative technologies for sample processing. Additional information about the ENVIRONAGE birth cohort can be obtained via the website: [www.environage.be].

PROFILE IN A NUTSHELL

- The ongoing population-based prospective ENVIRONAGE birth cohort study is designed with a strong focus on molecular mechanisms to bridge the gap between prenatal and neonatal environmental exposures and disease onset across the life course.
- From February 2010 until now we recruited 1,080 mother-child pairs from the East-Limburg Hospital in Genk, Belgium.
- The first follow-up (age four to six) comprising a detailed questionnaire, biological specimens collection, and clinical measurements commenced in January 2015 and is yet to be finalized. The participation rate is 70%.
- The dataset comprises detailed air pollution exposure assessment from pre-conception onwards, questionnaire and anthropometric data, extensive biological specimens collected at birth and during follow-up with in-depth molecular phenotyping and clinical measures.

- Contact principal investigator Tim Nawrot at the email address [tim.nawrot@uhasselt.be] to request access to data or to set up a collaboration.

Funding

The ENVIRONAGE birth cohort is supported by the European Research Council (ERC-2012-StG.310898), and by funds of the Flemish Scientific Research Council (FWO, N1516112 / G.0.873.11.N.10).

Acknowledgements

The authors are extremely grateful to the participating women and neonates, as well as the staff of the maternity ward, midwives, and the staff of the clinical laboratory of East-Limburg Hospital in Genk.

Conflict of interest

The authors declare they have no competing financial interests. None of the funding agencies had any role in the design and conduct of the study, nor in the collection, analysis, and interpretation of the data, nor in the preparation, review, or approval of the manuscript.

CHAPTER 3

VARIATION OF DNA METHYLATION IN CANDIDATE AGE-RELATED TARGETS ON THE MITOCHONDRIAL-TELOMERE AXIS IN CORD BLOOD AND PLACENTA

Bram G. Janssen,¹ Hyang-Min Byun,² Bianca Cox,¹ Wilfried Gyselaers,^{3,4}
Benedetta Izzi,⁵ Andrea A. Baccarelli,² Tim S. Nawrot^{1,6}

¹ Centre for Environmental Sciences, Hasselt University, Hasselt, Belgium

² Laboratory of Environmental Epigenetics, Exposure Epidemiology and Risk Program, Harvard School of Public Health, Boston, MA, USA

³ Department of Obstetrics, East-Limburg Hospital, Genk, Belgium

⁴ Department of Physiology, Hasselt University, Diepenbeek, Belgium

⁵ Department of Cardiovascular Sciences, Center for Molecular and Vascular Biology, Leuven University, Leuven, Belgium

⁶ Department of Public Health & Primary Care, Leuven University, Leuven, Belgium

ABSTRACT

Background: Epigenetics is tissue-specific and potentially even cell-specific, but little information is available from human reproductive studies about the concordance of DNA methylation patterns in cord blood and placenta, as well as within-placenta variations. We evaluated methylation levels at promoter regions of candidate genes in biological aging pathways (*SIRT1*, *TP53*, *PPARG*, *PPARGC1A*, and *TFAM*), a subtelomeric region (*D4Z4*) and the mitochondrial genome (*MT-RNR1*, *D-loop*).

Methods: Ninety individuals were randomly chosen from the ENVIRONAGE birth cohort to evaluate methylation concordance between cord blood and placenta using highly quantitative bisulfite-PCR pyrosequencing. In a subset of nineteen individuals, a more extensive sampling scheme was performed to examine within-placenta variation.

Results: The DNA methylation levels of the subtelomeric region and mitochondrial genome showed concordance between cord blood and placenta with correlation coefficients ranging from $r = 0.31$ to 0.43 , $p \leq 0.005$, and also between the maternal and fetal sides of placental tissue ($r = 0.53$ to 0.72 , $p \leq 0.05$). For the majority of targets, an agreement in methylation levels between four fetal biopsies was found (with intra-class correlation coefficients ranging from 0.16 to 0.72), indicating small within-placenta variation.

Conclusions: The methylation levels of the subtelomeric region (*D4Z4*) and mitochondrial genome (*MT-RNR1*, *D-loop*) showed concordance between cord blood and placenta, suggesting a common epigenetic signature of these targets between tissues. Concordance was lacking between the other genes that were studied. In placental tissue, methylation patterns of most targets on the mitochondrial-telomere axis were not strongly influenced by sample location.

INTRODUCTION

Epigenetic modifications represent a potential link between adverse insults and altered fetal development. Epigenetic changes, of which DNA methylation is the most commonly characterized, can occur throughout the course of life, but much of the epigenome is already established in germ cells and embryos as it appears to be particularly important for the regulation of embryonic growth and placental development.¹³⁹ Disruption of the epigenome has been demonstrated in human placenta-related pathologies such as intrauterine growth restriction¹⁴⁰ and preeclampsia.^{141,142}

Although cord blood and placental tissue are the most frequently used specimens in human reproductive studies due to their functional significance, non-invasive collection, and good accessibility, some challenges need to be taken into consideration. Gene methylation is tissue-specific and potentially even cell-specific.¹⁴³⁻¹⁴⁵ In this regard, little information is available about DNA methylation patterns in cord blood and placental tissue, as well as within-placenta variations. Considerable variation in the DNA methylation of imprinted genes is observed in tissues derived from monozygotic and dizygotic twin pairs (such as cord blood-derived mononuclear cells and granulocytes, umbilical vein endothelial cells, buccal epithelial cells and placental tissue), with greater discordance in dizygotic twin pairs.¹⁴⁵ These data highlight that both genetic and intrauterine exposure factors contribute to the establishment of the neonatal epigenome of different tissues.

Rationale of target selection

Due to the growing interest in biological aging processes *in utero*, a better understanding of epigenetic alterations of age-and mitochondrial-related targets in cord blood and placenta is desired. Within the ENVIRONAGE project (ENVIRONMENTal influence ON AGEing in early life), we impart new dimensions to the present understanding of human aging and its environmental influence from early life onwards. The integration of mitochondria into the 'core axis of aging' is supported by the premature aging conditions exemplified by telomere dysfunction as well as mutations or deficiencies in key regulators of mitochondrial biogenesis and function.¹⁴⁶ Our selected targets are partly based on the hypothesized aging model outlined by Sahin *et al.*¹⁴⁶ proposing that

telomere attrition activates the ‘guardian of the genome’, tumor protein 53 (*TP53*), resulting in a *TP53*-mediated mitochondrial dysfunction. We defined eight candidate targets that relate to the mitochondrial-telomere axis of aging (**Figure 1**).

Sirtuin 1 (*SIRT1*) is an important determinant of longevity in humans that influences telomerase activity¹⁴⁷ and also inactivates *TP53*.¹⁴⁸ On the other hand, *SIRT1* activates peroxisome proliferator-activated receptor γ-coactivator1α (*PPARGC1A*) boosting mitochondrial biogenesis.¹⁴⁹ Indeed, *SIRT1* activity has been found to decrease in aged tissues, and this might contribute to the increased *TP53* activity and suppressed *PPARGC1A* activity seen in aged mouse and human tissues.¹⁵⁰

In addition to its close relationship with *TP53*, *PPARGC1A* is involved in mitochondrial function by acting as a transcriptional co-activator of several nuclear-encoded transcription factors, including mitochondrial transcription factor A (*TFAM*) which regulates mitochondrial biogenesis.¹⁵¹ *PPARGC1A* is also a co-activator of peroxisome proliferator-activated receptor γ (*PPARG*), regulating trophoblast invasion and early placental development.¹⁵²

Recently, mitochondrial DNA methylation has been shown to control mitochondrial gene transcripts¹⁵³ and therefore identifies a novel series of targets for *in utero* changes. The displacement loop (*D-loop*) is particularly important in this regard because it contains promoters for mitochondrial DNA transcription and nearly the entire mitochondrial genome transcribes from this region. In addition, 12S ribosomal RNA (*MT-RNR1*) encodes for a protein that facilitates the formation of RNA secondary structures, assembly of the mitochondrial ribosome, and mitochondrial translation.¹⁵⁴

Finally, we studied the non-satellite tandem repeat *D4Z4* in the subtelomeric region. In contrast to mammalian telomeric repeats (TTAGGG), the subtelomeric region has a high density of CpG sequences. *In vitro* and *in vivo* mouse studies indicate a conserved link between telomere length and the epigenetic status of subtelomeres.¹⁵⁵⁻¹⁵⁷ Evidence from human studies shows an inverse correlation between DNA methylation of the subtelomeric *D4Z4* repeat and average telomere length in a panel of cancer cell lines,¹⁵⁸ while a positive correlation with telomere length is observed in patients with dyskeratosis congenital.¹⁵⁹ These

data thereby demonstrate that the epigenetic status of the telomeric region is affected by disease conditions. Variations in telomere length among adults may already be established *in utero* and might be linked to epigenetic changes induced by environmental factors, making early life an important time of susceptibility to change.^{160,161}

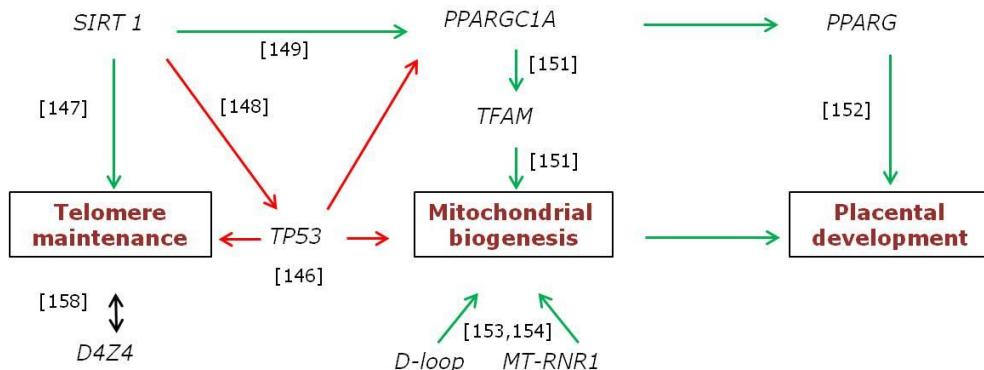


Figure 1. Scheme of candidate targets playing a role in the core axis of aging. We selected eight candidate age-related targets on the mitochondrial-telomere axis that can be categorized in three groups: (i) (sub)telomere maintenance-related genes or region (*D4Z4*, *SIRT1*, and *TP53*); (ii) mitochondrial biogenesis-related genes (*TP53*, *PPARG*, *PPARGC1A*, and *TFAM*); (iii) regions of mitochondrial DNA (*MT-RNR1* and *D-loop*). The arrows indicate a positive (green) or negative (red) relationship between the targets, and numbers refer to articles in the reference list.

Focus of the study

Few studies have investigated within-placenta variation of (global) DNA methylation levels and these only focus upon a specific set of genes.^{72,143,162,163} Since the placenta shows a remarkable amount of normal variability in size and structure, these results cannot be extrapolated to other genes. To provide a representative snapshot of placental biomarkers, it requires multiple samples to be taken from a single placenta. However, it is not always feasible given the relatively large number of samples or subjects under investigation in an epidemiological context and the related costs for the epigenetic measurements. Therefore, the number of study participants versus the numbers of sampling sites or biological replicates is an important consideration in an molecular epidemiological study design. A solution to minimize the impact of regional differences in methylation or gene expression patterns within a mother's placenta is to standardize the sampling method by selecting one specific site

from which biopsies are taken.¹⁶⁴ In this study, we evaluated the concordance of DNA methylation patterns in cord blood and placental tissue, as well as within-placenta variations, at specific regions of the aforementioned eight candidate-target regions that were selected due to their relevance to aging.

MATERIALS AND METHODS

Study population

We randomly selected cord blood and placental tissue from the ongoing ENVIRONAGE birth cohort. In this cohort, we recruit mother-newborn pairs from the East-Limburg Hospital in Genk, Belgium of which extensive medical and lifestyle data are available as described previously.^{130,133} All subjects provided informed consent, and all procedures were approved by the Ethical Committee of Hasselt University and the East-Limburg Hospital. We used a subset of ninety placenta-cord blood samples (sample set A) to examine the relationship between DNA methylation in those two tissues. In a subset of nineteen placentas (sample set B), a more extensive sampling scheme was performed to examine within-placenta variation in DNA methylation.

Cord blood collection and placental sampling

Umbilical cord blood was collected immediately after delivery in Vacutainer® Plus Plastic K2EDTA Tubes (BD, Franklin Lakes, NJ, USA). Samples were centrifuged at 3,200 rpm for 15 min to retrieve buffy coats and instantly frozen, first at -20°C and later at -80°C. Placentas were deep-frozen within ten minutes of delivery. Afterwards, we took biopsy samples of approximately 1 to 2 cm³ for DNA extraction using a standardized protocol as described by Adibi *et al.*¹⁶⁵ Four distinct sites from nineteen placentas were sampled from the fetal side across the middle region of the placenta approximately four cm away from the umbilical cord and 1-1.5 cm below the chorio-amniotic membrane (**Figure 2**). An extra biopsy was taken from the maternal side. Chorio-amniotic membrane contamination was avoided by careful visual examination and dissection. Via histological examination (hematoxylin & eosin staining), we compared four fetal biopsies from four placentas. This confirmed that biopsies were taken from chorionic villous tissue with normal architecture composed of trophoblasts.

In the fetal biopsies we could identify terminal and intermediate villi with cytотrophoblasts and syncytотrophoblasts, vessels, fibrin and the intervillous space. We did not observe any consistent differences in the histology or cell type composition between the fetal samples, nor between the four placentas.

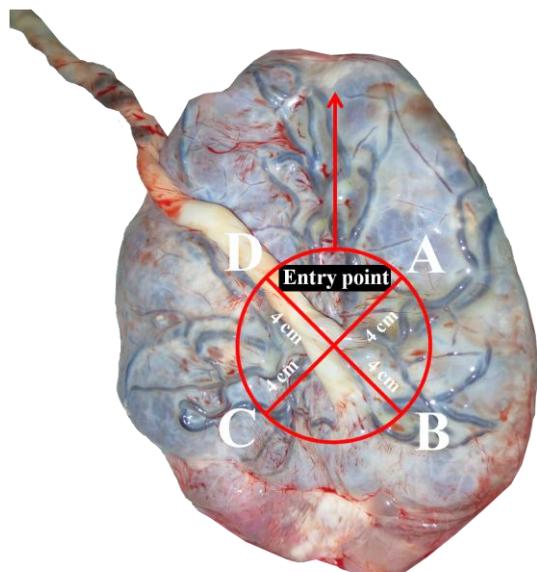


Figure 2. Placental sampling scheme to minimize site variability. The largest of two umbilical cord arteries was used to identify the site of entry into the fetal side of the placenta. This entry point was used to orientate the placenta and faced upwards. The first biopsy (A) was taken to the right of the entry point, four cm away from the cord and 1–1.5 cm below the chorio-amniotic membrane. The three other biopsies (B, C, D) were taken in the remaining quadrants of the placenta and an extra biopsy (A2) from the maternal side corresponding with biopsy A. Care was taken with the visual examination and dissection to avoid the chorio-amniotic membrane contamination.

DNA methylation analyses

Genomic DNA was isolated from buffy coat of cord blood and from placental tissue using the QIAamp DNA mini kit (Qiagen, Inc., Venlo, the Netherlands). We performed DNA methylation analysis by highly quantitative bisulfite-PCR pyrosequencing. Bisulfite conversions were performed using 1 µg of extracted genomic DNA with the EZ-96 DNA methylation Gold kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. The final elution volume was 40 µl, using M-elution buffer. We interrogated CpG sites within promoter regions (*SIRT1*, *TP53*, *PPARG*, *PPARGC1A*, *TFAM*) or other specific regions (subtelomere: *D4Z4*; mitochondrial genome: *MT-RNR1*, *D-loop*) of targets on the mitochondrial-telomere axis. We combined data from the literature and data derived from the UCSC Genome Browser (<http://genome.ucsc.edu>, assembly GRCh37/hg19), such as transcription factor binding sites, DNase I hypersensitive sites and histone modifications to identify regions with potentially methylated CpG sites. The MethPrimer program (<http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi>) was used for PCR primer design. The assays for *D4Z4*, *MT-RNR1* and *D-loop* have been previously described.^{166,167} Detailed information regarding primer sequences and genomic regions is given in **Supplemental Material, Table S1**. Methylation levels of the mitochondrial regions *MT-RNR1* and *D-loop* showed a strong correlation in placental tissue ($n = 81$, $r = 0.85$, $p < 0.0001$) and cord blood ($n = 82$, $r = 0.68$, $p < 0.0001$).

PCR amplification of regions of interest prior to pyrosequencing was performed in a total reaction volume of 30 µl, using 15 µl GoTaq Hot Start Green Master Mix (Promega, Madison, WI, USA), 10 pmol forward primer, 10 pmol reverse primer, 1 µl bisulfite-treated genomic DNA, and water. One primer was biotin-labeled in order to purify PCR products in conjunction with Sepharose beads. PCR products were bound to Streptavidin Sepharose High Performance beads (Amersham Biosciences, Uppsala, Sweden) via the biotin label, and immobilized on the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Inc., Westborough, MA, USA) before being purified, washed, denatured using 0.2 mol/L NaOH solution, and washed again according to the manufacturer's protocol. 0.3 µmol/L of pyrosequencing primer was annealed to the purified single-stranded PCR product and pyrosequencing was performed using the PyroMark Q96 MD

Pyrosequencing System (Qiagen, Inc., Germantown, MD, USA). We used 0% (PSQ-T oligo: 5'-TTGCGATACAACGGGAACAAACGTTGAATT-3') and 100% (PSQ-C oligo: 5'-TTGCGATACGACGGGAACAAACGTTGAATT-3') DNA methylation control oligos. The sequencing primer for the control oligo is: 5'-AACGTTGTTCCCGT-3'. We mixed the PSQ-C oligo (or PSQ-T oligo) with the sequencing oligo in PyroMark Annealing Buffer (Qiagen, Inc., Valencia, CA, USA) and performed pyrosequencing with the sequencing entry C/TGTAT.¹⁶⁶ The degree of methylation was expressed as the percentage of methylated cytosines over the sum of methylated and unmethylated cytosines. The efficiency of the bisulfite-conversion process was assessed using non-CpG cytosine residues within the sequence.

Statistical analysis

Multiple CpGs within each region of interest were interrogated and we calculated the average across all the CpGs for the analysis. Spearman correlation coefficients were applied to assess the association between methylation levels in cord blood and placental tissue of ninety participants (sample set A). The Wilcoxon signed rank test was performed to compare methylation levels between the two different tissues.

In nineteen individuals (sample set B), we measured DNA methylation of the eight targets in the four quadrants of the placenta. We computed the intra-class correlation coefficient (ICC) from a variance components mixed model to evaluate within- versus between-placenta variability. Mixed models take into account regional differences of each individual placenta and calculate the proportion of variation that is explained by the variance between individuals. Additionally, we tested for differences between placental biopsies using the Friedman's test and conducted post-hoc analysis with the Wilcoxon signed rank test. The Bonferroni method was applied to correct for multiple pairwise comparisons. All statistical analysis were performed using the SAS software program (version 9.2; SAS Institute Inc., Cary, NC, USA).

RESULTS

The study included two sample sets; sample set A ($n = 90$) for the correlation of methylation levels between cord blood and placental tissue and sample set B ($n = 19$) for the assessment of within-placenta variation. Characteristics of the two samples are reported in **Table 1**.

Table 1. Characteristics of the two sample sets. Sample set A: Ninety individuals for the correlation between cord blood and placental tissue. Sample set B: Nineteen individuals for within-placenta variation analysis. Data are presented as mean \pm SD or range and number (%)

Variables	Sample set A ($n = 90$)	Sample set B ($n = 19$)
Maternal age, y	29.0 (19-40)	27.8 (18-38)
Pre-pregnancy BMI, kg/m ²	25.5 \pm 5.1	26.0 \pm 6.6
Smoking status		
Never-smoker	64 (71.1%)	10 (52.7%)
Past-smoker	11 (12.2%)	7 (36.8%)
Smoker	15 (16.7%)	2 (10.5%)
Alcohol		
No	69 (76.6%)	12 (63.2%)
Yes	21 (23.3%)	7 (36.8%)
Parity		
1	44 (48.9%)	11 (57.9%)
2	36 (40.0%)	6 (31.6%)
≥ 3	10 (11.1%)	2 (10.5%)
Newborn's gender		
Male	47 (52.2%)	7 (36.8%)
Female	43 (47.8%)	12 (63.2%)
Gestational age, w	39.2 (35-42)	38.9 (36-41)
Apgar score after 5 min		
6	1 (1.1%)	-
7	2 (2.2%)	-
8	2 (2.2%)	1 (5.3%)
9	20 (22.2%)	7 (36.8%)
10	65 (72.2%)	11 (57.9%)
Birth weight, g	3462 \pm 461	3515 \pm 381
Birth length, cm	50.4 \pm 1.8	50.2 \pm 2.0
Caesarean section	5 (5.6%)	4 (21.0%)
Epidural anaesthesia	64 (71.1%)	13 (68.4%)

Concordance between DNA methylation in cord blood and placenta

Low methylation levels, ranging from 1.7% to 11.7%, were observed in both tissues for all targets except *D4Z4*, which had mean methylation levels of 65.2% in cord blood and 45.1% in placenta (**Table 2**). Mean cord blood methylation levels at the interrogated regions of *D4Z4*, *SIRT1*, *PPARG*, *MT-RNR1*, and *D-loop* were significantly higher than those observed in placenta ($p \leq 0.01$). *PPARGC1A* showed significantly lower methylation levels ($p < 0.0001$) in cord blood compared with placental tissue. The examined CpGs in the promoter region of *PPARGC1A* in placental tissue showed strong correlation ($r \geq 0.60$) but not in cord blood. No significant differences in mean methylation levels at the promoter regions of *TP53* and *TFAM* were observed between tissues.

The correlation between methylation levels in cord blood and placental tissue was significant for three of the eight targets (*D4Z4*: $r = 0.43$, $p < 0.0001$; *MT-RNR1*: $r = 0.36$, $p = 0.0006$; *D-loop*: $r = 0.31$, $p = 0.005$). *SIRT1*, *TP53*, *PPARG*, *PPARGC1A*, and *TFAM* showed no significant correlation between cord blood and placental tissue (**Table 2**). The correlation between tissues did not alter after adjustment for maternal age, gender, gestational age or parity.

Table 2. Summary of the correlation between cord blood and placental tissue methylation (sample set A)

Target	n	Mean (%) \pm SD cord blood	Mean (%) \pm SD placenta	Spearman correlation
<i>D4Z4</i>	88	65.1 \pm 8.4	45.1 \pm 7.9†	0.43*
<i>SIRT1</i>	78	2.7 \pm 2.0	2.0 \pm 1.1†	-0.08
<i>TP53</i>	89	2.6 \pm 0.4	2.6 \pm 0.5	0.02
<i>PPARG</i>	88	3.2 \pm 3.8	2.8 \pm 4.2†	-0.13
<i>PPARGC1A</i>	89	2.4 \pm 0.6	8.2 \pm 6.7†	0.06
<i>TFAM</i>	87	1.8 \pm 0.3	1.7 \pm 0.24	0.08
<i>MT-RNR1</i>	86	11.7 \pm 0.6	9.5 \pm 0.4†	0.36*
<i>D-loop</i>	82	4.0 \pm 1.1	3.7 \pm 1.3†	0.31*

† Statistically significant difference in methylation between cord blood and placental tissue (Wilcoxon signed rank test; p -value ≤ 0.05).

* p -value ≤ 0.05

Within-placenta variation in DNA methylation

We measured DNA methylation of the eight targets in four quadrants of nineteen placentas and evaluated the within-placenta variation in DNA methylation using the ICC and Friedman test (χ^2_3). Between-placenta variability, exemplified by the ICC, was higher than within-placenta variability for *D4Z4* (72% vs. 28%, $p = 0.003$), *TP53* (55% vs. 45%, $p = 0.04$), *PPARG* (60% vs. 40%, $p = 0.006$), *PPARGC1A* (64% vs. 37%, $p = 0.005$), *MT-RNR1* (58% vs. 42%, $p = 0.009$), and *D-loop* (61% vs. 39%, $p = 0.01$), but not for *SIRT1* (32% vs. 68%, $p = 0.16$) and *TFAM* (16% vs. 84%, $p = 0.42$) (**Figure 3**). For *PPARGC1A*, two placentas showed much higher methylation levels (>20%) compared to the other placentas. For the *D-loop* assay, all four biopsies from two placentas failed pyrosequencing twice and were excluded from analysis.

The methylation levels between different biopsies of each individual, exemplified by the χ^2_3 statistic, showed no significant difference for *D4Z4* ($p = 0.60$), *SIRT1* ($p = 0.14$), *TP53* ($p = 0.17$), *PPARG* ($p = 0.18$), *PPARGC1A* ($p = 0.14$), and *TFAM* ($p = 0.32$). Only for *MT-RNR1* and *D-loop* there was a borderline significant difference between biopsies observed (both $p = 0.05$). Pairwise comparisons showed no significant differences between biopsies after Bonferroni adjustment. Mean methylation levels of each biopsy averaged by all individuals are shown in **Supplemental Material, Figure S1**.

Additionally, we compared the methylation levels of all the targets between biopsy A, taken from the fetal side, and the corresponding biopsy A2, taken from the maternal side. No significant difference in methylation levels was observed between the maternal and fetal side for any of the targets ($p \geq 0.1$). However, the correlation between the fetal and maternal side was only significant for the same targets that also correlated between cord blood and placental tissue (i.e. *D4Z4*: $r = 0.72$, $p = 0.005$; *MT-RNR1*: $r = 0.53$, $p = 0.05$; *D-loop*: $r = 0.60$, $p = 0.04$).

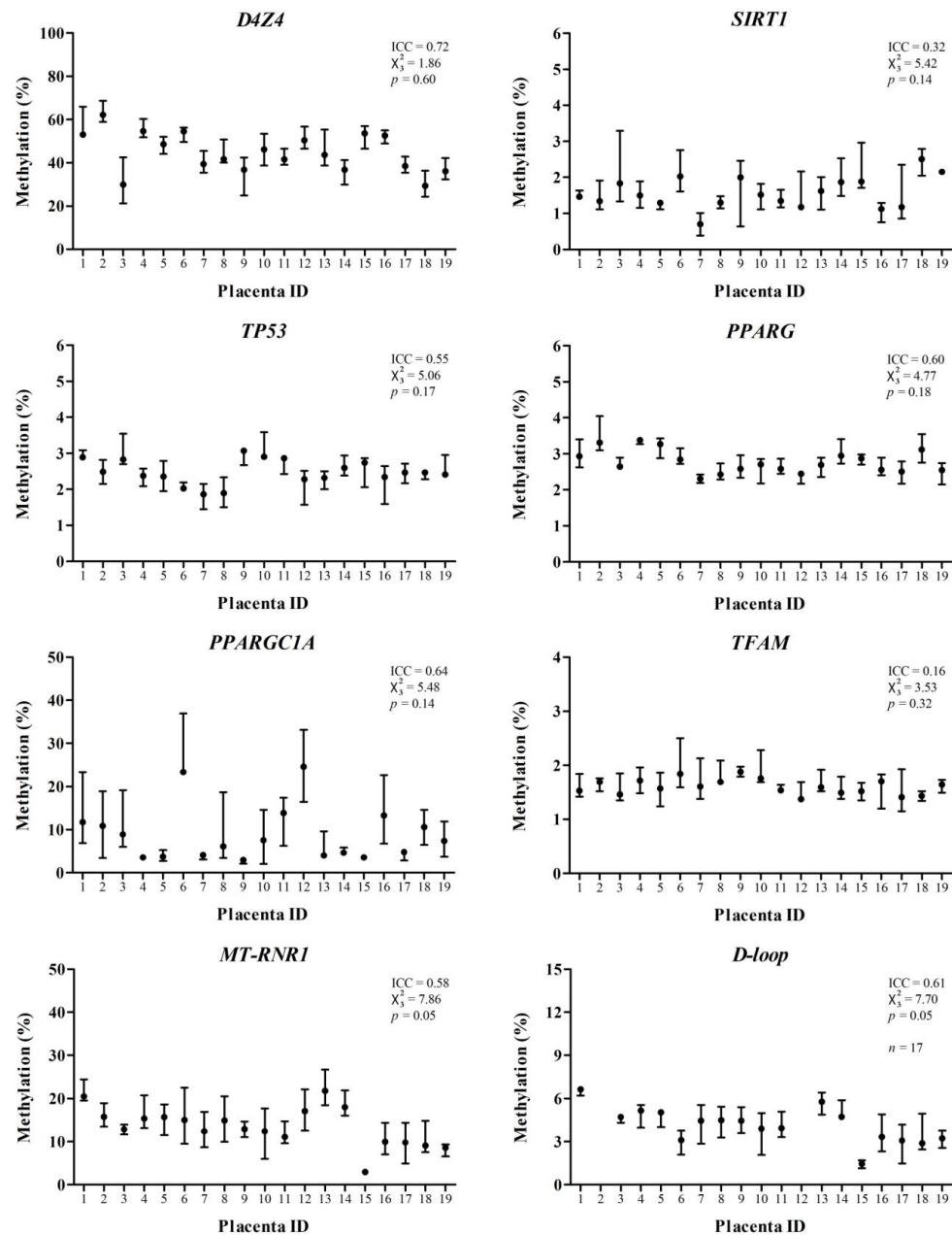


Figure 3. Within- versus between- placenta variability. Methylation levels of four placental biopsies for each individual are presented as box plots (median with range). The intra-class correlation coefficient (ICC) and Friedman's test statistic (χ^2_S) with additional p-value are shown in the right upper corner of each panel. Data are presented for nineteen individuals (sample set B), except for D-loop for which two placentas were excluded.

DISCUSSION

As suggested by Non and colleagues,⁷² researchers interested in epigenetic or gene expression analysis should evaluate each gene of interest across placental sampling sites, as each gene may display site-specific differences. Within the ENVIRONAGE birth cohort, integration of targets that reflect or determine the biological aging process, including mitochondrial biogenesis, telomere length, and candidate genes in placental development, will be investigated to understand the environmental influence on the 'core axis of aging' from early life onwards. In this study, we found that: 1/ methylation levels of the subtelomeric region (*D4Z4*) and mitochondrial genome (*MT-RNR1* and *D-loop*) showed concordance between umbilical cord blood and placental tissue, while promoter regions of candidate genes on the mitochondrial-telomere axis showed tissue-specific methylation patterns; 2/ most of our selected candidate targets, which play a potential role in biological aging, did not display major differences in methylation levels across several biopsies in placental tissue.

Concordance between DNA methylation in cord blood and placenta

The mean methylation levels of some targets in our study differed between cord blood and placental tissue (i.e. *D4Z4*, *SIRT1*, *PPARG*, *PPARGC1A*, *MT-RNR1*, and *D-loop*). Similar results have been reported by other studies on imprinted genes and global methylation levels of cord blood and the placenta.^{144,168} It is noteworthy to mention that the promoter region of *PPARGC1A* was largely unmethylated in cord blood but not in placental tissue. In addition, much higher methylation levels in all four biopsies were observed in two out of nineteen placentas from sample set B. This is of particular interest because our interrogated region is a putative binding site for CREB transcription factors. The transcriptional activity of CREB is critical for the establishment and maintenance of energy homeostasis in mice neonates¹⁶⁹ and appears to be involved in the regulation of *PPARGC1A*, which is a key regulator of mitochondrial genes and energy metabolism. Methylation of the *PPARGC1A* promoter region may therefore present one mechanism by which the genes could be differentially regulated between cord blood and placental tissue.

Most of our studied genes had low methylation levels in both cord blood and placental tissue. Despite the decreased ability to identify significant methylation changes in unmethylated genomic contexts due to technical limitations, small changes in promoter gene methylation have been extensively described in several fields,¹⁷⁰ including the one of environmental epigenetics.¹⁷¹⁻¹⁷⁴ Small changes in regional methylation as well as single CpG sites have been described to interfere with gene/protein expression¹⁷⁵ via mechanisms that include alteration of affinity of transcription factors to their binding sites or chromosome looping events.¹⁷⁶ Even when small changes in methylation would not impact gene expression, they might still serve as markers of exposure or outcome.¹⁷⁰

Correlation of the subtelomeric region and mitochondrial genome between tissues

As described above, methylation levels in umbilical cord blood may not always reflect those in the placenta and vice versa. In our study, we observed significant correlations between tissues at the subtelomeric region (*D4Z4*) and mitochondrial genome (*MT-RNR1* and *D-loop*). Our results expand upon those from a previous study investigating global and gene-specific DNA methylation across multiple tissues in early pregnancy¹⁷⁷ which reported that only the repetitive element *AluY8b*, used as proxy for global DNA methylation, correlated between placental tissue and cord blood. However, global DNA methylation levels analyzed by LUMA showed no correlation between the umbilical cord blood and placental tissue.¹⁶⁸ Our observations regarding subtelomeric methylation levels extends previous observations of concordance in telomere length among multiple organs of the human fetus.^{161,178} Together, these data indicate that methylation patterns in mitochondria and repetitive elements, such as *D4Z4* and *Alu*, are likely to reflect a common epigenetic signature between cord blood and placental tissue. Indeed, methylation levels at the ends of chromosomes and the mitochondrial genome may be more susceptible to changes as these 'hotspots' are more sensitive to oxidative stress in comparison to other parts of the genome.

Correlation between the maternal and fetal side of placental tissue

The methylation levels of the subtelomeric region and mitochondrial genome correlated between the maternal and fetal side of placental tissue. This was in addition to our findings of a correlation between fetal placental tissue and cord blood, thereby suggesting that the methylation status of these regions was consistent from maternal to fetal side to cord blood. A previous study of placental methylation variation showed good correlation ($r = 0.76$, $p < 0.0001$) for *LINE-1* between the fetal and maternal sides, but no such correlation existed for stress-related genes.⁷²

Within-placenta variation in DNA methylation

Placental tissue, as with cord blood, is composed of a complex population of cells that makes this organ shows high variability in overall DNA methylation compared with other tissues.¹⁷⁹ Cytotrophoblasts and fibroblasts are the two main cell types in the placenta and most genes exhibit similar promoter methylation patterns. Although some specific genes show differential promoter methylation between these cell types, the methylation status of placental villi mainly reflects the profile of the cytotrophoblast cells.¹⁴³ Avila and colleagues concluded that the within-placenta variability for several genes generally showed less sample-to-sample variation for DNA methylation than gene expression, and different placental sites and depths showed consistent methylation patterns.¹⁶² Our findings of low variation between four sampled biopsies of most targets is largely consistent with the results of Avila *et al.*¹⁶² and Non *et al.*⁷² We can conclude that methylation variation is probably not due to sample location but rather due to cell composition differences between samples. Nevertheless, these authors found that methylation patterns of some genes differed across placental locations, but these differences were of limited magnitude. Since we did not observe differences between sample locations, our chosen biopsy location provides a representative biological measure from each individual placenta. However, future studies on placental methylation may consider pooling multiple sites across the middle region of the placenta to further reduce sampling variability.¹⁶⁴

Analysis of within-placenta variability in DNA methylation

In our study we used two parameters, Friedman's statistic and ICC, which provided different information regarding within-placenta variation. An advantage of this combination is that we were able to evaluate the methylation levels across biopsies together with the agreement between biopsies. The higher the ICC, the more reliably a biopsy measure reflects true between-individual differences. Although there is considerable variation in methylation levels between individuals, as seen with the large range of methylation values, all targets showed similar methylation levels across the four biopsies (Friedman's test). However, both regions of the mitochondrial genome, *MT-RNR1* and *D-loop*, showed borderline significant differences compared between biopsies, although not significant after Bonferroni correction. This observation was not reflected in the ICC, which was fairly high (58% and 61% for *MT-RNR1* and *D-loop* respectively), therefore showing a good agreement between biopsies. One remark on the usage of the ICC as a reliability statistic is that it is usually applied to assess the reproducibility of measurements in replicates, which normally yield high ICC values. In our study we applied the ICC to different biopsies from the same organ, assuming that some biological variation is already present and will therefore yield lower ICCs. Variation across sites in an organ as complex as the placenta is within its nature and even a small deviation in methylation level in one biopsy influences the separate biopsy correlations and subsequently the ICC. The low methylated genes *TFAM* and *SIRT1* lacked correlation between biopsies, possibly as a result of the relatively higher variation on account of the low absolute methylation levels. We did not observe any obvious differences in the histology or cell type composition between the fetal samples sites across the middle region of the placenta nor between placentas.

CONCLUSION

The methylation of the subtelomeric region and mitochondrial genome showed concordance between the different tissues (cord blood and the fetal and maternal side of the placenta). However, the other selected candidate targets showed tissue-specific methylation patterns at promoter regions, as exemplified

by lack of correlation between cord blood and placental tissue. These data indicate that methylation patterns in mitochondria and repeated elements are likely to reflect a common epigenetic signature between cord blood and placental tissue. Within the placenta, all our selected methylation targets on the mitochondrial-telomere axis showed similar methylation levels, and for these targets it is amenable that sample location does not affect DNA methylation. The strength of this study is that we took multiple biopsies at a fixed locations in a large number of placentas to assess the within-placenta variability using a highly sensitive bisulfite-pyrosequencing approach. Furthermore, we assessed within-placenta variation using two parameters in order to evaluate methylation levels across biopsies together with the agreement between biopsies. This information may guide future molecular epidemiological research that investigate the epigenetics of targets operating within the core axis of aging.

Acknowledgements

The authors thank the participating women, as well as the staff of the maternity ward (Anja Moors, Christine De Bruyn, Caroline Dielen, Noelia Dony, Julie Faes, Carmen Kerkhofs, Elisabeth Vandendriessche), midwives, and the staff of the clinical laboratory of East-Limburg Hospital in Genk. We thank dr. Timothy Barrow for proofreading the manuscript. The ENVIRONAGE birth cohort is supported by the EU Program ‘Ideas’ (ERC-2012-StG 310898), by the Flemish Scientific Fund (FWO, N1516112/G.0.873.11.N.10) and Bijzonder Onderzoeks Fonds of Hasselt University (BOF). This work was also supported by funding from the National Institute of Environmental Health Sciences (R01ES021733 and R01ES021357). Benedetta Izzi has a postdoctoral fellowship (PDMK/13/163).

SUPPLEMENTAL MATERIAL**Table S1.** Bisulfite-Pyrosequencing primer sequence information based upon Assembly GRCh37/hg19 of the UCSC genome browser

Target	Chr	Amplicon start-end	Primer set (Forward / Reverse / Sequence)	Amplicon (bp)	Annealing temp (°C)	Target CpGs	Reference†
<i>D4Z4</i> ‡	4q35	/	F: 5'-GGTTTTTGTTGTATTTG-3' R: 5'- <u>CAAATCTAACCTAAACTC</u> -3' S: 5'-GGATAGTATTTTT-3'	308	60	4	[1]
	10q26	/					
<i>SIRT1</i>	10	69644144-	F: 5'-TAGTTGAAAGAGAAGTTGAGAAAG-3'	248	53	5	[2]
		69644392	R: 5'-AAAAATTAAACCCCATCA-3' S: 5'-AAATTAAACCCCATCA-3'				
<i>TP53</i>	17	7590838-	F: 5'-TATATGGGGAAAATTAAATT-3'	244	53	4	[3]
		7591082	R: 5'- <u>CCAAAATAATTCCACCAATTCTAC</u> -3' S: 5'-AATTATTAATTGGT-3'				
<i>PPARG</i>	3	12328971-	F: 5'-TTGGAAAGAATTTGGGAAGA-3'	164	53	5	[4]
		12329135	R: 5'- <u>ACCCAAAAAAATCCCATTC</u> -3' S: 5'-GAATATTTGGGAAGA-3'				
<i>PPARGC1A</i>	4	23891766-	F: 5'-TTTTTGTGAGTTGTTTTAA-3'	231	53	3	[5]
		23891997	R: 5' <u>TTACAAAAAATTAAATTATATAACCA</u> -3' S: 5'-AGTTTGTGTTTAATT-3'				[6]
<i>TFAM</i>	10	60144975-	F: 5'-GGGGATAGAGGTGGTTTAAG-3'	282	55.9	6	[7]
		60145257	R: 5'- <u>AACAATACACAACCAACATCAC</u> -3' S: 5'-AAATCTACTAACATC-3'				
<i>MT-RNR1</i>	M	1191-	F: 5'-TTTTAGAGGAGTTGTTGTAAT-3'	176	58.3	2	[8]
		1366	R: 5'- <u>ATAACCCATTCTTACCAACCTCATA</u> -3' S: 5'-AGTTTGTGTTGTAAT-3'				
<i>D-loop</i>	M	6-	F: 5'-TGTGAGATTTAATTGTTATTA-3'	254	54.1	3	[8]
		259	R: 5'- <u>CAAATCTATCACCTATTAACCAC</u> -3' S: 5'-TAATTAATTATATATT-3'				

Legend of Table S1

† The reference articles do not necessarily examine exactly the same region as we report, but rather just show that these candidate regions were previously evaluated and described in the literature.

‡ The proximal subtelomeric region of chromosome 4 harbours the polymorphic *D4Z4* tandem repeat array (one repeat of 3.3 kb). The number of repeated units in chromosome 4 varies from 11 to more than 100 in normal individuals [9]. The subtelomere of chromosome 10 is highly homologous (> 95%) to chromosome 4 and also contains the *D4Z4* repeat array. *D4Z4* primers were designed to a consensus sequence for the repetitive element and amplify a global pool of repetitive elements rather than a single element or genomic locus. As the *D4Z4* repeat is a tandem repeat about 3.3 kb in size, theoretically it is possible to amplify products of 308 bp to 3608 bp, but the PCR extension time was minimized to ensure only products of 308 bp were amplified. [1]

Reference list Table S1

- [1] Choi SH, Worswick S, Byun HM, Shear T, Soussa JC, Wolff EM, et al. Changes in DNA methylation of tandem DNA repeats are different from interspersed repeats in cancer. *Int J Cancer* 2009;125(3):723-9.
- [2] Liu T, Liu PY, and Marshall GM. The Critical Role of the Class III Histone Deacetylase SIRT1 in Cancer. *Cancer Research* 2009;69(5):1702-5.
- [3] Hou L, Zhang X, Tarantini L, Nordio F, Bonzini M, Angelici L, et al. Ambient PM exposure and DNA methylation in tumor suppressor genes: a cross-sectional study. *Part Fibre Toxicol* 2011;8:25.
- [4] Pancione M, Sabatino L, Fucci A, Carafa V, Nebbioso A, Forte N, et al. Epigenetic silencing of peroxisome proliferator-activated receptor gamma is a biomarker for colorectal cancer progression and adverse patients' outcome. *PLoS ONE* 2010;5(12):e14229.
- [5] Barrès R, Osler ME, Yan J, Rune A, Fritz T, Caidahl K, et al. Non-CpG methylation of the PGC-1 α promoter through DNMT3B controls mitochondrial density. *Cell Metab* 2009;10(3):189-98.
- [6] Barrès R, Yan J, Egan B, Treebak JT, Rasmussen M, Fritz T, et al. Acute Exercise Remodels Promoter Methylation in Human Skeletal Muscle. *Cell Metabolism* 2012;15(3):405-11.
- [7] Choi YS, Kim S, Kyu Lee H, Lee K-U, and Pak YK. In vitro methylation of nuclear respiratory factor-1 binding site suppresses the promoter activity of mitochondrial transcription factor A. *Biochemical and Biophysical Research Communications* 2004;314(1):118-22.
- [8] Byun HM, Panni T, Motta V, Hou L, Nordio F, Apostoli P, et al. Effects of airborne pollutants on mitochondrial DNA Methylation. *Particle and Fibre Toxicology* 2013;10(1):18.
- [9] van Overveld PG, Lemmers RJ, Deidda G, Sandkuijl L, Padberg GW, Frants RR, et al. Interchromosomal repeat array interactions between chromosomes 4 and 10: a model for subtelomeric plasticity. *Hum Mol Genet* 2000;9(19):2879-84.

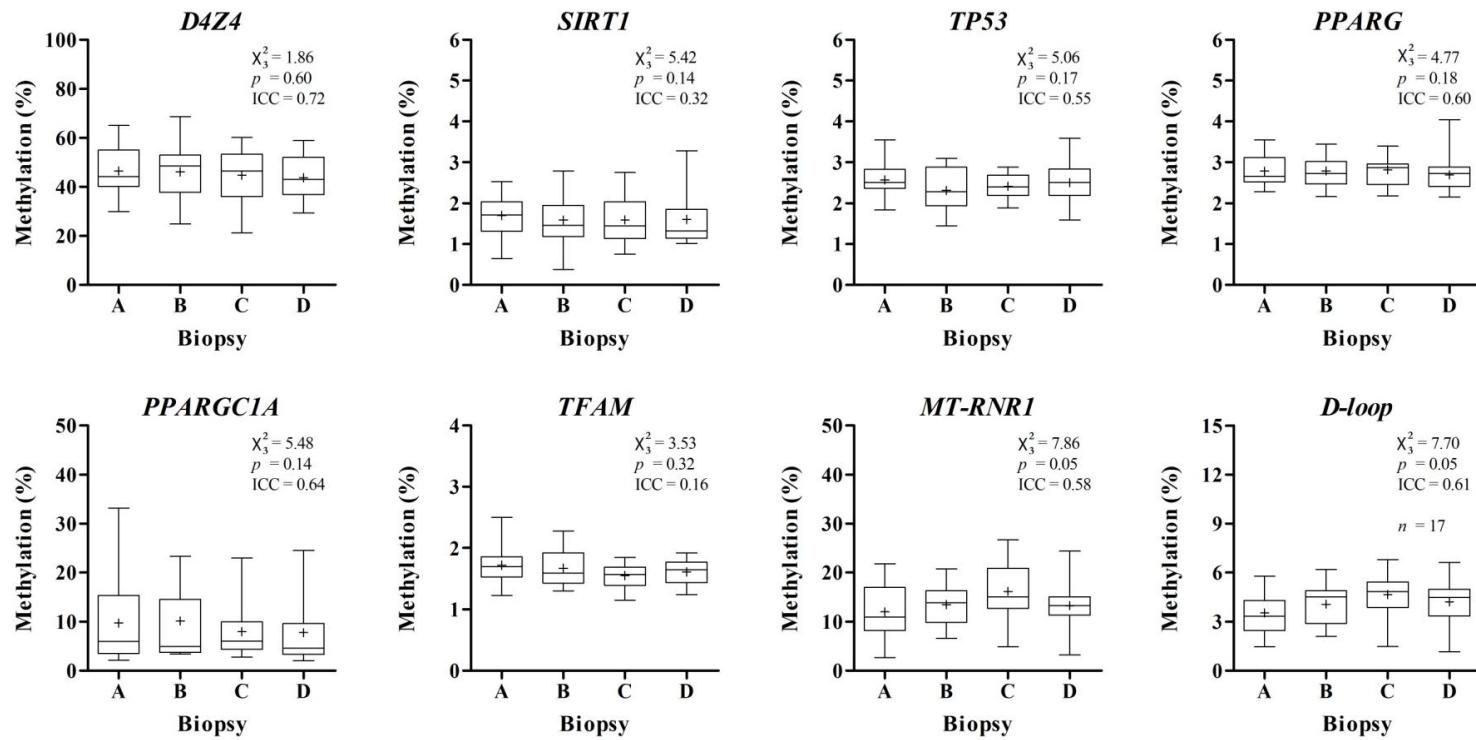


Figure S1. Methylation levels of each biopsy averaged by all individuals. All four biopsies (A-D) for each of the eight targets are represented by box plots displaying the median with range and mean (+) of methylation levels (%). The Friedman's test statistic (χ^2_3) with additional p-value and intra-class correlation coefficient (ICC) are shown in the right upper corner of each panel. All data is presented for nineteen individuals (sample set B) with the exception of D-loop ($n = 17$).

CHAPTER 4

PLACENTAL MITOCHONDRIAL DNA AND *CYP1A1* METHYLATION AS POTENTIAL BIOMARKERS FOR LOW BIRTH WEIGHT IN SMOKING PREGNANT WOMEN

Bram G. Janssen,¹ Wilfried Gyselaers,^{2,3} Hyang-Min Byun,⁴ Harry A. Roels,^{1,5} Ann Cuypers,¹ Andrea A. Baccarelli,⁴ Tim S. Nawrot^{1,6}

¹ Centre for Environmental Sciences, Hasselt University, Hasselt, Belgium

² Department of Obstetrics, East-Limburg Hospital, Genk, Belgium

³ Department of Physiology, Hasselt University, Diepenbeek, Belgium

⁴ Laboratory of Environmental Epigenetics, Exposure Epidemiology and Risk Program, Harvard School of Public Health, Boston, MA, USA

⁵ Louvain Centre for Toxicology and Applied Pharmacology (LTAP), Université catholique de Louvain, Brussels, Belgium

⁶ Department of Public Health & Primary Care, Leuven University, Leuven, Belgium

In preparation

ABSTRACT

Background: Maternal smoking during pregnancy results in an increased risk of low birth weight through perturbations in the *utero*-placental exchange. Epigenetics and mitochondrial function in fetal tissues might be responsive molecular signatures to *in utero* tobacco smoke exposure.

Methods: In the framework of the ENVIRONAGE birth cohort, we investigated the effect of self-reported tobacco smoke exposure during pregnancy on birth weight and the relation of fetal tissue markers including 1/ relative mitochondrial DNA (mtDNA) content, determined by real-time quantitative PCR, 2/ DNA methylation of mtDNA, and 3/ DNA methylation of the biotransformation gene *CYP1A1*, both determined by bisulfite-pyrosequencing. The total sample included 255 non-smokers, 65 former-smokers defined as those who had quit smoking before pregnancy, and 62 smokers.

Results: Mothers who continued smoking during pregnancy delivered newborns with an average reduced birth weight of -208 g (95% confidence interval [CI]: -318 to -99 g, $p = 0.0002$) compared with mothers that did not smoke during pregnancy. The relative mtDNA content was lower (-21.6%, 95% CI: -35.4 to -4.9%, $p = 0.01$), while absolute mtDNA methylation levels were higher (+0.62%, 95% CI: 0.21 to 1.02%, $p = 0.003$) in the exposed group. Lower absolute CpG-specific methylation of *CYP1A1* (-4.57%, 95% CI: -7.15 to -1.98%, $p < 0.0001$) was observed in placental tissues of smokers compared with non-smokers. We observed no sign of mediation of placental molecular signatures between the association of smoking and birth weight.

Conclusions: mtDNA content, mtDNA methylation, and *CYP1A1* methylation in placental tissue can serve as molecular signatures between the association of gestational tobacco smoke exposure and low birth weight.

INTRODUCTION

The consequences of adverse insults during the *in utero* period on childhood's health, and later in life, is a growing area of research interest with major public health implications. It is well known that maternal smoking during pregnancy increases the risk of low birth weight^{180,181} and preterm delivery,^{111,182} which is probably due to perturbations in the fetoplacental exchange.¹⁸³ The precise mechanisms underlying these adverse effects remain unclear, but emerging data suggests that biochemical, genetic, and epigenetic activities respond to and are modified by *in utero* tobacco exposure in fetal tissues.

Tobacco smoke is made up of more than 7,000 chemicals, consisting of particle and gaseous phases, and includes over 70 substances known to cause cancer.¹⁸⁴ Constituents of tobacco smoke such as polycyclic aromatic hydrocarbons (PAHs) enter cells and activate genes involved in detoxification such as *CYP1A1* (cytochrome P450, family 1, subfamily A, polypeptide 1) via the aryl hydrocarbon (Ahr) signaling pathway, resulting in a cellular oxidative imbalance. Mitochondrial DNA (mtDNA), which resides as multiple double stranded circular copies in mitochondria, is extremely vulnerable and responsive to tobacco-induced oxidative stress.^{132,185,186} As a result, alterations in mtDNA content, i.e. mtDNA copies, is an indication of dysfunctional or damaged mitochondria.⁸⁶ The inter-genomic crosstalk between mitochondria and the nucleus is complex and growing evidence suggest that mitochondrial dysfunction has an effect upon the epigenetic landscape of the nuclear genome.^{74,75} DNA methylation is the most intensively studied epigenetic modification. Exposures to adverse environmental factors are important determinants for methylation programming during early life.¹⁸⁷ Global^{73,188-190} and gene-specific (e.g. *CYP1A1*)^{172,191-197} DNA methylation differences have been demonstrated in cord blood and placental cells of neonates from mothers who smoked during pregnancy. Disruption of the fetal methylome has been associated with adverse pregnancy outcomes and could provide an underlying mechanism through which smoking affects fetal growth.^{192,195,198}

While several independent studies described the effect of maternal smoking during pregnancy on birth weight, mitochondrial DNA, and DNA methylation, we integrated all these facets into the framework of the ENVIRONAGE birth cohort.

We hypothesized that gestational tobacco smoke exposure impacts all these molecular signatures in placental tissue and might mediate the effects on birth weight.

MATERIALS AND METHODS

Study population

In the present study, 382 mother-newborn pairs were enrolled in the ENVIRONAGE birth cohort in Belgium (acronym for ENVIROnmental influence ON early AGEing). All procedures were approved by the Ethical Committee of Hasselt University and East-Limburg Hospital in Genk, Belgium. The study design and procedures were previously described in detail.¹³³ Briefly, written informed consent was obtained from each participant who gave birth in the East-Limburg Hospital. Questionnaires and medical records consulted after birth provided information on maternal age, maternal education, smoking status, ethnicity, pre-pregnancy body mass index (BMI), gestational age, newborn's gender, Apgar scores, birth weight and length, parity, and ultrasonographic data. Maternal education was coded as 'low' (no diploma or primary school), 'middle' (high school) or 'high' (college or university degree). Based on the native country of the newborn's grandparents we classified his/her ethnicity as European-Caucasian when two or more grandparents were European, or non-European when at least three grandparents were of non-European origin. We asked the mothers whether they consumed alcohol during pregnancy, used medication, and how many times per week they exercised for at least 20 minutes. Information about maternal tobacco smoke exposure was self-reported and obtained by asking whether mothers smoked during pregnancy, whether they smoked at any time during their life or if they never smoked in their life. Mothers who had ever smoked had to fill out the amount of smoking years and cigarettes per day, before and during pregnancy. Furthermore, we have data on passive smoke exposure (indoor smoking by somebody else) and we asked whether the mother continued smoking before she knew she was pregnant and how long (weeks). Three exposure classes were defined for this analysis: non-smokers ($n = 255$), past-smokers ($n = 65$), active-smokers ($n = 62$). For this study, the only inclusion criterion was that mothers had to be able to fill out

questionnaires in Dutch and enrollment was equally spread over all seasons of the year.

Sample collection

Placentas were deep-frozen within 10 minutes after delivery. Placental specimens were taken for DNA extraction by minimally thawing the placentas. We took villous tissue (1 to 2 cm³) at a fixed location from the fetal side of the placenta, approximately 1-1.5 cm below the chorio-amniotic membrane, and preserved the biopsies at -80°C.¹²⁵ Genomic DNA was isolated from placental tissue using the QIAamp DNA mini kit (Qiagen, Inc., Venlo, the Netherlands) and stored at -80°C until further use.

DNA methylation analysis

We performed DNA methylation analysis by highly quantitative bisulfite-PCR pyrosequencing as previously described in detail.¹²⁵ Bisulfite conversions were performed using 1 µg of extracted genomic DNA with the EZ-96 DNA methylation Gold kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. We interrogated four CpG sites within the promoter region of the *CYP1A1* gene, two CpG sites in the *MT-RNR1* region, and three CpG sites in the *D-loop* region. Detailed information regarding primer sequences is given in **Supplemental Material, Table S1**. PCR amplification of regions of interest prior to pyrosequencing was performed in a total reaction volume of 30 µl, using 15 µl GoTaq Hot Start Green Master Mix (Promega, Madison, WI, USA), 10 pmol forward primer, 10 pmol reverse primer, 1 µl bisulfite-treated genomic DNA, and water. PCR products were purified and sequenced by pyrosequencing using the PyroMark Q96 MD Pyrosequencing System (Qiagen, Inc., Germantown, MD, USA). The degree of methylation was expressed as the percentage of methylated cytosines over the sum of methylated and unmethylated cytosines. The efficiency of the bisulfite-conversion process was assessed using non-CpG cytosine residues within the sequence. We assessed within-placenta variability in a random subset of nineteen placentas as previously described.¹²⁵

Mitochondrial DNA content analysis

mtDNA content was measured by determining the ratio of two mitochondrial gene copy numbers (*MTF3212/R3319* and *MT-ND1*) to two single-copy nuclear control genes (*RPLP0* and *ACTB*) using a quantitative real-time polymerase chain reaction (qPCR) assay as previously described¹³³ but with small modification. Isolated genomic DNA (12.5 ng) was added to 7.5 µl mastermix consisting of Fast SYBR® Green I dye 2x (5 µl/reaction), forward and reverse primer (each 0.3 µl/reaction) and RNase free water (1.9 µl/reaction), for a final volume of 10 µl per reaction. Primer sequences (**Supplemental Material, Table S1**) were diluted to a final concentration of 300 nM in the master mix. Samples were run in triplicate in 384-well format. qPCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with following thermal cycling profile: 20 s at 95°C (activation), followed by 40 cycles of 1 s at 95°C (denaturation) and 20 s at 60°C (annealing/extension), ending with melting curve analysis (15 s at 95°C, 15 s at 60°C, 15 s at 95°C). qBase software (Biogazelle, Zwijnaarde, BE) was used to normalize data and correct for run-to-run differences.¹⁹⁹

Statistical analysis

We used SAS software (version 9.2; SAS Institute Inc., Cary, NC, USA) for database management and statistical analysis. mtDNA content was log₁₀-transformed to improve normality. One-way ANOVA procedures and chi-square tests were used to examine the relationship between smoking and continuous or categorical characteristics respectively. We applied a conventional multiple linear regression to estimate the association between maternal smoking status and birth weight, length, and placental mtDNA content. The pyrosequencing-based DNA methylation analysis produced a methylation value (%) for each CpG of *CYP1A1* (four CpGs), *MT-RNR1* (two CpGs), and the *D-loop* region (three CpGs). Correlations between adjacent CpG sites within one gene or region were tested with Pearson correlation coefficients. With mixed-effect models, we took into account each CpG dinucleotide position and tested the association between maternal smoking status and gene-specific DNA methylation. We applied a Dunnett's test to correct for multiple comparisons of smokers and past-smokers to the reference group (non-smokers). Maternal alcohol consumption,

medication use, physical activity, maternal education, ethnicity, maternal age, pre-pregnancy BMI, parity, gestational age, and newborn's gender were all considered as possible confounders but only those associated with maternal smoking ($p \leq 0.05$) and that potentially could influence birth weight and length, mtDNA content or DNA methylation were considered for entry in the model. Newborn's gender, maternal age, gestational age, ethnicity, parity, and pre-pregnancy BMI were forced into the model regardless of the p -value, in addition to maternal education, and alcohol consumption. Q-Q plots of the residuals were used to test the assumptions of all linear models.

In a sensitivity analysis, Pearson correlation coefficients were calculated between the smoke- and pack-years or amount of cigarettes smoked during pregnancy and birth weight or length. Furthermore, we used mediation analysis to investigate whether our interrogated molecular signatures underlie the association between gestational tobacco smoke exposure and birth weight.²⁰⁰

RESULTS

Participant demographic and lifestyle factors

Demographic characteristics and perinatal factors of 382 mother-newborn pairs are reported in **Table 1**. The newborns, among them 194 girls (50.8%), had a mean gestational age of 39.2 weeks (range, 35-42) and comprised 200 (52.4%) primiparous and 142 (37.2%) secundiparous newborns. The mean (SD) birth weight of the newborns was 3,429 (426) g and birth length 50.3 (1.9) cm. About 90% ($n = 332$) of the newborns were Europeans of Caucasian ethnicity. Mean maternal age was 29.0 years (range: 18–42 yr). Most women (66.7%, $n = 255$) never smoked cigarettes and 65 women (17.0%) stopped smoking before pregnancy, whereas 62 mothers (16.2%) reported to have smoked during pregnancy (on average 7.8 cigarettes per day, inter quartile range [IQR]: 5-10). A fair number of mothers ($n = 73$, 19.1%) occasionally consumed alcohol during their pregnancy.

In addition to birth weight and length, we observed that maternal age was lower (27.7 ± 4.8 vs. 29.4 years ± 4.5 , $p = 0.008$) and not as much women were high educated (22.6% vs. 59.6%, $p < 0.0001$) in the smoking group compared with

non-smokers. Alcohol consumption was higher in the former smoking group compared with non-smokers (30.8% vs. 16.5%, $p = 0.01$).

Table 1. Characteristics of mother-newborn pairs according to gestational tobacco smoke exposure

Variable	All (n = 382)	Self-reported non-smokers (n = 255)	Self-reported past-smokers (n = 65)	Self-reported smokers (n = 62)	p-value*
Newborn					
Gender					0.47
Male	188 (49.2%)	120 (47.1%)	34 (52.3%)	34 (54.8%)	
Female	194 (50.8%)	135 (52.9%)	31 (47.7%)	28 (45.2%)	
Ethnicity					0.73
European-Caucasian	332 (86.9%)	223 (87.4%)	57 (87.7%)	52 (83.9%)	
Non-European	50 (13.1%)	32 (12.6%)	8 (12.3%)	10 (16.1%)	
Gestational age, w	39.2 ± 1.2	39.3 ± 1.2	39.2 ± 1.3	39.2 ± 1.2	0.82
Birth weight, g	3,429 ± 426	3,472 ± 424	3,437 ± 423	3,247 ± 395	0.0009
Birth length, cm	50.3 ± 1.9	50.5 ± 2.0	50.5 ± 1.7	49.5 ± 1.8	0.0007
Maternal					
Age, y	29.0 ± 4.7	29.4 ± 4.5	28.9 ± 4.9	27.7 ± 4.8*	0.03
Pre-pregnancy BMI, kg/m ²	24.3 ± 4.5	24.2 ± 4.4	24.8 ± 5.3	24.2 ± 4.1	0.65
Maternal education					<0.0001
Low	51 (13.3%)	26 (10.2%)	6 (9.2%)	19 (30.6%)	
Middle	131 (34.3%)	77 (30.2%)	25 (38.5%)	29 (46.8%)	
High	200 (52.4%)	152 (59.6%)	34 (52.3%)	14 (22.6%)	
Medication use†					0.57
None	134 (37.8%)	92 (39.5%)	21 (32.3%)	21 (37.5%)	
Alcohol consumption					0.03
Occasionally	73 (19.1%)	42 (16.5%)	20 (30.8%)	11 (17.7%)	
Physical activity (> 20 min)‡					0.40
< 1 times per week	122 (33.2%)	82 (33.3%)	19 (29.7%)	21 (36.8%)	
1 times per week	86 (23.4%)	63 (25.6%)	15 (23.4%)	8 (14.0%)	
> 2 times per week	159 (43.3%)	101 (41.1%)	30 (46.9%)	28 (49.2%)	
Parity					0.42
1	200 (52.4%)	132 (51.8%)	36 (55.4%)	32 (51.6%)	
2	142 (37.2%)	91 (35.7%)	25 (38.5%)	26 (41.9%)	
≥ 3	40 (10.5%)	32 (12.5%)	4 (6.1%)	4 (6.5%)	
Cigarettes before pregnancy	-		11.0 ± 6.9	10.3 ± 6.7	
Cigarettes during pregnancy	-	-		7.8 ± 4.6	

Data are presented as arithmetic means ± standard deviation (SD) or number (%).

† Medication use consistent of occasionally use of paracetamol or antibiotics (28 missing data).

‡ Missing data for 15 subjects.

* p-value derived from one-way ANOVA or chi-square tests in case of continuous or categorical variables respectively.

Smoking status and birth parameters

Mothers who continued smoking during pregnancy delivered newborns with lower birth weight (-208 g; 95% CI: -318 to -99 g, $p = 0.0002$) and smaller length (-1.0 cm 95% CI: -1.5 to -0.5 cm, $p < 0.0001$ respectively) compared with non-smoking mothers independently of maternal age, gestational age, newborn's gender, maternal education, ethnicity, parity pre-pregnancy BMI, and alcohol consumption. Mothers who stopped smoking before pregnancy did not show altered birth weight ($p = 0.55$) or length ($p = 0.87$).

Smoking status and mtDNA in placental tissue

In placental tissue, mtDNA content was strongly correlated with mtDNA methylation ($r = -0.63$, $p < 0.0001$). After adjustment for the aforementioned covariates and confounders, the relative mtDNA content in placental tissue was -21.6% (95% CI: -35.4 to -4.9%, $p = 0.01$) lower in smoking mothers, but not in past-smokers ($p = 0.72$), compared with non-smokers (**Figure 1**). In contrast, absolute methylation levels of the mitochondrial genome at the *MT-RNR1* gene were higher in mothers who continued smoking during pregnancy (+0.62%, 95% CI: 0.21 to 1.02%, $p = 0.003$) and marginally higher in mothers who stopped smoking prior to pregnancy (+0.37%, 95% CI: -0.02 to 0.75%, $p = 0.06$) compared with non-smokers (**Figure 1**). We found no interaction between smoking status and CpG site of *MT-RNR1* ($p_{int} = 0.94$) and methylation levels at the *D-loop* region did not differ between groups ($p = 0.85$).

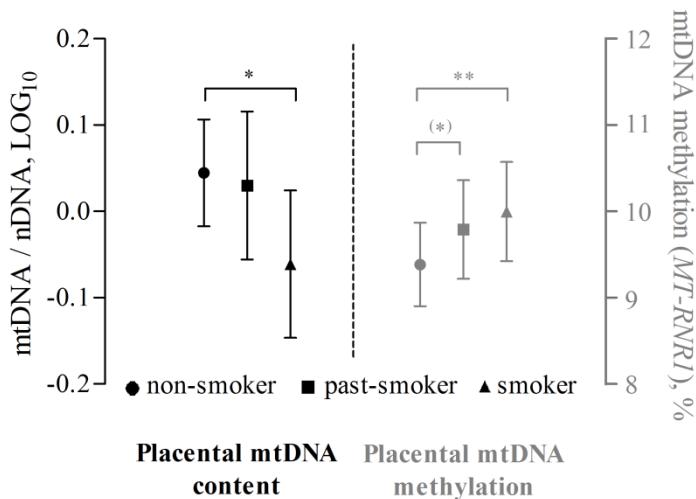


Figure 1. Estimated mean levels of mtDNA content and mtDNA methylation in placental tissue of non-smokers ($n = 255$), past smokers ($n = 65$), and current smokers ($n = 62$). The bars represent the estimated means with 95% confidence intervals for the non-smoking (●), past-smoking (■), and smoking group (▲). mtDNA content levels, displayed in black, are \log_{10} -transformed and methylation of the *MT-RNR1* gene, displayed in grey, are absolute methylation levels. Both the generalized linear model for mtDNA content and the mixed model for mtDNA methylation were adjusted for maternal age, gestational age, newborn's gender, maternal education, ethnicity, parity, pre-pregnancy BMI, and alcohol consumption. * p -value < 0.05; ** p -value < 0.005; (*) p -value = 0.06 difference compared to the non-smoking reference group.

Smoking status and gene-specific *CYP1A1* methylation in placental tissue

The examined CpGs in the promoter region of *CYP1A1* showed strong correlation in placental tissue ($r = 0.71$ to 0.93 , $p < 0.0001$) (**Supplemental Material, Figure S1**). Unadjusted mixed modeling revealed an interaction effect between smoking status and CpG sites in placental tissue ($p_{int} < 0.0001$) (**Figure 2**). Placental methylation levels at CpG3 were significantly lower in mothers who continued smoking during pregnancy compared with non-smoking mothers (-4.57%, 95% CI: -7.15 to -1.98, $p < 0.0001$), independently of maternal age, gestational age, newborn's gender, maternal education, ethnicity, parity, pre-pregnancy BMI, and alcohol consumption (**Table 2**). We did not observe significant differences in methylation levels in mothers who stopped smoking before pregnancy.

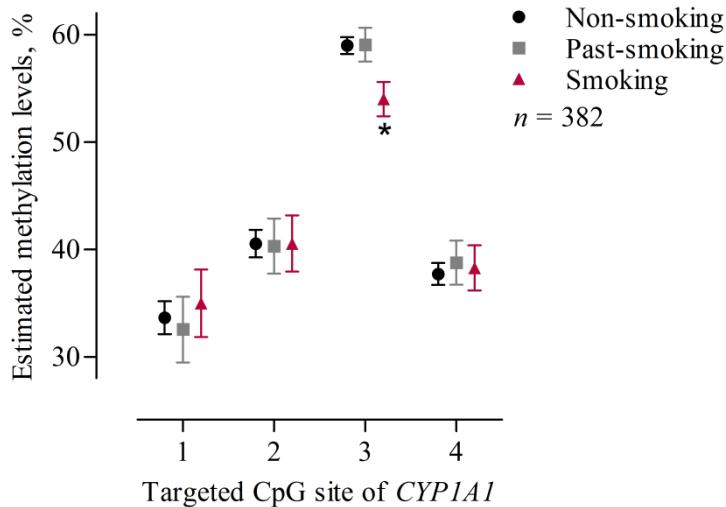


Figure 2. Unadjusted estimated methylation levels in percentage (%) at four targeted CpG sites within the *CYP1A1* promoter region of placental tissue ($n = 382$). Estimated methylation levels at each CpG are indicated for each smoking category (black line: non-smokers [$n = 255$]; grey line: past-smokers [$n = 65$]; red line: smokers [$n = 62$]). The error bars display the 95% confidence intervals.

Table 2. Effect of gestational tobacco smoke of CpG sites of *CYP1A1* in placental tissue ($n = 382$)

<i>CYP1A1</i> methylation	Non- smoking		Past-smoking		Smoking	
		% (95% CI)		p-value	% (95% CI)	p-value
Placental tissue†						
CpG 1	Ref	-1.16 (-5.90 to 3.56)	0.99	1.75 (-3.09 to 6.60)	0.95	
CpG 2	Ref	-0.30 (-4.25 to 3.66)	0.99	0.43 (-3.63 to 4.49)	0.99	
CpG 3	Ref	0.02 (-2.47 to 2.51)	0.99	-4.57 (-7.15 to -1.98)	< 0.0001	
CpG 4	Ref	1.00 (-2.11 to 4.11)	0.98	0.98 (-2.22 to 4.19)	0.99	

† Estimated percentage change in methylation levels for each CpG of *CYP1A1* compared to the non-smoking group (reference). The 95% CI and p-values are adjusted values of the Dunnett's procedure.

Mixed models are adjusted for maternal age, gestational age, newborn's gender, maternal education, ethnicity, parity, pre-pregnancy BMI, and alcohol consumption.

Sensitivity analysis

As anticipated, we observed a clear dose-response relation with between smoke-and pack-years or the amount of cigarettes smoked during pregnancy and birth weight and length. We did not observe a significant difference in birth weight or length between mothers who stopped smoking for a longer period, nor between mothers that stopped prior to pregnancy, compared to non-smokers. We observed a positive association between *CYP1A1* methylation levels and placental mtDNA content ($r = 0.14, p = 0.005$), while a negative association was observed between with placental mtDNA methylation ($r = -0.11, p = 0.02$) (**Figure 3**). Furthermore, we observed no sign of mediation of *CYP1A1* methylation, or any other investigated molecular signature, between the association of tobacco smoke exposure and birth weight (data not shown).

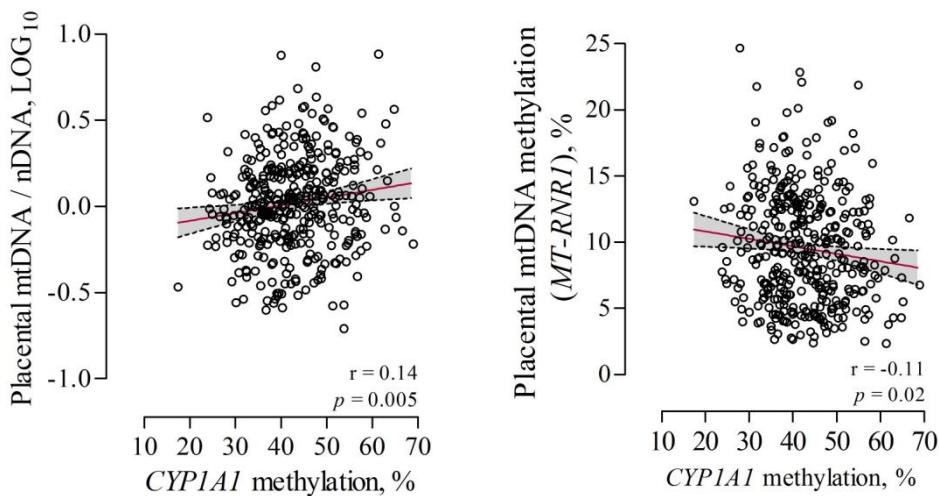


Figure 3. Correlation between *CYP1A1* methylation levels (%) and mtDNA content (\log_{10}) or mtDNA methylation (%) in placental tissue. The correlation plot depicts 95% CI as dashed lines.

DISCUSSION

In the present study, we showed that women who smoked during pregnancy had 1/ neonates with lower birth weight, 2/ lower mtDNA content, 3/ higher mtDNA methylation, and 4/ higher CpG-specific methylation levels of *CYP1A1* in placental tissue. The study was embedded in the Belgian ENVIRONAGE birth cohort, a population-based prospective cohort study from early life onwards.

Despite a limited number of (epi)genomic studies in placental tissue and cord blood, we are getting a better picture on the molecular pathways underlying the association between gestational tobacco smoke exposure and low birth weight. Suter and colleagues employed side-by-side gene expression and epigenome-wide methylation arrays and showed that the expression of 623 genes and the methylation of 1,024 CpG dinucleotides were significantly altered among placentas of smokers.¹⁹⁷ 438 genes revealed a significant correlation between methylation and gene expression and their potential functions or mechanisms were investigated using an Ingenuity Pathway Analysis. The authors found that the gene list was enriched for genes involved in functional pathways such as mitochondrial dysfunction, oxidative phosphorylation, and hypoxia pathways. Indeed, mitochondria, the ‘powerhouses’ of our cells, provide cellular energy via oxidative phosphorylation and are very sensitive to exposures that induce oxidative stress. The double stranded circular mtDNA that resides in multiple copies in mitochondria is vulnerable to reactive oxygen species (ROS) due to an inefficient DNA repair capacity and close proximity to the electron transport chain.²⁰¹ The estimated mutation rate of mtDNA is 5-10 times higher compared to nuclear DNA.⁸² Given the vital role of placental mitochondria, we showed that mtDNA content and mtDNA methylation were responsive to gestational tobacco smoke which affirms mtDNA as a sensitive marker of mitochondrial damage and dysfunction as proposed by Sahin *et al.*⁸⁶ Although there are other studies reporting changes in placental mtDNA content in smoking mothers^{132,185} or mothers exposed to air pollution,¹³³ we provide the first epidemiological evidence of altered methylation levels at the mitochondrial genome of placental tissue, but not of cord blood, in response to gestational tobacco exposure. We suggest that pollution-induced epigenetic modifications of the mitochondrial genome may prime alterations in mtDNA content by regulating mitochondrial function and biogenesis.¹³⁵ Damaged or non-functioning mitochondria are

specifically degraded through mitophagy and could result in a depletion of mtDNA,²⁰² which moreover, lead to changes in methylation patterns of a number of nuclear genes.⁷⁵ In our sensitivity analysis, we observed that mtDNA content and mtDNA methylation correlated with methylation of *CYP1A1* in placental tissue, which indeed, could be indicative of a relationship between mitochondrial dysfunction and the epigenetic landscape of the nuclear genome.⁷⁴ Whether mitochondrial dysfunction affects gene expression and methylation patterns of other genes needs to be determined.

An expanding body of evidence suggest that the epigenetic system of placental tissue and cord blood is sensitive to environmental exposures.¹⁸⁷ Epigenome-wide methylation studies are used to examine the epigenetic state of our genome at many different loci in a number of individuals and to assess whether any of these loci, or CpGs, are associated with a trait or environmental pollutant.¹⁷⁰ A 450K epigenome-wide methylation study performed by Joubert *et al.* demonstrated differentially methylated detoxifying genes (*AHRR* and *CYP1A1*) in cord blood of newborns exposed to tobacco smoke during pregnancy.¹⁷² This finding was confirmed in an independent population of infants by analyzing whole blood obtained by heel sticks.¹⁹⁶ More specifically, maternal smoking, assessed by both self-reporting and cotinine levels, displayed higher methylation levels at different CpGs of *CYP1A1* in cord blood which is in accordance with our results in cord blood. Conversely, in placental tissue of smokers, Suter and colleagues¹⁹¹ observed that the CpG dinucleotides proximal to a xenobiotic response element (XRE) were hypomethylated, whereas those distal from such elements did not demonstrate differential methylation. The authors calculated the total percentage of methylation for a distinct region of the promoter (-1411 to -1295 bp from the transcription start site), without taking the CpGs separately into account like we did in our study. We observed lower methylation levels at a specific CpG site that lies adjacent to a XRE site in placental tissue of mothers who smoked during pregnancy. It is important to note that this specific CpG site harbors a C/G single nucleotide polymorphism (SNP: rs3809585 with allele frequencies C: 1.717% and G: 98.283%). We are confident that this SNP did not affect DNA methylation since all pyrograms confirmed a G nucleotide in the analyzed sequence. Interestingly, the study of Joubert *et al.* in cord blood, the study of Suter *et al.* in placental tissue, and our

study in placental tissue, interrogated approximately the same region and CpGs although with different detection methods (**Figure 4**). With our candidate bisulfite pyrosequencing approach, we confirmed that there are highly CpG-specific responses at the *CYP1A1* gene in placental tissue that are opposite to that of cord blood reported by Joubert and colleagues.¹⁷² Although we lacked meaningful gene expression data of *CYP1A1* in our study, Suter and colleagues previously showed that lower methylation levels in a region covering the XRE site were correlated with increased expression of *CYP1A1* in placental tissue.¹⁹¹ Moreover, other studies demonstrate increased *CYP1A1* mRNA²⁰³ and protein²⁰⁴ expression in human placentas in response to tobacco smoke exposure. Constituents of tobacco smoke such as PAHs enter cells and are recognised by the aryl hydrocarbon receptor (Ahr) causing its translocation to the nucleus and the formation of a heterodimer with the Ahr nuclear translocator protein (ARNT). This complex binds to genes with a XRE within the promoter and initiates expression of detoxifying enzymes involved in phase I and II xenobiotic metabolism.²⁰⁵

The main limitation of the study is the chance of exposure misclassification. Information about maternal smoking during pregnancy was self-reported by a questionnaire and cannot be verified. A possibility to overcome this limitation is the usage of maternal urinary cotinine, however, previous studies demonstrated that this method is not superior to self-reported smoking.¹⁸¹ Lastly, we acknowledge the fact that we cannot fully exclude residual or unmeasured confounding by other factors that could be associated with both tobacco smoke exposure and placental molecular signatures. Although a causal relationship exists between prenatal tobacco smoke and low birth weight or preterm birth, not all infants exposed to tobacco smoke develop these adverse perinatal outcomes. It is therefore reasonable to assume that several interactions exist between tobacco smoke exposure and biochemical, genetic, and epigenetic factors, which make the fetus more susceptible to changes in fetal programming. Taken together, mitochondrial responses and the methylation burden can be used to evaluate the effect of tobacco smoke exposure on the fetal methylome.

CONCLUSION

This study provides epidemiological evidence of molecular changes in placental tissue that can serve as biomarkers of exposure to tobacco smoke during pregnancy. Whether the molecular signatures described in our study are related to developmental defects in Belgian children will be investigated in an ongoing study follow-up study of the ENVIRONAGE birth cohort.

Acknowledgments

The authors thank the participating women and neonates, as well as the staff of the maternity ward, midwives, and the staff of the clinical laboratory of East-Limburg Hospital in Genk. The ENVIRONAGE birth cohort is supported by the EU Program 'Ideas' (ERC-2012-StG 310898), and by the Flemish Scientific Fund (FWO, N1516112/G.0.873.11.N.10).

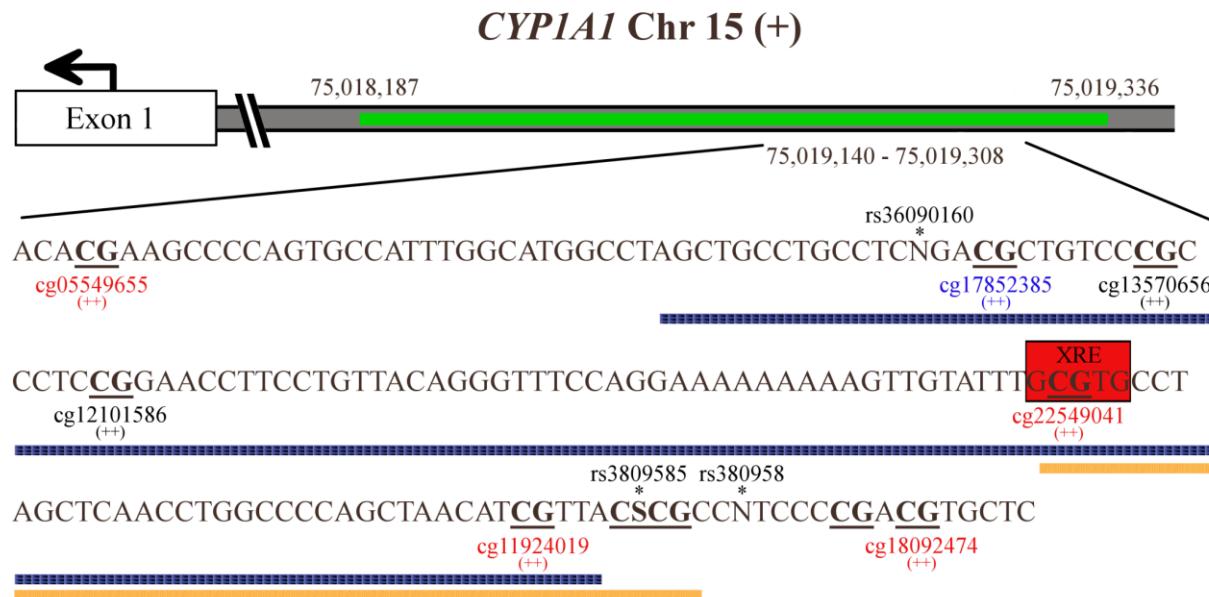


Figure 4. CpG sites located on the shore of a CpG island in a bidirectional regulatory region of the **CYP1A1 gene.** The CpG island is depicted in green with a distinct portion magnified (chr15:75,019,140-75,019,308). CpG sites are denoted in bold and underlined whereas possible SNPs are indicated with an asterisk. The orange bar represents the analyzed sequence in our study and includes four CpG sites. The blue bar represents the analyzed sequence in placental tissue derived from the article of Suter *et al.*¹⁹¹ and includes five CpG sites. The cg probes that were investigated in the 450K study of Joubert *et al.* in cord blood¹⁷² are displayed with the color representing the statistical significance of the association between cotinine and methylation of the probe (blue: $p > 1 \times 10^{-5}$; black: $1 \times 10^{-5} \geq p \geq 1 \times 10^{-7}$; red: $p < 1 \times 10^{-7}$) and the magnitude of effect (++: higher methylation). The information on the figure is based on the UCSC Genome Browser on Human Feb. 2009, GRCh37/hg19.

SUPPLEMENTAL MATERIAL

Table S1. Bisulfite-pyrosequencing, mitochondrial and nuclear primer sequence information based upon Assembly GRCh37/hg19 of the UCSC genome browser

Gene symbol	Chr	Amplicon start-end	Primer set (Forward / Reverse / Sequence) [†]	Amplicon (bp)	Target CpGs
Pyrosequencing					
<i>CYP1A1</i>	15	75013061- 75017877	F: 5'-TGTATAGGGTTTTAGGAAAAA-3' R: 5'- <u>AAATTATTTCTAACCTAACCAAC</u> -3' S: 5'-AAAAAAAGTTGTATTG-3'	147	4
<i>MT-RNR1</i>	M	1191- (+)	F: 5'-TTTTAGAGGAGTTGTTGTAAT-3' R: 5'- <u>ATAACCCATTCTTACCACCTCATA</u> -3' S: 5'-AGTTTGTTTGTAAAT-3'	176	2
<i>D-loop</i>	M	6- (-)	F: 5'-TGTGTAGATATTAATTGTTATT-3' R: 5'- <u>CAAATCTATCACCTATTAACCAC</u> -3' S: 5'-TAATTAATTAATATATT-3'	254	3
mtDNA content					
<i>MTF3212/R3319</i>	M	3213- 3320	F: 5'-CACCAAGAACAGGGTTGT-3' R: 5'-TTAACAAACATACCCATGGCCA-3'	108	-
<i>MT-ND1</i>	M	3314- 3428	F: 5'-ATGCCAACCTCTACTCCT-3' R: 5'-AAAGGCCAACGTTGTAG-3'	115	-
<i>RPLP0</i>	12	120636904- 120636988	F: 5'-CCCAATTGTCCTTACCT-3' R: 5'-GAACACAAGCCCACATCC-3'	85	-
<i>ACTB</i>	7	5567833- 5567934	F: 5'-ACTCTCCAGCCTCTTCC-3' R: 5'-TGTGGAAGCTAAGTCCTGCC-3'	102	-

[†] The underlined sequence is the biotinylated primer.

Abbreviations: Cytochrome P450, family 1, subfamily A, polypeptide 1 (*CYP1A1*); Mitochondrial region RNR1 (*MT-RNR1*), Displacement loop (*D-loop*); mitochondrial forward primer from nucleotide 3212 and reverse primer from nucleotide 3319 (*MTF3212/R3319*); Mitochondrial encoded NADH dehydrogenase 1 (*MT-ND1*); Acidic ribosomal phosphoprotein P0 (*RPLP0*); Beta actin (*ACTB*).

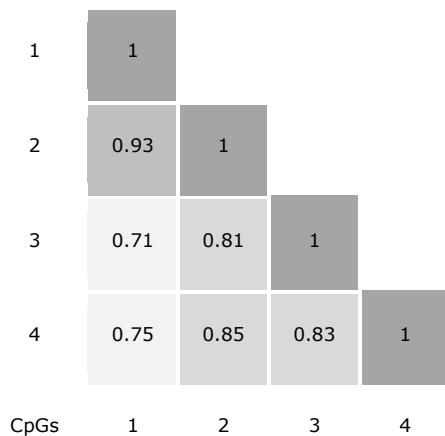


Figure S1. Pearson correlation coefficients between CpG sites of *CYP1A1* gene in placental tissue ($n = 382$). All p -values were < 0.0001 .

CHAPTER 5

PLACENTAL DNA HYPMETHYLATION IN ASSOCIATION WITH PARTICULATE AIR POLLUTION IN EARLY LIFE

Bram G. Janssen,¹ Lode Godderis,^{2,3} Nicky Pieters,¹ Katrien Poels,² Michał Kiciński,¹ Ann Cuypers,¹ Frans Fierens,⁴ Joris Penders,^{5,6} Michelle Plusquin,¹ Wilfried Gyselaers,^{6,7} Tim S. Nawrot^{1,2}

¹ Centre for Environmental Sciences, Hasselt University, Hasselt, Belgium

² Department of Public Health & Primary Care, Leuven University, Leuven, Belgium

³ Idewe, External Service for Prevention and Protection at Work, Heverlee, Belgium

⁴ Belgian Interregional Environment Agency, Brussels, Belgium

⁵ Laboratory of Clinical Biology, East-Limburg Hospital, Genk, Belgium

⁶ Department of Physiology, Hasselt University, Diepenbeek, Belgium

⁷ Department of Obstetrics, East-Limburg Hospital, Genk, Belgium

ABSTRACT

Background: There is evidence that altered DNA methylation is an important epigenetic mechanism in prenatal programming and that developmental periods are sensitive to environmental stressors. We hypothesized that exposure to fine particles ($PM_{2.5}$) during pregnancy could influence DNA methylation patterns of the placenta.

Methods: In the ENVIRONAGE birth cohort, levels of 5'-methyl-deoxycytidine (5-mdC) and deoxycytidine (dC) were quantified in placental DNA from 240 newborns. Multiple regression models were used to study placental global DNA methylation and *in utero* exposure to $PM_{2.5}$ over various time windows during pregnancy.

Results: $PM_{2.5}$ exposure during pregnancy averaged (25th-75th percentile) 17.4 (15.4-19.3) $\mu g/m^3$. Placental global DNA methylation was inversely associated with $PM_{2.5}$ exposures during whole pregnancy and relatively decreased by 2.19% (95% confidence interval (CI): -3.65, -0.73%, $p = 0.004$) for each 5 $\mu g/m^3$ increase in exposure to $PM_{2.5}$. In a multi-lag model in which all three trimester exposures were fitted as independent variables in the same regression model, only exposure to $PM_{2.5}$ during trimester 1 was significantly associated with lower global DNA methylation (-2.13% per 5 $\mu g/m^3$ increase, 95% CI: -3.71, -0.54%, $p = 0.009$). When we analyzed shorter time windows of exposure within trimester 1, we observed a lower placental DNA methylation at birth during all implantation stages but exposure during the implantation range (6-21d) was strongest associated (-1.08% per 5 $\mu g/m^3$ increase, 95% CI: -1.80, -0.36%, $p = 0.004$).

Conclusions: We observed a lower degree of placental global DNA methylation in association with exposure to particulate air pollution in early pregnancy, including the critical stages of implantation. Future studies should elucidate genome-wide and gene-specific methylation patterns in placental tissue that could link particulate exposure during *in utero* life and early epigenetic modulations.

INTRODUCTION

The human placenta forms the interface between the fetal and maternal circulation and plays a critical role in the regulation of fetal growth and development through controlled nutrient supply. Fetal adaptations and developmental plasticity arising from perturbations in utero-placental exchange to meet fetal requirements, 'program' the fetus for an increased risk of developing cardiovascular disease and diabetes in adult life.^{1,52} Epigenetic modifications, described as heritable changes in genes function that cannot be explained by changes in the underlying DNA sequence, are believed to play an essential role in this process. With exception of imprinted genes, all DNA methylation patterns are established during embryogenesis, and play an important role in gene regulation which could comprise a biologically plausible link between *in utero* exposures and disease risks during adulthood.¹²⁹

Several studies support evidence of detrimental effects of particulate matter (PM) on the health outcomes of fetuses,^{206,207} neonates^{49,208} and is later in life associated with cardiovascular morbidity and mortality.^{6,209,210} DNA methylation is, besides histone modification and non-coding RNAs, a well-characterized epigenetic modification that may provide an attractive mechanism linking particulate air pollution in early life and health consequences in adulthood.²¹¹ Indeed, data from the Dutch Hunger Winter (1944-45) indicate that prenatal environmental conditions can cause epigenetic changes in humans that persist throughout life.⁶⁶ In addition to these observational data, animal studies showed that certain transient environmental influences during *in utero* life could produce persistent changes in epigenetic marks that have life-long consequences.^{63,65}

Alterations in DNA methylation patterns are mediated by several factors and have been associated with many different health outcomes.²¹² Evidence from animal and human studies in adults indicate that particulate air pollution may affect global and gene-specific methylation.⁶⁷⁻⁷⁰ A number of studies⁷¹⁻⁷³ describe DNA methylation patterns in placental tissue but the association between particulate air pollution and DNA methylation in placental tissue has never been investigated.

Within the ENVIRONAGE birth cohort, we determined whether exposure to ambient particulate matter ($PM_{2.5}$) during different periods of prenatal life is associated with differences in global DNA methylation of placental tissue at birth.

MATERIALS AND METHODS

Study population and data collection

The Ethics Committee of Hasselt University and East-Limburg Hospital approved the protocol of the ENVIRONAGE birth cohort study.¹³³ Between Friday 1200 hours and Monday 0700 hours from 10 February 2010 until 21 January 2012, we recruited 258 mother-infant pairs (only singletons) in the East-Limburg Hospital in Genk, Belgium. The catchment area of the hospital includes the province Limburg in Belgium and combines both urban and suburban to rural areas with population densities between the municipalities ranging from 82 to 743 inhabitants/km².

The placenta could not be collected from six newborns, DNA yield was insufficient for ten placentas and two newborns had missing data for $PM_{2.5}$ exposure. We ended with a final sample size of 240 newborns. The only inclusion criterion was that mothers had to be able to fill out questionnaires in Dutch. Enrollment was spread equally over all seasons of the year. The overall participation rate of eligible mothers was 56%. Participating mothers provided written informed consent when they arrived at the hospital for delivery, and completed study questionnaires in the postnatal ward after delivery to provide detailed information on age, pre-pregnancy body mass index (BMI), maternal education, occupation, smoking status, alcohol consumption, place of residence, use of medication, parity, and newborn's ethnicity. Maternal education was coded as 'low' (no diploma or primary school), 'middle' (high school) or 'high' (college or university degree). Past-smokers were defined as those who had quit before pregnancy and smokers as having smoked before and during pregnancy.

Samples of placental tissue were collected immediately after delivery, along with perinatal parameters such as newborn's gender, birth date, birth weight and length, gestational age (range, 35-42 weeks), Apgar score, and ultrasonographic data. All neonates were assessed for congenital anomalies immediately after

birth and were considered healthy with an Apgar score after 5 min ranging between 7 and 10. No neonate was delivered in the Neonatal Intensive Care Unit. The ENVIRONAGE birth cohort generally consists of mothers with normal pregnancies without complications and with healthy neonates.

Sample collection

Placentas were obtained from 252 mothers and deep-frozen within 10 minutes after delivery. Afterwards, we thawed placentas to take tissue samples (each biopsy was approximately 1 to 2 cm³) for DNA extraction following a standardized protocol as described by Adibi *et al.*¹⁶⁵ Briefly, villous tissue, protected by the chorio-amniotic membrane, was biopsied from the fetal side of the placenta and preserved at -80°C. We assessed within-placenta variability in a random subset of 7 placentas by comparing biopsies taken at four standardized sites across the middle region of the placenta, approximately 4 cm away from the umbilical cord. The first biopsy was taken to the right of the main artery and the three other biopsies in the remaining quadrants of the placenta. Methylation levels within each placenta varied by a mean of 4.5% (CV) across the quadrants (ICC = 0.25). To minimize the impact of within-placental variability, biopsies were all taken 1-1.5 cm below the chorio-amniotic membrane at a fixed location by using a device to orientate the fetal side of the placenta in relation to the umbilical cord. Care was taken by visual examination and dissection to avoid the chorio-amniotic membrane contamination.

Exposure estimates

We interpolated the regional background levels of PM_{2.5} for each mother's residential address using a spatial temporal interpolation method (Kriging) that uses land cover data obtained from satellite images (CORINE land cover data set) in combination with monitoring stations ($n = 34$).^{120,123,213} This model provides interpolated PM_{2.5} values from the Belgian telemetric air quality networks in 4 × 4 km grids. Based on 34 different locations validation statistics of the interpolation tool gave a temporal explained variance (R²) for hourly averages PM_{2.5} > 0.80 and a spatial R² for annual mean PM_{2.5} > 0.80.¹²³ To explore potentially critical exposures during pregnancy, individual mean PM_{2.5} concentrations (micrograms per cubic meter) were calculated for various

periods, for which the date of conception was estimated based on ultrasound data: each of the three trimesters of pregnancy, with trimesters being defined as: 1-13 weeks (trimester 1), 14-26 weeks (trimester 2) and 27 weeks to delivery (trimester 3); and early pregnancy stages, with windows being defined as: 1-5 days (pre-implantation), 6-12 days (implantation), 6-21 days (implantation range) and 22-28 days (post-implantation week 4). Also, the whole pregnancy exposure was calculated as the mean of all pregnancy days. We have complete residential information during and before pregnancy. For those that moved during pregnancy ($n = 14$; 5.8%), we calculated exposure windows accounting for the address changes during the period. Previously, our long-term exposure estimates have been validated by the association between modeled air pollution and carbon load in lung macrophages.²¹³ Additionally, NO₂ and maximum 8-hour average O₃ exposures were interpolated using the same methods as PM_{2.5} exposure.

The Royal Meteorological Institute (Brussels, Belgium) provided mean daily temperatures and relative humidity for the study region which were averaged using the same exposure windows as for PM_{2.5}. Apparent temperature was calculated by using the following formula: $-2.653 + (0.994 \times T_a) + (0.0153 \times T_d^2)$, where Ta is air temperature and Td is dew-point temperature (in degrees Celsius).^{214,215}

Global DNA methylation analysis

Genomic DNA was isolated from placental tissue using the MagMAX™ DNA Multi-Sample kit (Applied Biosystems, Foster City, CA, USA). Mean DNA yield was 80.0 ng/ μ l with purity values of 1.9 for A260/280 ratio and 2.0 for A260/230 ratio.

We determined global DNA methylation as previously published.^{216,217} Briefly, isolated genomic DNA samples were hydrolyzed to individual deoxyribonucleosides in a simplified one-step procedure.²¹⁸ A digest mix was prepared by adding 300 mU Phosphodiesterase I (Sigma Aldrich, P3134-100MG), 200 U alkaline phosphatase (Sigma Aldrich, P7923-2KU) and 250 U Benzonase® Nuclease (Sigma Aldrich, E1014-5KU) to 5 ml Tris-HCl buffer (pH 7.9, 20 mM) containing 100 mM NaCl and 20 mM MgCl₂. Extracted DNA (1 μ g diluted in 50 μ l HPLC-grade water) was hydrolyzed by adding 50 μ l digest mix

and incubating at 37°C for 24 h. After hydrolysis, water (HPLC-grade) was added to the samples up to a total volume of 1 ml. Reference standards for 5'-methyl-deoxycytidine (5-mdC) and deoxycytidine (dC) were purchased from Jena Bioscience (N-1044) and Sigma (D3897-1G) respectively. Stock solutions of 5-mdC and dC were prepared by dissolving the purchased solid reference standards in pure water (HPLC-grade). Using these stock solutions, a series of calibration solutions was prepared for 5-mdC and dC in a range of 0.1-10 ng/mL and 1-100 ng/mL respectively. The same calibration standards were used in all of the experiments. Global DNA methylation was obtained by quantifying 5-mdC and dC using ultra-pressure liquid chromatography (UPLC), in combination with tandem mass spectrometry (MS-MS). LC/MS-MS analysis of the samples was conducted on a Waters® Acquity UPLC™, coupled to a Waters® Micromass Quattro Premier™ Mass Spectrometer, using electro spray ionization (ESI). Injections were performed on a Waters® UPLC column (BEH C₁₈, 50 mm x 2.1 mm, 1.7 µm) which was held at a temperature of 40°C during analysis.

The global DNA methylation is expressed as a percentage of 5-mdC versus the sum of 5-mdC and dC [5-mdC / (5-mdC + dC)] %. We measured samples in duplicate to account for technical variation which resulted in a R² of 0.8 (ICC = 0.90). The average methylation value of both measurements was used in the statistical analysis.

Statistical analysis

We used SAS software (version 9.2; SAS Institute Inc., Cary, NC, USA) for database management and statistical analysis. Categorical data were presented as frequencies (%) and numbers, continuous data as mean and standard deviation. Spearman correlation coefficients and linear regression were used to assess the association of global DNA methylation from placental tissue with PM_{2.5} exposure. The unadjusted association between the average concentrations over 91 days periods and global methylation was estimated using restricted cubic splines²¹⁹ with 5 knots located at the 5th, 25th, 50th, 75th, and 95th percentiles. We performed multiple linear regression to determine the independent effect size of PM_{2.5} exposure during pregnancy on global methylation. Covariates considered for entry in the model ($p \leq 0.10$) using single regression procedures were newborn's gender, maternal age (years), pre-pregnancy BMI (kg/m²), net

weight gain (kg), maternal education (low/middle/high), newborn's ethnicity (European/non-European), smoking status (never-smoker/past-smoker/smoker), prenatal alcohol use (yes/no), prenatal acetaminophen (yes/no), delivery method (vaginal/Caesarean), gestational age (weeks), parity (1/2/ \geq 3), season at conception, and trimester-specific apparent temperature. Newborn's gender, maternal age, maternal education, smoking status, gestational age, parity, season at conception, and trimester-specific apparent temperature were forced into the model regardless of the *p*-value. In final models we introduced time-specific exposure to NO₂ and maximum 8-hour average O₃. Finally, we explored potential effect modification between trimester-specific PM_{2.5} exposure and birth weight (continuous), newborn's gender and trimester-specific apparent temperature. Estimates are given as a relative difference from the mean methylation in the whole newborn population. We plotted the studied covariates and global methylation patterns to ensure that there was no threshold phenomenon and that linear regression techniques were appropriate. Q-Q plots of the residuals were used to test the assumptions of all linear models.

RESULTS

Characteristics and exposure levels of the study population

The study included 240 mother-newborn pairs (mean maternal age, 29.1 yr; range, 18-42 yr). Demographic and prenatal lifestyle factors are reported in **Table 1**. Briefly, mean (\pm SD) pre-pregnancy BMI of the participating mothers was 24.4 (\pm 4.5) kg/m². 39 mothers (16.2%) reported to have smoked during pregnancy and smoked a mean number of 7.2 (\pm 4.2) cigarettes per day. Most women (67.6%, *n* = 162) never smoked cigarettes. More than 50% of the mothers were high educated. The newborn population, including 130 girls (54.2%), had a mean gestational age of 39.2 weeks (range, 35-42), 96.2% were term born infants and included a vast majority of primiparous (53.3%, *n* = 128) or secundiparous (36.7%, *n* = 88) newborns.

Table 1. Characteristics of mother-newborn pairs ($n = 240$)

Characteristics	Mean \pm SD or range and number (%)
Maternal	
Age, y	29.1 (18-42)
Pre-pregnancy BMI, kg/m ²	24.4 \pm 4.5
Net weight gain, kg	14.6 \pm 6.4
Maternal education	
Low	30 (12.5%)
Middle	84 (35.0%)
High	126 (52.5%)
Smoking status	
Never-smoker	162 (67.6%)
Past-smoker	39 (16.2%)
Smoker	39 (16.2%)
Acetaminophen	
No	136 (56.7%)
Yes	104 (43.3%)
Alcohol†	
No	194 (82.5%)
Yes	41 (17.5%)
Parity	
1	128 (53.3%)
2	88 (36.7%)
≥ 3	24 (10.0%)
Apparent temperature, °C	
Trimester 1	9.4 \pm 6.0
Trimester 2	8.3 \pm 6.2
Trimester 3	8.6 \pm 5.9
Newborn	
Newborn's gender	
Female	130 (54.2%)
Ethnicity‡	
European	206 (86.5%)
Non-European	32 (13.5%)
Gestational age, w	39.2 (35-42)
Season§	
Fall	56 (23.3%)
Winter	60 (25.0%)
Spring	51 (21.3%)
Summer	73 (30.4%)
Apgar score after 5 min	
7	2 (0.8%)
8	11 (4.6%)
9	74 (30.8%)
10	153 (63.8%)
Birth weight, g	3,401 \pm 422
Birth length, cm	50.2 \pm 1.9
Placental global DNA methylation, %	4.6 \pm 0.4

† Data available for 235 and ‡ 238 subjects.

§ Season determined for date of conception.

Table 2 presents the mean outdoor exposure to PM_{2.5} averaged for the implantation windows and for each of the three trimesters of pregnancy. Average (25th-75th percentile) trimester-specific PM_{2.5} exposure was 16.7 (12.3-20.0) µg/m³ for the first trimester, 17.4 (12.0-22.1) µg/m³ for the second trimester, 18.2 (12.8-22.9) µg/m³ for the third trimester and 17.4 (15.4-19.3) µg/m³ for the whole pregnancy. Nitrogen dioxide (NO₂) and maximum 8-hour average ozone (O₃) for the specific exposure windows are presented in **Supplemental Material, Table S1**.

Table 2. Exposure characteristics (*n* = 240)

PM _{2.5} , µg/m ³	Mean	SD	25 th percentile	75 th percentile
Pre-implantation (1-5d)	16.9	11.0	10.4	19.3
Implantation (6-12d)	16.9	9.8	10.1	20.1
Implantation range (6-21d)	16.7	8.1	11.3	20.4
Post-implantation (22-28d)	17.3	10.4	10.1	20.4
Trimester 1 (1-13w)	16.7	5.9	12.3	20.0
Trimester 2 (14-26w)	17.4	6.2	12.0	22.1
Trimester 3 (27w-delivery)	18.2	6.3	12.8	22.9
Whole pregnancy	17.4	3.6	15.4	19.3

Predictors and correlates of placental global DNA methylation

Mean (\pm SD) global DNA methylation levels of placental tissue was 4.6% (\pm 0.4). First, we identified potential predictors of global DNA methylation levels in placental tissue and/or factors that may confound the association between methylation levels and PM_{2.5} exposure (**Table 3**). Placental global DNA methylation was lower in girls as compared with boys (β = -0.108 \pm 0.049, p = 0.03) and was lower in past-smokers [*n* = 39] (β = -0.133 \pm 0.067, p = 0.05) and smokers [*n* = 39] (β = -0.102 \pm 0.068, p = 0.13) as compared with never-smokers [*n* = 162] (Reference). Placental methylation levels correlated positively with intake of acetaminophen during pregnancy (β = 0.129 \pm 0.049, p = 0.009). Placental methylation levels correlated with season at conception; the levels were highest in spring (β = 0.351 \pm 0.070, p < 0.0001) and lowest in fall (Reference). In addition, placental methylation levels correlated with apparent temperature averaged over the first trimester (β = 0.019 \pm 0.004, p < 0.0001), second trimester (β = 0.006 \pm 0.004, p = 0.15) and third trimester (β = -0.021 \pm 0.004, p < 0.0001).

Table 3. Predictors of placental global methylation in mother-newborn pairs ($n = 240$)

Variables	β^*	SE	p-value
Maternal characteristics			
Age, y	0.0005	0.005	0.93
Pre-pregnancy BMI, kg/m ²	0.002	0.005	0.68
Net weight gain, kg	0.002	0.004	0.54
Maternal education			
Low	Ref	-	-
Middle	-0.071	0.081	0.38
High	-0.076	0.077	0.33
Smoking			
Never-smoker	Ref	-	-
Past-smoker	-0.133	0.067	0.05
Smoker	-0.102	0.068	0.13
Acetaminophen			
No	Ref	-	-
Yes	0.129	0.049	0.009
Alcohol†			
No	Ref	-	-
Yes	-0.036	0.065	0.58
Parity			
1	Ref	-	-
2	0.054	0.053	0.31
≥ 3	0.074	0.085	0.38
Newborn characteristics			
Newborn's gender			
Male	Ref	-	-
Female	-0.108	0.049	0.03
Ethnicity‡			
European	Ref	-	-
Non-European	-0.020	0.074	0.78
Gestational age, w	0.028	0.019	0.15
Birth weight, g	0.0001	0.00006	0.06
Birth length, cm	0.026	0.013	0.05
Time-related characteristics			
Season§			
Fall	Ref	-	-
Winter	0.096	0.067	0.15
Spring	0.351	0.070	< 0.0001
Summer	0.097	0.065	0.14
Apparent temperature, °C			
Trimester 1	0.019	0.004	< 0.0001
Trimester 2	0.006	0.004	0.15
Trimester 3	-0.021	0.004	< 0.0001

* β-estimate is an absolute change in percentage of global DNA methylation.

† Data available for 235 and ‡ 238 subjects.

§ Season determined for date of conception.

Placental DNA methylation at birth in association with PM_{2.5} exposure

Although maternal age, maternal education, gestational age, and parity were not significantly associated with global DNA methylation, we forced these variables into the multiple regression models, together with newborn's gender, smoking status, prenatal acetaminophen use, season at conception, and trimester-specific apparent temperature. Both before (**Figure 1 A-C**) and after adjustment (**Figure 2**) for the aforementioned variables, placental global DNA methylation was inversely associated with PM_{2.5} exposures during the whole pregnancy, which was mainly driven by the exposures during the first trimester. Because the inter-quartile range of the PM_{2.5} exposure of the different exposure windows differed only slightly, the reported effect estimates over the different periods were not explained by differences in the window specific distribution of PM_{2.5}. Overall, placental methylation relatively decreased by 2.19% (95% confidence interval [CI]: -3.65, -0.73%, $p = 0.004$) for each 5 $\mu\text{g}/\text{m}^3$ increase in exposure to PM_{2.5}. Looking into different exposure windows during pregnancy showed significantly and inverse associations between global DNA methylation at birth with exposures during first trimester (-2.41% per 5 $\mu\text{g}/\text{m}^3$ increase, 95% CI:

-3.62, -1.20%, $p = 0.0001$) and second trimester (-1.51% per 5 $\mu\text{g}/\text{m}^3$ increase, 95% CI: -2.66, -0.36%, $p = 0.01$), while no significant association was observed with PM_{2.5} exposure of the third trimester (-0.45% per 5 $\mu\text{g}/\text{m}^3$ increase, 95% CI: -1.72, 0.82%, $p = 0.49$). Further, we ran an additional model in which time-specific exposure windows for PM_{2.5} were combined with time-specific maximum 8-hour average O₃ and NO₂ (**Figure 2**). This did not alter our previous reported findings on PM_{2.5} (effect-size for global methylation relatively decreased by 2.52%, 95% CI: -4.27, -0.76%, $p = 0.005$ for each 5 $\mu\text{g}/\text{m}^3$ increase in PM_{2.5} of trimester 1). Next, we built a multi-lag model in which all the three trimester exposures were fitted as independent variables in the same regression model (**Table 4**). Only exposure to PM_{2.5} during trimester 1 remained significantly associated with a relative decrease of 2.13% per 5 $\mu\text{g}/\text{m}^3$ increase (95% CI: -3.71, -0.54%, $p = 0.009$) in global DNA methylation of placental tissue.

Within trimester 1, we analyzed shorter time windows specifically drawn to target the critical stages of DNA methylation. We observed a decrease of

placental global DNA methylation with $\text{PM}_{2.5}$ exposure during all the implantation stages (**Figure 2**) but exposure during the implantation range (6-21d) was strongest associated with placental global DNA methylation at birth (-1.08% per $5 \mu\text{g}/\text{m}^3$ increase, 95% CI: -1.80, -0.36%, $p = 0.004$). The association between placental global DNA methylation and $\text{PM}_{2.5}$ exposure from the post-implantation window onwards weakens compared with the implantation exposure windows (-0.59% per $5 \mu\text{g}/\text{m}^3$ increase, 95% CI: -1.17, -0.005%, $p = 0.05$) and appeared not significant after additional adjustment for the corresponding NO_2 and O_3 exposure period (-0.58% per $5 \mu\text{g}/\text{m}^3$ increase, 95% CI: -1.34, 0.18%, $p = 0.14$). Studying all different weeks of trimester 1 (week 1-13), we found in addition to the implantation period (**Figure 2**), a significant inverse association between $\text{PM}_{2.5}$ residential exposure estimates only for week 7 adjusted for NO_2 and O_3 and placental global DNA methylation (-1.03% per $5 \mu\text{g}/\text{m}^3$ increase CI: -1.68, -0.38%, $p = 0.002$).

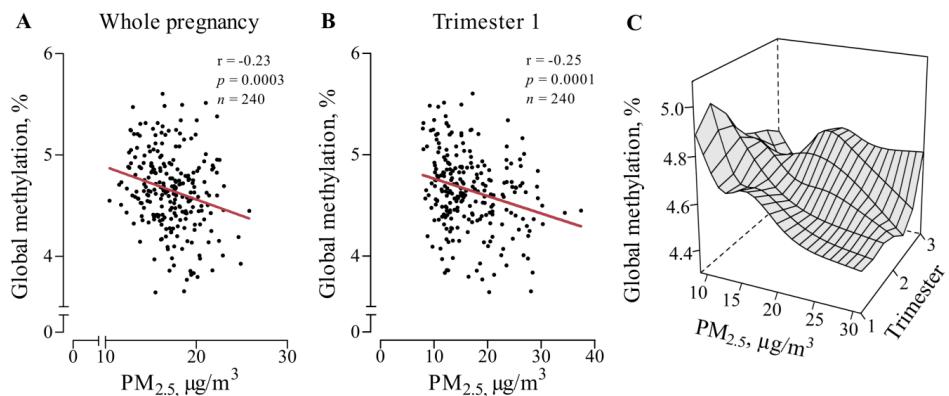


Figure 1. Bivariate analysis of placental global DNA methylation in association with prenatal exposure to fine particulate air pollution ($\text{PM}_{2.5}$). $\text{PM}_{2.5}$ exposure ($\mu\text{g}/\text{m}^3$) are presented with spearman correlation coefficients for the whole pregnancy (A) and first trimester (B). In panel C, the association between average $\text{PM}_{2.5}$ concentrations and placental global DNA methylation is given. The effect of the average concentrations over 91 days periods was estimated using restricted cubic splines with 5 knots located at the 5th, 25th, 50th, 75th, and 95th percentiles.

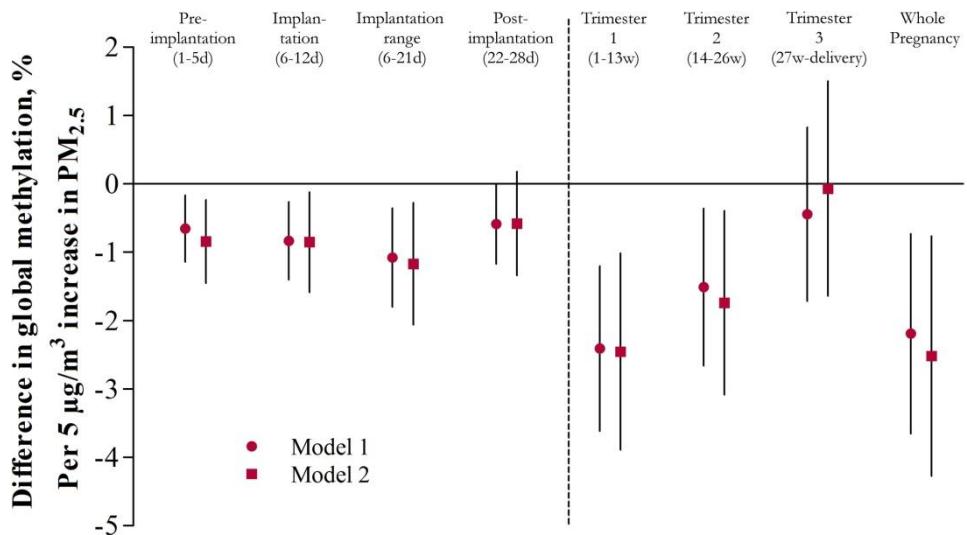


Figure 2. Relative difference in placental global DNA methylation in association with *in utero* exposure to particulate air pollution (PM_{2.5}) during various time windows (*n* = 240). The effect size is a relative difference (95% CI) in mean placental global DNA methylation for each 5 µg/m³ increase of PM_{2.5} exposure (µg/m³). Model 1 (●) is adjusted for newborn's gender, maternal age, gestational age, parity, maternal education, smoking status, prenatal acetaminophen use, season at conception, and trimester-specific apparent temperature. Model 2 (■) is additionally adjusted for the corresponding NO₂ and maximum 8-hour average O₃ exposure.

Table 4. Relative difference in global DNA methylation of placental tissue in association with *in utero* exposure to particulate air pollution (PM_{2.5}) (*n* = 240)

Multi-lag model, PM _{2.5} †‡	Relative difference	95% CI	p-value
Trimester 1 (1-13w)	-2.13%	-3.71 to -0.54%	0.009
Trimester 2 (14-26w)	-0.43%	-1.84 to 0.98%	0.55
Trimester 3 (27w-delivery)	0.74%	-0.85 to 2.33%	0.36

† All the three trimester exposures were fitted as independent variables in the same regression model. The effect size is a relative difference (95% CI) in mean placental global DNA methylation for each 5 µg/m³ increase of PM_{2.5} exposure (µg/m³).

‡ Adjusted for newborn's gender, maternal age, gestational age, parity, maternal education, smoking status, prenatal acetaminophen use, season at conception, and trimester-specific apparent temperature.

Sensitivity analysis

Variation in method of delivery can affect release of stress hormones that may influence methylation.⁷² Restricting our analysis to only vaginal partus (excluding 9 Caesarean deliveries) did not alter the observed effects for any exposure window. Models in which we replaced the classification of smoking (never-smokers/past-smokers/smokers) by either never-smoker/smoker, pack years or amount of cigarettes during pregnancy showed comparative results. Since the weeks after conception might be particularly critical for DNA methylation, we also evaluated the effect of tobacco smoke on methylation levels of mothers who stopped smoking upon learning of being pregnant but no difference in methylation level was seen ($n = 11$, $p = 0.28$). Finally, we did not observe effect-modification by newborn's gender ($p > 0.22$) and birth weight ($p > 0.37$) on the association between global DNA methylation and PM_{2.5} during the different exposure windows. We also did not observe effect-modification by trimester-specific apparent temperature on the placental DNA methylation and PM_{2.5} exposure during trimester 1 (interaction term $p \geq 0.19$).

DISCUSSION

The placenta plays a pivotal role in nutrient transfer, growth, and organ development of the embryo. Epigenetic modification may provide a plausible link between particulate air pollution and alterations in gene expression that might lead to disease phenotypes related to fetal programming. The key finding of our study is that exposure to particulate air pollution from fertilization up to and including embryo implantation was associated with lower global DNA methylation levels in placental tissue at birth. This observation persisted after adjustment for newborn's gender, maternal age, gestational age, parity, smoking, maternal education, prenatal exposure to acetaminophen, season at conception, trimester-specific apparent temperature or any other covariate studied.

DNA methylation patterns are established in two developmental periods (germ cells and early embryos) and are likely needed to generate cells with a broad developmental potential and correct initiation of embryonic gene expression.⁵⁷ In this regard, epigenetic reprogramming of imprinted genes in germ cells and

early embryos appear to be particularly important for the regulation of embryonic growth and placental development.¹³⁹ It has been hypothesized that regulation of imprinted gene expression is less stable in the placenta than in the fetus itself which may aid the placenta in adapting to changing physiological conditions.^{140,179} This leads to speculation that perturbations in DNA methylation patterns or sporadic loss-of-imprinting in the early stages of development lie at the basis of altered gene expression and contribute to abnormal placental or fetal development.¹⁴⁰ Indeed, research suggests that transplacental exposure to environmental toxicants during critical developmental periods lead to disease pathogenesis in later life.^{65,66,220,221} Both in animal and human cells, there is direct evidence for the role of hypomethylation for inducing genomic and chromosomal instability.²²²⁻²²⁴

The sensitivity of the epigenetic system to environmental factors occurs primarily during the period of developmental plasticity because this is the time when epigenetic marks undergo critical modifications.⁵⁹ After fertilization and prior to implantation, DNA methylation patterns are largely erased but are re-established by *de novo* DNA methyltransferases (DNMTs) in the blastocyst stage.⁶⁰ The placenta develops from the outer layer of the blastocyst upon implantation into the maternal endometrium.¹⁸⁹ Our results show that exposure to particulate air pollution during the implantation window is associated with the methylation profile of placental tissue. The finding of lower methylation levels from the beginning of placental formation is of critical interest in development considering that disturbance of maintenance DNA methylation in placental tissue is associated with abnormal embryonic development in the mouse model²²⁵ and genetic inactivation of DNMTs is lethal to developing mouse embryos.²²⁶ Experimental evidence showed that oxidative DNA damage could interfere with the capability of methyltransferases to interact with DNA resulting in lower methylation of cytosine residues at CpG sites.²²⁷ Since trophoblast differentiation is most important early in pregnancy when the placenta is initially being constructed²²⁸ and maternal air pollution exposure may influence markers of placental growth and function,²²⁹ it could well be that altered global DNA methylation during early pregnancy influences placental development. Maternal tobacco smoke, a personalized form of air pollution, has shown to alter placental methylation levels^{73,197} and underlie changes to placental function that may lead

to altered fetal development and programming⁷³ or pregnancy pathologies such as impaired fetal growth¹⁸⁰ and preterm delivery.^{111,182} Our relative estimates of lower global DNA methylation levels for an increase of 5 µg/m³ in the first trimester is associated with a decrease of 2.13% ($p = 0.009$) in global DNA methylation, compares with -2.17% ($p = 0.13$) in active smokers and -2.84% ($p = 0.05$) in past smokers. Our observations in smokers are much smaller compared with the estimates in cord blood assessed by ELISA in the study of Guerrero-Preston and colleagues showing a -48.5% ($p < 0.01$) lower global DNA methylation among newborns with smoking mothers compared with their nonsmoking counterparts.¹⁹⁰ However, differences in tissue and techniques make direct comparison of methylation status difficult. The mechanisms of air pollution-induced health effects involve oxidative stress and inflammation.^{230,231} The associations we observed in our current study may be part of the systemic consequences of induced inflammatory conditions both in mother lungs as well as in placental tissue. An alternative hypothesis is that inhaled particles may translocate directly from the lung into the blood stream where these fine particles induce oxidative stress in blood cells and potentially in placental tissue.^{48,232}

Although this is the first study investigating the effect of PM_{2.5} on DNA methylation in early life, several other studies have examined the role of environmental factors on DNA methylation levels in adults. Baccarelli and colleagues showed that blood DNA methylation in the LINE-1 repetitive element was decreased in elderly individuals of the Normative Aging Study with recent exposure to high levels of traffic particles including PM_{2.5}, whereas no association was observed between methylation of the Alu repetitive element and particle levels.⁶⁹ Another study within the same elderly cohort found that prolonged exposure to black carbon and sulfate particles is associated with hypomethylation of Alu and LINE-1 in leukocytes respectively.⁶⁸ Besides surrogate markers of global DNA methylation, several studies also report associations of gene-specific DNA methylation in leukocytes and exposure to airborne polycyclic aromatic hydrocarbons and PM.^{70,220} In contrast to particulate exposure, arsenic was positively associated with DNA methylation in LINE-1 repeated element in both maternal and fetal leukocytes.¹⁷³

A first limitation of this study is that placental tissue is composed of a complex population of cells (syncytiotrophoblasts/cytotrophoblasts, mesenchymal cells, Hofbauer cells, fibroblasts). Also maternal blood is a major constituent of placental tissue which makes that this organ shows high variability in overall DNA methylation compared to other tissues.¹⁷⁹ However, within-placenta variability for several genes showed generally less sample-to-sample variation for DNA methylation than gene expression levels and different placental sites and depths show consistent methylation patterns.^{72,162} In our study, the coefficient of variation of global methylation between sample spots from different quadrants of the placenta was 4.5% with an intraclass correlation coefficient (ICC) of 0.25. To minimize the impact of regional differences in methylation patterns within a mother's placenta, we standardized our method and chose one spot. Most of the methylation variation is not due to sample location but rather cell composition differences between samples. Heterogeneity in cell types in placental tissue may also contribute to inter-individual variation.¹⁸⁹ Global methylation status measured by quantifying 5-mdC and dC using ultra-pressure liquid chromatography in combination with tandem mass spectrometry, gives a good estimate of global methylation because it averages total methylation of all cell types. Although most genes present in the two main cell types of the placenta (cytotrophoblasts and fibroblasts) exhibit similar promoter methylation patterns, some specific genes show differential promoter methylation.¹⁴³ Methylation status of placental villi reflects mainly the profile of the cytotrophoblast cells. We did not observe any obvious differences in the histology or cell type composition between the fetal samples taken at four standardized sites across the middle region of the placenta (approximately 4 cm away from the umbilical cord) nor between placentas. Regardless of the limitations, the placenta can be used as a proxy for methylation changes in the fetus as it is derived from the outer layer of the blastocyst. The organ has a great plasticity to a range of intrauterine conditions/exposures and the question remains if the fetus is affected in a direct manner or indirectly by adaptations in its function. Variables interfering with placental integrity may predispose to placenta-related gestational complications such as preeclampsia, fetal growth restriction, and abruption.²³³ Fetuses adapt their mitochondrial structure and metabolism when the supply of nutrients is limited. Changes in mitochondrial

DNA content, may represent a biological effect along the path linking air pollution to effects on the unborn. Recently we showed that mitochondrial DNA content in placental tissue, but not cord blood, was influenced by PM₁₀ exposure during the last trimester of pregnancy. The effects of these molecular changes must be further elucidated.¹³³ Secondly, although our results were consistent after multiple adjustments, we cannot exclude the possibility of residual confounding by some unknown factor that is associated with both placental methylation levels and ambient air pollution. Ambient exposure does not account for indoor exposure, but we obtained information on environmental tobacco smoke. Season and apparent temperature were taken into account as epigenetic adaptive changes to season have been reported in aquatic species.²³⁴ We found the highest methylation levels in placental tissue for conceptions at spring and the lowest in fall, which corresponds with observations in blood from adults by Baccarelli and colleagues.⁶⁹ Thirdly, the resolution of our interpolation model (4 x 4 km) may not represent perfect PM_{2.5} exposure at the individual level, however our exposure model has good validation statistics with an explained variance higher than 80%¹²³ and also validation regarding to personal exposure by measuring carbon load in lung macrophages.²¹³ Our study was not designed to evaluate temporal changes of DNA methylation during pregnancy and may be hampered by the fact that assays of term placentas may not reflect *in vivo* methylation patterns occurring earlier at critical points of development. Nevertheless, our associations were robust and strong in the context of environmental epidemiology.

Generally, there are three approaches to study DNA methylation patterns: 1/ gene-specific through a candidate gene approach (e.g. bisulfite pyrosequencing), 2/ gene-specific through a genome-wide approach (e.g. Illumina array), and 3/ globally as an average of the entire genome. In our study, we chose to measure global DNA methylation instead of surrogate markers of global DNA methylation. Gene-specific assays are crucial for integrating information about DNA methylation patterns with gene expression at promoter level but do not provide a global picture of DNA methylation changes within the genome.²³⁵ Genome-wide methylation assays and gene expression analysis are needed to complement our findings of lower global methylation levels. For example, investigating DNMTs should give more insight into possible

mechanisms that control epigenetic programming and thus placental development. Additional studies should also elucidate gene-specific methylation patterns since there is evidence that altered DNA methylation at the human *H19/IGF2* imprinting control region,¹⁴⁰ genes such as *TIMP3*,²³⁶ and disruption of imprinted genes in mouse models may be associated with abnormal placental outcomes and fetal development.²³⁷ Our findings give mechanistic plausibility to the hypothesis that air pollution is linked to fetal programming. Indeed, there is an increasing awareness that the placenta responds to and modulates perturbations in the maternal environment, thereby playing a key role in transmitting the programming stimuli to the fetus.²³⁸

The current study was performed in an European hotspot regarding particulate air pollution³² with 33 days in 2011 exceeding the European legislation of 50 µg/m³. Thanks to legislation, levels of urban air pollution have generally decreased over the course of the latter half of the 20th century in the United States and Western Europe. However, no such trend has taken place in many cities and megacities of developing countries.

CONCLUSION

We observed a lower degree of placental global DNA methylation in association with exposure to particulate air pollution during early pregnancy. More specifically, exposure from fertilization up to and including implantation, a critical period for methylation reprogramming, was a highly sensitive window for PM_{2.5} exposure on placental DNA methylation at birth. There is need to further investigate how environmental conditions such as particulate air pollution affect gene-specific DNA methylation and gene expression patterns during fetal development.

Competing interests

The authors declare they have no competing financial interests.

Author's contributions

T.S.N., W.G., B.G.J. and L.G. designed the study; B.G.J., N.P., M.P., W.G. did data collection; K.P., L.G. and B.G.J. performed analytical measurements; F.F.

and B.G.J. designed the exposure matrixes; B.G.J., M.K., and T.S.N. analyzed the data; B.G.J. and T.S.N. wrote the first draft of paper. All authors critical revised and approved the final version of the manuscript.

Acknowledgments

The ENVIRONAGE birth cohort is supported by grants from the European Research Council (ERC) and the Flemish Scientific Fund (FWO, 1.5.158.09.N.00/G.0753.10). Michał Kiciński has a Ph.D. fellowship of the Research Foundation – Flanders (FWO). We thank Dr. Van Robays for the histological examinations, the midwives of the maternity ward, and staff of the clinical laboratory of East-Limburg Hospital in Genk.

SUPPLEMENTAL MATERIAL

Table S1. Exposure characteristics of NO₂ and maximum 8-hour average O₃ (*n* = 240)

Pollution indicator	Mean	SD	25 th percentile	75 th percentile
NO₂, µg/m³				
Trimester 1	21.5	6.1	16.9	24.9
Trimester 2	22.1	6.6	16.8	26.5
Trimester 3	22.2	6.8	17.2	26.8
Whole pregnancy	21.9	4.9	18.8	24.8
O₃, µg/m³ †				
Trimester 1	63.3	19.8	44.0	80.9
Trimester 2	60.1	20.8	41.0	79.2
Trimester 3	61.2	20.6	41.8	79.3
Whole pregnancy	61.5	8.0	54.9	67.5

† Maximum 8-hour average ozone concentration.

CHAPTER 6.1

PLACENTAL MITOCHONDRIAL DNA CONTENT AND PARTICULATE AIR POLLUTION DURING *IN UTERO* LIFE

Bram G. Janssen,¹ Elke Munters,¹ Nicky Pieters,¹ Karen Smeets,¹ Bianca Cox,¹ Ann Cuypers,¹ Frans Fierens,² Joris Penders,^{3,4} Jaco Vangronsveld,¹ Wilfried Gyselaers,^{4,5} Tim S. Nawrot^{1,6}

¹ Centre for Environmental Sciences, Hasselt University, Hasselt, Belgium

² Belgian Interregional Environment Agency, Brussels, Belgium

³ Laboratory of Clinical Biology, East-Limburg Hospital, Genk, Belgium

⁴ Department of Physiology, Hasselt University, Diepenbeek, Belgium

⁵ Department of Obstetrics, East-Limburg Hospital, Genk, Belgium

⁶ Department of Public Health & Primary Care, Leuven University, Leuven, Belgium

ABSTRACT

Background: Studies emphasize the importance of particulate matter (PM) in the formation of reactive oxygen species and inflammation. We hypothesized that these processes can influence mitochondrial function of the placenta and fetus.

Objectives: We investigated the influence of PM₁₀ exposure during pregnancy on the mitochondrial DNA (mtDNA) content of the placenta and umbilical cord blood.

Methods: DNA was extracted from placental tissue ($n = 174$) and umbilical cord leukocytes ($n = 176$). Relative mtDNA copy numbers (i.e. mtDNA content) were determined by quantitative real-time PCR. Multiple regression models were used to link mtDNA content and *in utero* exposure to PM₁₀ over various time windows during pregnancy.

Results: In multivariate-adjusted analysis, a 10 µg/m³ increase in PM₁₀ exposure during the last month of pregnancy was associated with a 16.1% decrease (95% CI: -25.2 to -6.0%, $p = 0.003$) in placental mtDNA content. The corresponding effect-size for average PM₁₀ exposure during the third trimester was 17.4% (95% CI: -31.8 to -0.1%, $p = 0.05$). Furthermore, we found that each doubling in residential distance to major roads was associated with an increase in placental mtDNA content of 4.0% (95% CI: 0.4 to 7.8%, $p = 0.03$). No association was found between cord blood mtDNA content and PM₁₀ exposure.

Conclusions: Prenatal PM₁₀ exposure was associated with placental mitochondrial alterations, which may both reflect and intensify oxidative stress production. The potential health consequences of decreased placental mtDNA content in early life must be further elucidated.

INTRODUCTION

Particulate matter (PM) is a part of ambient air pollution and is most relevant to human health.^{6,42} PM has been associated with adverse health outcomes of the fetus^{206,239-243} and neonate.⁴⁹ In addition, the functional morphology of the placenta is also influenced by PM exposure in experimental animal models.²⁴⁴ The underlying mechanisms by which PM exposure may induce adverse fetal health effects are poorly understood. Several studies have emphasized the importance of PM and its associated metal components in the formation of reactive oxygen species (ROS)^{46,245} and inflammation.²⁴⁶

The placenta is a metabolically active organ that plays a role in nutrient transfer, growth, and organ development. Mitochondria play an important role in the regulation of these processes. These intracellular organelles are essential for cellular energy provision through the production of ATP via oxidative phosphorylation. Each cell contains approximately 200 to 2,000 mitochondria, each carrying 2-10 copies of mitochondrial DNA (mtDNA) that are bound to protein structures. The major difference between human nuclear DNA (nDNA) and mtDNA is that the latter lacks protective histones, chromatin structure, and introns. Additionally, the mtDNA repair mechanisms work less efficiently than that of nDNA.⁸³ Mitochondria are the major intracellular sources and primary targets of ROS and, therefore, mtDNA is particularly vulnerable to ROS-induced damage and has a high mutation rate.²⁰¹ Mitochondria compensate for these mutations, resulting in a change in mtDNA copy number (i.e. change in mtDNA content). Recently, mitochondrial function has been linked to various disease mechanisms and can be assessed by measuring the mtDNA content, an established marker of mitochondrial damage and dysfunction.^{86,247}

Developmental adaptations due to metabolic changes, including suboptimal fetal nutrition, permanently 'program' the fetus and may lead to adverse pregnancy outcomes that form the origin of diseases that may arise in adult life.^{52,53,248} Mitochondrial damage and dysfunction attributes to metabolic shifts and may represent a biological effect along the pathway linking PM to effects on the newborn. However, whether placental and cord blood mtDNA content is associated with PM₁₀ exposure during *in utero* life has never been studied. In the present study we investigated the association of placental and cord blood mtDNA

content with long- and short-term exposure to airborne PM₁₀ and residential distance to major roads.

MATERIALS AND METHODS

Study population and data collection

AGEing is a complex phenotype responsive to a plethora of ENVIRONMENTal exposures from early life onwards including particulate air pollution. The current study is part of a new initiated and ongoing birth cohort 'ENVIRONAGE'. We recruited 178 newborns (only singletons) from East-Limburg Hospital in Genk born between Friday 12 PM and Monday 7 AM from the 10th of February 2010 until the 3rd of April 2011. The only inclusion criterion was that mothers had to be able to fill out questionnaires in Dutch. Enrollment was equally spread over all seasons of the year. The overall participation rate of eligible mothers was 47%. During the first month of the campaign midwives recorded the reason of non-participation. The main reasons (in descending importance) were: forgotten to ask for participation, communication problems, or complications during labor. Participating mothers provided written informed consent when they arrived at the hospital for delivery, and completed study questionnaires in the postnatal ward after delivery to provide detailed information on age, socioeconomic status, ethnicity, smoking, place of residence, pre-pregnancy body mass index (BMI), and parity. Socioeconomic status was coded and condensed into a scale with scores ranging from 0 to 2 based on mother's education. Ethnicity was classified based on the native country of the newborn's grandparents as European (when two or more grandparents were European) or non-European (when at least three grandparents were of non-European origin). Current smokers were defined as having smoked before and during pregnancy. Before-smokers were defined as those who had quit prior to pregnancy, and never-smokers had never smoked.

Samples of placental tissue ($n = 174$) and umbilical cord blood ($n = 176$) were collected immediately after delivery, along with other perinatal parameters such as newborn's sex, birth date, birth weight and length, gestational age (range, 35-42 weeks), Apgar score, and ultrasonographic data. All neonates were assessed for congenital anomalies immediately after birth and all were

considered healthy. The Apgar score after 1 min ranged from 2 to 10 but improved up to values between 7 and 10 after 5 min for all participants. Birth date was condensed into a seasonal scale where a difference was made between cold periods (October to March) and warm periods (April to September). The study was conducted according to the principles outlined in the Helsinki declaration for investigation of human subjects. Written informed consent was provided by all study participants in accordance with procedures approved by the Ethical Committee of Hasselt University and East-Limburg Hospital.

Sample collection

Umbilical cord blood was collected immediately after delivery in Vacutainer® Plus Plastic K₂EDTA Tubes (BD, Franklin Lakes, NJ, USA). Blood cell counts (including platelet counts) and differential leukocyte counts were determined using an automated cell counter with flow differential (Cell Dyn 3500, Abbott Diagnostics, Abbott Park, IL USA). Samples were centrifuged at 3,200 rpm for 15 minutes to retrieve buffy coats and instantly frozen, first at -20°C and afterwards at -80°C.

Placentas were obtained for 174 mothers in the delivery room and deep-frozen within 10 minutes. Afterwards, placentas were thawed to take tissue samples for DNA extraction following a standardized protocol as described in Adibi *et al.*¹⁶⁵ Briefly, villous tissue, protected by the chorio-amniotic membrane, was biopsied from the fetal side of the placenta and preserved at -80°C. We assessed within placenta variability in a random subset of six placentas by comparing biopsies taken at four standardized sites across the middle region of the placenta, approximately four cm away from the umbilical cord. The first biopsy was taken to the right of the main artery and three other biopsies in the remaining quadrants of the placenta. mtDNA content within each placenta varied by a mean of 19.3% across the quadrants. To minimize the impact of within placental variability, biopsies used for mtDNA content assays were all taken 1-1.5 cm below the chorio-amniotic membrane at a fixed location by using a device to orientate the fetal side of the placenta in relation to the umbilical cord. Care was taken by visual examination and dissection to avoid the chorio-amniotic membrane contamination. Each biopsy was approximately 1 to 2 cubic

centimeters. Histological confirmation of cell type in ten placentas showed consistent results in all studied samples.

Exposure measurement

We calculated the regional background levels of PM₁₀ for each mother's home address using a kriging interpolation method^{120,213} that uses land cover data obtained from satellite images (CORINE land cover data set). This model provides interpolated PM₁₀ values from the Belgian telemetric air quality networks in 4 × 4 km grids. In order to explore potentially critical exposures during pregnancy, individual PM₁₀ concentrations ($\mu\text{g}/\text{m}^3$) were calculated for various periods: 0-7 days before delivery (Lag 0-7), the last month of pregnancy, and for each of the three trimesters of pregnancy, with trimesters being defined as: 1-13 weeks (trimester 1), 14-28 weeks (trimester 2) and 29 weeks to delivery (trimester 3). Also, the whole pregnancy exposure was calculated as the mean of three trimesters. The date of conception was estimated based on ultrasound data. Additionally, NO₂ exposure was interpolated using the same methods as PM₁₀ exposure and is used in a sensitivity analysis. Distances from the mother's residence to a major road were calculated through geocoding (the shortest distance being set at ten meters). A major road was defined as an N-road (major local traffic road with average total number of motor vehicles per 24h > 10,000) or an E-road (motorway/highway). The Royal Meteorological Institute (Brussels, Belgium) provided mean daily temperatures and relative humidity for the study region and are averaged using the same exposure windows as for PM₁₀. The temperature and relatively humidity averaged $10.1 \pm 1.4^\circ\text{C}$ and $80.9 \pm 10.1\%$, respectively. Apparent temperature ($8.4 \pm 1.6^\circ\text{C}$) was calculated by using the following formula: $-2.653 + (0.994 \times \text{Ta}) + (0.0153 \times \text{Td}^2)$, where Ta is air temperature and Td is dew-point temperature (in $^\circ\text{C}$).^{214,215}

Measurement of mtDNA content

DNA was extracted from white blood cells of the buffy coat and placental tissue cells using the MagMAX™ DNA Multi-Sample kit (Applied biosystems, Foster City, CA, USA) following the manufacturer's instructions. Briefly, this purification kit uses MagMAX™ magnetic bead-based nucleic acid isolation technology for

producing high quantities of purified DNA. RNA contamination was minimized with a RNase digestion step. The concentration of extracted DNA was measured at 260 nm with the Nanodrop spectrophotometer (ND-1000, Isogen Life Science, De Meern, NE). DNA yield (ng/ μ l) and purity ratios (A260/280 and A260/230) were determined. Extracted DNA was stored at -20°C until further use.

mtDNA content was measured in placental tissue and leukocytes of umbilical cord blood by determining the ratio of two mitochondrial gene copy numbers (*MTF3212/R3319* and *MT-ND1*) to three single-copy nuclear control genes (*RPLP0*, *ACTB*, and *HBB*)[†] using a quantitative real-time polymerase chain reaction (qPCR) assay. Extracted genomic DNA was diluted to a final concentration of 5 ng/ μ l in RNase free water, prior to the qPCR runs. PCR reactions were set up by aliquoting 7.5 μ l master mix into each well of a MicroAmp® Fast Optical 96-Well Reaction Plate compatible with the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), followed by 2.5 μ l of each experimental DNA sample, for a final volume of 10 μ l per reaction. The master mix consisted of Fast SYBR® Green I dye 2x (Applied Biosystems, Lennik, 5 μ l/reaction), forward (0.3 μ l/reaction) and reverse (0.3 μ l/reaction) primer, and RNase free water (1.9 μ l/reaction). Primer sequences (**Table 1**) were diluted to a final concentration of 300 nM in the master mix. Two non-template controls and six inter-run calibrators were carried along in each PCR plate. The thermal cycling profile was the same for all transcripts: 20 s at 95°C for activation of the AmpliTaq Gold® DNA-polymerase, followed by 40 cycles of 1 s at 95°C for denaturation, and 20 s at 60°C for annealing/extension. Amplification specificity and absence of primer dimers was confirmed by melting curve analysis at the end of each run (15 s at 95°C, 15 s at 60°C, 15 s at 95°C). After thermal cycling, raw data were collected and processed. C_T-values of the two mitochondrial genes were normalized relative to the three nuclear reference genes according to the qBase software (Biogazelle, Zwijnaarde, BE). The program uses modified software from the classic comparative C_T method ($\Delta\Delta C_T$) that takes multiple reference genes into account and uses inter-run calibration algorithms to correct for run-to-run differences.¹⁹⁹ Plate effects were minimized by measuring one gene for all 178 placenta or cord blood samples in one day. The coefficient of variation for the mtDNA content in inter-run samples was 4.2%.

[†] In a modified protocol, we use only *RPLP0* and *ACTB* as reference genes.

Table 1. Primer sequences for selected genes and their accession number

Gene symbol	Accession number	Nuclear/Mitochondria	Forward 5' – 3'	Reverse 5' – 3'	Primer efficiency (%)
<i>MTF3212/R3319</i>	NC_012920.1	M	CACCAAGAACAGGGTTGT	TGGCCATGGGTATGTTGTTAA	96.3
<i>MT-ND1</i>	NC_012920.1	M	ATGGCCAACCTCCTACTCCT	CTACAACGTTGGGCCTTT	99.3
<i>RPLP0</i>	NM_001002.3	N	GGAATGTGGGCTTGTGTTTC	CCCAATTGTCCTTACCTT	100.7
<i>ACTB</i>	NM_001101.3	N	ACTCTTCCAGCCTCCCTCC	GGCAGGACTTAGCTCCACA	96.8
<i>HBB</i>	NM_000518.4	N	GTGCACCTGACTCCTGAGGAGA	CCTTGATACCAACCTGCCAG	100.4

Abbreviations: Mitochondrial forward primer from nucleotide 3212 and reverse primer from nucleotide 3319 (*MTF3212/R3319*); Mitochondrial encoded NADH dehydrogenase 1 (*MT-ND1*); Acidic ribosomal phosphoprotein P0 (*RPLP0*); Beta actin (*ACTB*); Hemoglobin beta (*HBB*).

Statistical analysis

We used SAS software (version 9.2; SAS Institute Inc., Cary, NC, USA) for database management and statistical analysis. Continuous data were checked for normality and were presented as arithmetic means \pm SD or geometric means with interquartile range (IQR) when data was not normally distributed. Categorical data are presented as frequencies (%) and numbers. Pearson or Spearman correlation coefficients and linear regression were used to assess the relationship of mtDNA content from placental tissue or umbilical cord blood with PM_{10} exposure. Multiple linear regression was performed to determine the independent variables of mtDNA content. Covariates considered for entry in the model ($p \leq 0.10$) were newborn's sex, maternal age, pre-pregnancy BMI, net weight gain, socioeconomic status, ethnicity, smoking status, parity, gestational age, season, and time-specific apparent temperature. Newborn's sex, maternal age, smoking status, gestational age, and ethnicity were forced into the model regardless of the p -value. In addition, umbilical cord models were adjusted for white blood cell count, percentage of neutrophils, and platelet counts to account for cord blood cell distribution. Q-Q plots of the residuals were used to test the assumptions of all linear models.

RESULTS

Characteristics of the study population

Table 2 summarizes the characteristics of the 178 mother-newborn pairs. Maternal age averaged 29.1 years and ranged from 18 to 42 years. The mothers had a mean pre-pregnancy BMI of $24.3 \pm 4.8 \text{ kg/m}^2$. 15.7% of the mothers ($n = 28$) smoked during pregnancy, and 29.2% ($n = 52$) had ever smoked. The average pack-years for mothers who ever smoked was 6.1 ± 5.1 . The majority were working mothers (87.7%), who lived on average 15.5 km (IQR = 5-20 km) from their working places. The study population included 82 male newborns and 87.6% ($n = 149$) were classified as Europeans. Seven infants were born preterm (less than 37 weeks). Birth weight averaged $3,403 \pm 387 \text{ g}$. We determined the mtDNA content in cells from placental tissue and cord blood of 174 and 176 subjects respectively.

Table 2. Study population characteristics ($n = 178$)

Characteristic	Mean \pm SD, geometric mean (25 th -75 th percentile) or number (%)
Maternal	
Age, y	29.1 \pm 4.9
< 20	6 (3.4%)
20-29	88 (49.4%)
30-35	65 (36.5%)
\geq 35	19 (10.7%)
Socioeconomic status	
Low	28 (15.7%)
Middle	58 (32.6%)
High	92 (51.7%)
Smoking status	
Never	126 (70.8%)
Before pregnancy	24 (13.5%)
Before and during pregnancy	28 (15.7%)
Cigarettes / day	
0	124 (75.6%)
1 to 10	28 (17.1%)
\geq 11	12 (7.3%)
Pack-years of ever smokers	6.1 \pm 5.1
Pre-pregnancy BMI, kg/m ²	24.3 \pm 4.8
Net weight gain, kg	14.5 \pm 6.5
Parity	
1	101 (56.7%)
2	57 (32.0%)
\geq 3	20 (11.2%)
Daily apparent temperature, °C	8.4 \pm 1.6
Newborn	
Gestational age, w	39.2 (39-40)
Preterm delivery, < 37 w	
Yes	7 (3.9%)
No	171 (96.0%)
Sex	
Male	82 (46.0%)
Female	96 (53.9%)
Ethnicity	
European	156 (87.6%)
Non-European	22 (12.4%)
Season	
Cold period	104 (58.4%)
Warm period	74 (41.6%)
Apgar score	
1 min	8.4 (8-9)
5 min	9.5 (9-10)
Neonate birth weight, g	3,403 \pm 387
Neonate length, cm	50 (49-51)
Placental mtDNA content †	1.03 (0.6-1.63)
Umbilical cord mtDNA content ‡	1.02 (0.75-1.30)
White blood cells, $\times 10.e3/\mu\text{L}$	15.3 \pm 4.6
Neutrophils, %	52.3 \pm 8.5
Platelets, $\times 10.e3/\mu\text{L}$	289.4 \pm 95.4

Data are presented as arithmetic means \pm standard deviation (SD) or number (%). Not normally distributed values are presented as geometric means with 25th-75th percentiles. mtDNA content is determined as mtDNA copy number (mean of *MT3212/R3319* and *MT-ND1*) normalized to nDNA copy number (mean of *RPLP0*, *ACTB*, and *HBB*).

† Data available for 174 subjects. ‡ Data available for 176 subjects.

PM_{10} exposure during the seven days before delivery averaged $24.9 \pm 11.1 \mu\text{g}/\text{m}^3$, and $25.6 \pm 8.6 \mu\text{g}/\text{m}^3$ for the last month of pregnancy (**Table 3**). Average trimester-specific PM_{10} exposure was $21.5 \pm 5.1 \mu\text{g}/\text{m}^3$ for the first trimester, $22.3 \pm 4.3 \mu\text{g}/\text{m}^3$ for the second trimester and $24.4 \pm 5.7 \mu\text{g}/\text{m}^3$ for the third trimester. The average distance from the participant's home address to the nearest major road was 207 m (85–676 m).

Table 3. Exposure characteristics

Pollution indicator	Mean \pm SD	25 th percentile	75 th percentile
PM₁₀, $\mu\text{g}/\text{m}^3$			
Week (Mean Lag 0–7)	24.9 ± 11.1	17.5	30.5
Last month	25.6 ± 8.6	19.3	29.5
Trimester 1	21.5 ± 5.1	18.1	23.7
Trimester 2	22.3 ± 4.3	19.3	25.3
Trimester 3	24.4 ± 5.7	20.2	28.1
Whole pregnancy	22.7 ± 3.7	20.1	25.1
Traffic-related pollution			
Residential distance to major road (m)	207	85	676

† Arithmetic mean except for the residential distance to major road, which was not normally distributed, for which the geometric mean is given.

Predictors of mtDNA content

Placental mtDNA content was negatively associated with parity ($\beta = -0.064 \pm 0.027$, $p = 0.018$), also after adjusting for maternal age ($\beta = -0.063 \pm 0.029$, $p = 0.028$), negatively associated with cold season ($\beta = -0.243 \pm 0.040$, $p < 0.0001$), and positively associated with apparent temperature the week before delivery ($\beta = 0.018 \pm 0.003$, $p < 0.0001$). Birth weight adjusted for newborn's sex, gestational age, season, and apparent temperature during third trimester was not significantly associated with placental mtDNA content ($p = 0.71$) nor with PM_{10} exposure during the third trimester ($p = 0.33$).

mtDNA content in association with *in utero* PM_{10} exposure

Unadjusted analysis showed that placental mtDNA content was correlated with PM_{10} exposure during the whole pregnancy ($\beta = -0.01 \pm 0.006$, $p = 0.068$), but this was largely attributable to exposure during the last week, last month, and third trimester of pregnancy (all $p < 0.0001$, **Figure 1 A-C**). PM_{10} exposures

during the first and second trimester of pregnancy were not significantly ($p > 0.31$) associated with placental mtDNA content. We adjusted for relevant variables that may influence the mtDNA content outcome ($p \leq 0.10$). Although newborn's sex, maternal age, smoking status, gestational age, and ethnicity were not significantly associated with placental mtDNA content, we forced these variables into the regression model, together with parity, season, and time-specific apparent temperature. After adjustment for the aforementioned variables, placental mtDNA content remained negatively associated with PM₁₀ exposure during the last week, last month, and third trimester of pregnancy (**Figure 1D**). Each 10 µg/m³ increase in PM₁₀ was associated with a lower placental mtDNA content of 10.1% (95% CI: -17.6 to -1.9%, $p = 0.02$) when considering the average exposure during the last week of pregnancy, 16.1% (95% CI: -25.2 to -6.0%, $p = 0.003$) during the last month of pregnancy, and 17.4% (95% CI: -31.8 to -0.1%, $p = 0.05$) during the third trimester (**Table 4**). Placental mtDNA content at birth did not correlate with PM₁₀ exposure during the first and second trimester.

In contrast to placental mtDNA content, none of these pollution windows were significantly associated with cord blood mtDNA content, either before or after adjusting for potential confounders as in the previous models plus including platelet counts, neutrophils, and total number of white blood cells (**Table 4**, **Figure 1D**). Although we adjusted for cord blood cell distribution, mtDNA content was not significantly associated with cord blood platelets ($p = 0.97$), neutrophils ($p = 0.47$), white blood cells ($p = 0.18$) or the white blood cell/platelet ratio ($p = 0.15$).

Markers of traffic-related air pollution

Distance to a major road is an exposure marker that can be used as a surrogate for traffic-related air pollution.²⁴⁹ Adjusted estimates showed that distance to major roads was significantly associated with placental mtDNA content [a 4% increase in mtDNA content with each doubling of the distance (95% CI: 0.4 to 7.8%, $p = 0.03$)]. No association was observed between cord blood mtDNA content and distance to major roads.

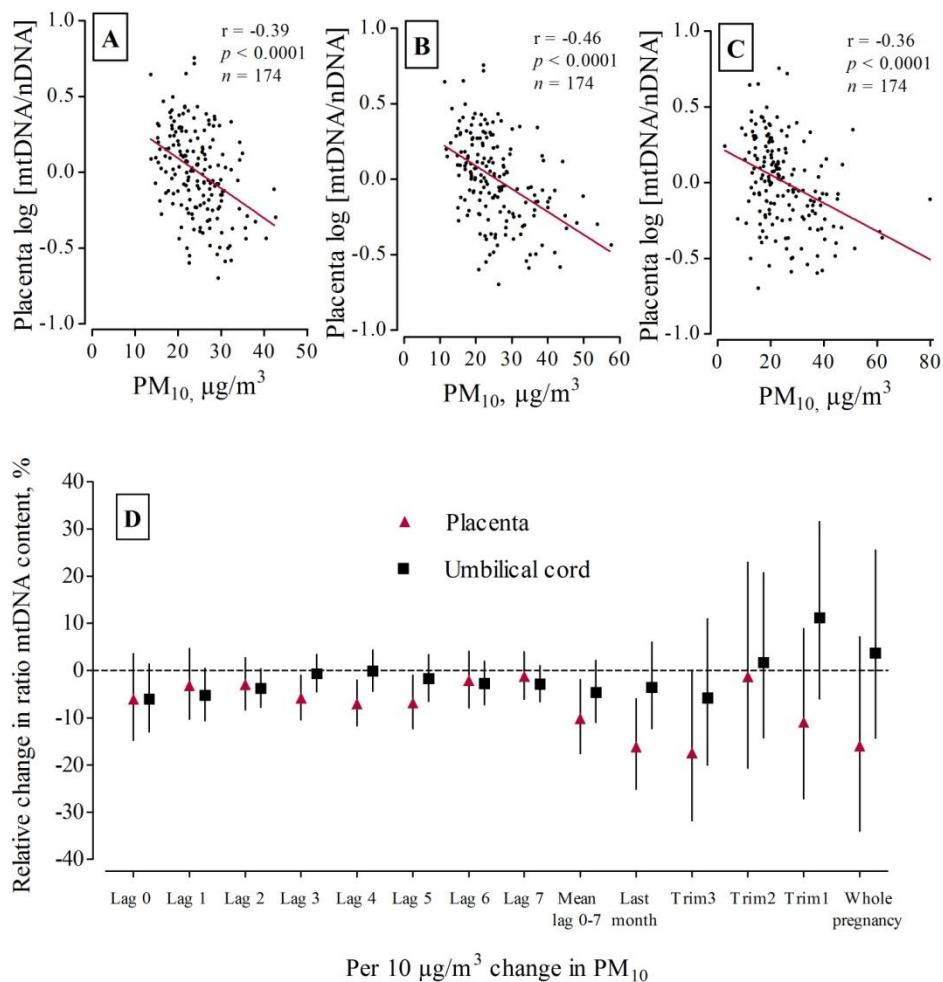


Figure 1. Association between mtDNA content in placental tissue ($n = 174$) or cord blood ($n = 176$) and PM_{10} exposure. Three correlation plots are given, each indicating PM_{10} exposure during a period of pregnancy: third trimester (A), the last month of pregnancy (B), and mean of seven days before delivery (C). In panel D, the relative percentage change (95% CI) in mtDNA content of both placental tissue and cord blood for each $10 \mu\text{g}/\text{m}^3$ increase of PM_{10} exposure is shown. The model is adjusted for newborn's sex, maternal age, parity, gestational age, ethnicity, smoking status, season, and time-specific apparent temperature. Additionally, umbilical cord blood was adjusted for blood cell count (# of white blood cells, % neutrophils, and # of platelets). PM_{10} concentrations are given in $\mu\text{g}/\text{m}^3$. Values of mtDNA content are \log_{10} -transformed.

Table 4. Estimated change in placental and umbilical cord blood mtDNA content in association with PM₁₀ during pregnancy or distance from residence to nearest major road

Variable	Placental tissue (n = 174)			Umbilical cord blood (n = 176)		
	Percentage change	95% CI	p-value	Percentage change	95% CI	p-value
Time window, PM₁₀ †,‡						
Trimester 1	-10.9%	-27.1 to 8.9%	0.26	11.2%	-6.0 to 31.6%	0.22
Trimester 2	-1.3%	-20.7 to 22.9%	0.91	1.8%	-14.2 to 20.8%	0.84
Trimester 3	-17.4%	-31.8 to -0.1%	0.05	-5.8%	-20.0 to 11.0%	0.48
Last month	-16.1%	-25.2 to -6.0%	0.003	-3.6%	-12.3 to 6.0%	0.45
Week (Mean Lag 0-7)	-10.1%	-17.6 to -1.9%	0.02	-4.6%	-11.0 to 2.2%	0.20
Traffic						
Distance from residence to a major road †,§	4.0%	0.4 to 7.8%	0.03	-2.0%	-4.8 to 0.9%	0.17

† Effect size was estimated for each 10 µg/m³ increase in PM₁₀ exposure at the mother's residence during the corresponding period.

‡ Adjusted for newborn's sex (boy/girl), maternal age (years), parity (continues), gestational age (weeks), ethnicity (European/non-European), smoking status (never, before, current) season (cold/warm period), and time-specific apparent temperature (°C). Additionally, umbilical cord blood was adjusted for blood cell count (# of white blood cells, % neutrophils, and # of platelets).

§ Percentage was calculated for each doubling in distance from residence to major road (based on a model with log₁₀ distance and log₁₀ mtDNA content).

Sensitivity analysis

Excluding women with preeclampsia ($n = 1$) or other pregnancy complications ($n = 2$) did not alter the reported changes between PM_{10} exposure and mtDNA content of placental tissue or cord blood. The expression of the nuclear genes, used as internal controls to quantify mtDNA content in qPCR assays, was not significantly associated with PM_{10} exposure during the various time windows ($p > 0.28$). Models in which we replaced PM_{10} exposure with NO_2 exposure (**Supplemental Material, Table S1** and **S2**) showed significant negative associations with placental mtDNA content for a $10 \mu\text{g}/\text{m}^3$ increase in NO_2 during the last month (-14.4%, 95% CI: -26.5 to -0.3%, $p = 0.05$) and third trimester (-21.8%, 95% CI: -32.1 to -9.8%, $p = 0.0009$) of pregnancy. The other time periods were not significantly associated, and no significant associations between NO_2 and cord blood mtDNA content during any time period were observed.

DISCUSSION

The placenta plays a pivotal role in nutrient transfer, growth, and organ development and these processes are regulated by mitochondria. Placental mitochondria also play an important role in the proper formation and functioning of the placenta and, therefore, are essential for fetal health. Urban PM has adverse effects on the functional morphology of the placenta in experimental animal models²⁴⁴ and has been associated with adverse health outcomes of the fetus,^{206,239-243} but the molecular changes have barely been studied. The key finding of our study is that placental mtDNA content, a molecular marker of mitochondrial damage and mitochondrial inflammation, is associated with *in utero* exposure to PM_{10} , especially during the last period of pregnancy. We also assessed the association between proximity of the mother's home to major roads, as a surrogate of traffic-related air pollution and the placental mtDNA content. The placental mtDNA content was positively associated with an increase in residential distance to major roads. These associations persisted with adjustment for newborn's sex, maternal age, smoking status, gestational age, ethnicity, parity, season, and time-specific apparent temperature or any other

covariate studied. In contrast to placental mtDNA content, none of these average pollution levels correlated with mtDNA content from cord blood.

Our observation that exposure to PM₁₀ and traffic-related air pollutants during pregnancy appears to modulate mtDNA replication in a negative manner is consistent with two studies on the effect of smoking. A decrease in the mtDNA content in the lung of smokers has been observed which was attributed to the oxidative stress induced by smoking.¹⁸⁶ Moreover, Bouhours-Nouet *et al.*,¹³² showed that maternal smoking is associated with mtDNA depletion in placental tissue from newborns. In our study, we did not confirm a significant association between smoking during pregnancy and a lower mtDNA content ($p = 0.40$) in the whole study population, possibly due to a lack of power.

The biological mechanisms by which air pollution may affect fetal health outcomes are poorly understood but the formation of ROS and inflammation due to PM is thought to be of importance. In addition to ROS formed as a by-product of mitochondrial respiration,²⁵⁰ ROS may also be present in mitochondria of placental tissue in response to maternal smoking.¹³² However, ROS are not only formed in placental mitochondria but also in mitochondria of endothelial cells, lining the inside of maternal and fetal capillary surface areas of the placenta. It has been shown that PM exposure, particularly to pro-oxidative combustion particles, influences endothelial function.^{251,252} The observation that smoke exposure during pregnancy causes a direct increase in the vascular resistance of the placenta from the fetal side^{253,254} suggests that PM exposure may lead to an increased resistance of umbilical-placental circulation which may impair oxygen and nutrient exchange across placenta. Mitochondria respond to energy deficiency by synthesizing more copies of their mtDNA and increase their abundance.²⁴⁷ However, mtDNA is particularly vulnerable to ROS-induced damage and has a high mutation rate.²⁰¹ mtDNA replication can be a compensatory mechanism in response to inefficient mitochondrial function due to mutations, resulting in a vicious circle of more ROS formation from defective cells.²⁵⁵ In time, the bio-energetic and replicative functions of defective mitochondria decline resulting in further depletion of mtDNA content and loss of mitochondrial function.⁸⁹

Pollutants may interfere differently with placental development during different gestational periods. During the first trimester and late pregnancy, the placenta expresses several cytochrome P450 enzymes, although only a few of them are active, indicating that metabolism of PM may be reduced.¹³ Mutations in placental mtDNA may occur in early pregnancy, leading to an onset of mitochondrial dysfunction in later trimesters. The strongest association we observed between placental mtDNA content and different PM₁₀ exposure windows during pregnancy was for the last period of pregnancy, suggesting that this might be a potential window for susceptibility to PM₁₀ exposure. Indeed, first and third trimester air pollution exposures have been implicated as having the most relevance for low birth weight and preterm birth.²⁵⁶ The study of Morello-Frosch *et al.*²⁴³ revealed a decrease in birth weight of 7.7 g for each 10 µg/m³ increase in PM₁₀ in the third trimester, although the international collaboration on air pollution and pregnancy outcomes reported heterogeneity in estimated effects of air pollution on birth weight among different locations.²⁵⁷ We found neither a significant association between birth weight and PM₁₀ exposure nor an association between birth weight and placental or cord blood mtDNA content.

Mitochondrial dysfunction can be caused by a change in mtDNA content and may be related to the development of multiple forms of disease. Decreased mtDNA content of white blood cells has been shown in type-2 diabetes,⁸⁸⁻⁹⁰ breast cancer^{91,92} and low birth weight.²⁴⁰ Alternatively, low-dose benzene exposure in various occupational groups and PM exposure in steel workers was associated with damaged mitochondria, as exemplified by increased mtDNA copy numbers in whole blood and white blood cells, respectively.^{247,258} In contrast to these observations, our results are consistent with an earlier report¹³² on maternal smoking (a personalised form of air pollution) and a lower mtDNA content. We must bear in mind that mtDNA content fluctuates during aging, under the influence of different environmental factors, and the tissue investigated.^{78,255} Experimental evidence shows that short telomeres trigger a decline in mitochondrial mass that induces additional telomere shortening.⁸⁶

The fact that we observed associations with mtDNA content in placental tissue but not in umbilical cord blood demands consideration. First, umbilical cord blood has a separate circulation that may not be representative of other tissues. For example, Gemma *et al.*²⁴⁰ postulated that umbilical cord blood is not

representative for fetal tissue. Also, they found no association between maternal leukocyte mtDNA content and umbilical cord mtDNA content, indicating that leukocyte mtDNA of cord blood may not be a good indicator of mtDNA in maternal tissue or that other *in utero* factors influence mtDNA content of cord blood. Some authors attributed variation in mtDNA in human blood cells due to variation in platelets.^{259,260} Platelet contamination increases mtDNA without contributing to nDNA and affects the mtDNA content. However, we adjusted our cord blood models for blood cell count (including platelet count). A second consideration is that the movement of pollutants into the fetal compartments can be blocked or facilitated by placental transporters.^{228,244} Pollutants that do not traverse the placenta will not contribute to effects in the cord blood but may impact placental cells, including effects on active nutrient transfer and vascular development that may adversely affect fetal development. Complex vascular alterations are considered to be the main cause of placental abnormalities in the second and third trimester and PM-induced effects may comprise a mechanism by which these alterations occur.²⁶¹ Finally, differences in turnover rates of mtDNA between tissues have been documented^{262,263} which might also contribute to different effects on cord blood and placental tissue.

Morphological changes in placental structure and vasculature occur throughout whole pregnancy.¹³ Peroxisome proliferator activated receptor protein gamma (PPARG), a nuclear transcription factor, and its transcriptional coactivator PPARy-coactivator alpha (PPARGC1A) are essential in basic placental development and function through the regulation of genes involved in trophoblast differentiation, angiogenesis, fatty acid transport, and inflammation.¹⁶⁵ In addition, PPARGC1A also controls other nuclear receptors and transcription factors that are essential in mitochondrial biogenesis and energy metabolism.²⁶⁴ We postulate that PM-induced oxidative stress may have effects on the expression of PPARG and PPARGC1A, resulting in changes in expression of PPARG-dependent genes and genes controlling mitochondrial biogenesis and mtDNA content of placental mitochondria.

Several limitations of the present study warrant consideration. Although our results were consistent after multiple adjustments, we cannot exclude the possibility of residual confounding by some unknown factor that is associated with both mitochondrial function and ambient air pollution. Ambient exposure

does not account for indoor exposure, but we obtained information on environmental tobacco smoke. mtDNA content showed considerable variation within placenta (19.3%), therefore we took biopsies at a fixed location to minimize variation of placental mtDNA content attributed to differences across placental regions.

CONCLUSION

In conclusion, *in utero* PM₁₀ exposure during the last period of pregnancy was associated with mitochondrial damage as exemplified by placental mtDNA content. This might suggest a potential window for susceptibility to PM₁₀ exposure. The potential health consequences of decreased mtDNA content in early life must be further elucidated.

Acknowledgments

This work was supported by grants from the Flemish Scientific Fund (FWO, G.0.873.11.N.10/1516112N), BOF and tUL-impulse financing (Transnational University Limburg, Hasselt-Maastricht Impuls Financing).

SUPPLEMENTAL MATERIAL

Table S1. Exposure characteristics of nitrogen dioxide

Pollution indicator	Mean [†] ± SD	25 th percentile	75 th percentile
Nitrogen dioxide (NO₂), µg/m³			
Week (Mean Lag 0-7)	22.0 ± 7.8	16.5	26.7
Last month	22.9 ± 7.5	17.5	27.8
Trimester 1	20.1 ± 5.9	16.2	23.6
Trimester 2	21.6 ± 5.9	17.4	25.5
Trimester 3	22.9 ± 7.0	18.4	27.2
Whole pregnancy	21.5 ± 5.2	18.6	24.5

[†] NO₂ values are presented as arithmetic mean with standard error and 25th-75th percentile.

Table S2. Estimated change in placental and umbilical cord blood mtDNA content in association with NO₂ during pregnancy or distance from residence to nearest major road

Variable	Placental tissue (n = 174)			Umbilical cord blood (n = 176)		
	Percentage change	95% CI	p-value	Percentage change	95% CI	p-value
Time window, NO₂ †,‡						
Trimester 1	14.1%	-2.5 to 33.4%	0.10	6.0%	-6.1 to 20.0%	0.38
Trimester 2	-9.6%	-23.0 to 6.1%	0.22	6.8%	-6.0 to 20.9%	0.30
Trimester 3	-21.8%	-32.1 to -9.8%	0.0009	4.4%	-6.6 to 16.7%	0.44
Last month	-14.4%	-26.5 to -0.3%	0.05	6.2%	-5.5 to 19.3%	0.29
Week (Mean Lag 0-7)	-3.0%	-16.0 to 12.0%	0.68	-2.3%	-12.4 to 9.0%	0.68

† Effect size was estimated for each 10 µg/m³ increase in NO₂ exposure at the mother's residence during the corresponding period.

‡ Adjusted for newborn's sex (boy/girl), maternal age (years), parity (continues), gestational age (weeks), ethnicity (European/non-European), smoking status (never, before, current) season (cold/warm period), and time-specific apparent temperature (°C). Additionally, umbilical cord blood was adjusted for blood cell count (# of white blood cells, % neutrophils, and # of platelets).

CHAPTER 6.2

PLACENTAL MITOCHONDRIAL METHYLATION AND EXPOSURE TO AIRBORNE PARTICULATE MATTER IN THE EARLY LIFE ENVIRONMENT: AN ENVIRONAGE BIRTH COHORT STUDY

Bram G. Janssen,¹ Hyang-Min Byun,² Wilfried Gyselaers,^{3,4} Wouter Lefebvre,⁵
Andrea A. Baccarelli,² Tim S. Nawrot^{1,6}

¹ Centre for Environmental Sciences, Hasselt University, Hasselt, Belgium

² Laboratory of Environmental Epigenetics, Exposure Epidemiology and Risk Program, Harvard School of Public Health, Boston, MA, USA

³ Department of Obstetrics, East-Limburg Hospital, Genk, Belgium

⁴ Department of Physiology, Hasselt University, Diepenbeek, Belgium

⁵ Flemish Institute for Technological Research (VITO), Mol, Belgium

⁶ Department of Public Health & Primary Care, Leuven University, Leuven, Belgium

ABSTRACT

Background: Most research to date has focused on epigenetic modifications in the nuclear genome, with little attention devoted to mitochondrial DNA (mtDNA). Placental mtDNA content has been shown to respond to environmental exposures that induce oxidative stress including airborne particulate matter (PM). Damaged or non-functioning mitochondria are specifically degraded through mitophagy, exemplified by a lower mtDNA content, and could be primed by epigenetic modifications in the mtDNA.

Methods: We investigated placental tissue from 381 mother-newborn pairs that were enrolled in the ENVIRONAGE birth cohort. We determined mtDNA methylation by bisulfite-pyrosequencing in two regions, i.e., the *D-loop* control region and 12S ribosomal RNA (*MT-RNR1*) and measured mtDNA content by qPCR. PM_{2.5} exposure was calculated for each participant's home address using a dispersion model.

Results: An interquartile range (IQR) increment in PM_{2.5} exposure over the entire pregnancy was positively associated with mtDNA methylation (*MT-RNR1*: +0.91%, $p = 0.01$ and *D-loop*: +0.21%, $p = 0.05$) and inversely associated with mtDNA content (relative change of -15.60%, $p = 0.001$) in placental tissue. mtDNA methylation was estimated to mediate 54%, $p = 0.01$ (*MT-RNR1*) and 27%, $p = 0.06$ (*D-loop*) of the inverse association between PM_{2.5} exposure and mtDNA content.

Conclusion: This study provides new insight into the mechanisms of altered mitochondrial function in the early life environment. Epigenetic modifications in the mitochondrial genome, especially in the *MT-RNR1* region, substantially mediate the association between PM_{2.5} exposure during gestation and placental mtDNA content which could reflect signs of mitophagy and mitochondrial death.

INTRODUCTION

Prenatal life is an important window of susceptibility to adverse effects of environmental exposures. It is well established that exposure to airborne particulate matter (PM) and tobacco smoke during gestation increases the risk of low birth weight and preterm birth in epidemiological studies.^{9,111,207} While the specific mechanisms behind fetal programming are still poorly understood, a large body of evidence suggests the human placenta as a main target organ since it serves as the gatekeeper between mother and fetus.¹³ Indeed, the placenta is the first organ to be fully developed during pregnancy. It is in contact with all nutritional, hormonal, and other environmental stress factors throughout pregnancy. During its nine month lifespan, the human placenta records morphological, functional, genetic, and epigenetic information, representing thus a molecular ‘footprint’ that can reveal the impact of *in utero* exposures on the fetus.¹⁹⁷

The placenta is a unique vascular organ that requires a constant and abundant source of energy. Mitochondria are intracellular organelles providing cellular energy through the production of adenosine-5'-triphosphate (ATP) via oxidative phosphorylation. In humans, these powerhouses contain multiple copies of double stranded, circular mitochondrial DNA (mtDNA). Mitochondria, and associated mtDNA copies, may change in number or mass due to mitophagy, a specific form of autophagy in which dysfunctional or damaged mitochondria are removed, and thus, are critical for maintaining proper cellular function.^{265,266} Analyzing mitochondrial mass by qPCR (i.e. mtDNA content) has been used as a quantitative method for specifically monitoring the last step of the degradation process of mitophagy in mammalian cells.²⁰² As such, alterations in mtDNA content are a marker of mitochondrial damage and dysfunction⁸⁶ and has been identified as an etiological determinant in a variety of human diseases such as diabetes, obesity, cardiovascular disease, and cancer.⁸⁷ Changes in fetal mtDNA content may represent a biological effect along the path linking air pollution to effects on the fetus, such as birth weight. Previously, we showed a lower mtDNA content in placental tissue in association with *in utero* exposure to PM₁₀ (PM with aerodynamic diameter $\leq 10 \mu\text{m}$),¹³³ which reflects signs of mitophagy and mitochondrial death.

Most research to date has focused on epigenetic modifications in the nuclear genome, with little attention devoted to mtDNA. Not until the discovery of a mitochondrial isoform of nuclear-encoded DNA methyltransferase enzyme 1 (mtDNMT1) by Shock *et al.*,¹⁵³ there was controversy and inconclusive evidence of DNA methylation in the mitochondrial genome. Although mtDNA methylation, by means of 5-methylcytosine and 5-hydroxymethylcytosine, has been increasingly well established after this breakthrough and led to several significant disease-related studies,^{103,105} such as cervical¹⁰⁰ and colorectal cancer,¹⁰¹ non-alcoholic fatty liver disease,¹⁰² and aging,¹⁰⁴ there are still conflicting reports in the literature regarding the existence and function of mtDNA methylation.^{267,268} Indeed, the functional consequences of this epigenetic mark are still under investigation but emerging evidence suggests a role in modifying the transcription of the mitochondrial genome.^{101,102} For example, Bellizzi *et al.*²⁶⁹ confirmed that mtDNA is methylated at the displacement loop (*D-loop*), a crucial promoter region from which mtDNA replication and transcription is initiated. Furthermore, some authors have suggested that aberrant DNA methylation in the mitochondrial *RNR1* (*MT-RNR1*) sequence, encoding for the mitochondrial ribosomal 12S ribosomal RNA (12S rRNA), could affect normal function and integrity of the mitochondrial ribosome.¹⁶⁶ Structural changes of 12S rRNA lead to instability of the small subunit of the ribosome and abolishes translation of mtDNA-encoded RNAs into proteins.²⁷⁰ Therefore, it is plausible that epigenetic modifications at important hotspots in the mtDNA can regulate replication and/or transcription of the mtDNA.

Whether exposure to PM during gestation influences methylation patterns of the mitochondrial genome in placental tissue is not known. In the framework of the ENVIRONAGE birth cohort (ENVIROnmental influence ON early AGEing), we hypothesized that, in addition to mtDNA content, methylation at the *D-loop* and *MT-RNR1* region (**Figure 1**) in placental tissue is associated with airborne PM_{2.5} exposure during gestation. To elucidate possible mechanisms that regulate mitochondrial function, we also examined mtDNA methylation as potential mediator between early life exposure to airborne PM and placental mtDNA content.

MATERIALS AND METHODS

Study population

We recruited 400 mother-newborn pairs (only singletons) from the ongoing ENVIRONAGE birth cohort as previously described.¹³³ The study participants lived in the Flemish region of Belgium. The placenta could not be collected from ten newborns, DNA yield was insufficient for four placentas and five newborns had missing data for PM exposure (lived outside Belgium). Therefore, the final sample included 381 newborns. Written informed consent was obtained from all participating mothers when they arrived at the hospital for delivery and was in accordance with procedures approved by the Ethical Committee of Hasselt University and East-Limburg Hospital in Genk. The study was conducted according to the principles outlined in the Helsinki Declaration for investigation of human subjects. The participation rate of eligible mothers (able to fill out a Dutch language questionnaire) was 56% and enrollment was spread equally over all seasons of the year. Study questionnaires were completed in the postnatal ward after delivery and provided detailed information on age, place of residence, maternal education, occupation, pre-pregnancy BMI, smoking status, use of medication, alcohol consumption, parity, mode of delivery (vaginal or caesarian section), and newborn's ethnicity. Past-smokers were defined as those who had quit before pregnancy and smokers as having smoked before and during pregnancy. Maternal education was coded as 'low' (no diploma or primary school), 'middle' (high school) or 'high' (college or university degree). Perinatal parameters such as birth date, gestational age (range, 35–42 weeks), newborn's gender, birth weight and length, Apgar score, and ultrasonographic data were collected after birth.

Exposure measurement

For each mother's residential address, we interpolated the regional background levels of PM_{2.5} (micrograms per cubic meter) using a spatial temporal interpolation method (Kriging)¹²⁰ that uses pollution data collected in the official fixed site monitoring network ($n = 34$) and land cover data obtained from satellite images (CORINE land cover data set) in combination with a dispersion model (dispersion modeling described by Lefebvre *et al.*).^{121,122} This model chain

provides daily PM_{2.5} values using data both from the Belgian telemetric air quality network, point sources, and line sources which are then interpolated to a high-resolution receptor grid. In the Flemish region of Belgium, the interpolation tool explained more than 80% of the temporal and spatial variability (R²).¹²³ The exposure during the entire pregnancy was calculated as the mean of all pregnancy days. The date of conception was estimated based on ultrasound data. We explored potential critical windows of exposures during pregnancy using daily mean PM_{2.5} concentrations averaged over various periods. The exposure windows of interest included each of the three trimesters of pregnancy, with trimesters being defined as: 1-13 weeks (first trimester), 14-26 weeks (second trimester), and 27 weeks to delivery (third trimester). Address changes during the period of pregnancy were taken into account when calculating the exposure windows ($n = 38$; 9.9%).

Placental sampling

Placentas were obtained in the delivery room and deep-frozen within ten minutes. As described previously,¹³⁰ we took villous tissue (1 to 2 cm³) at a fixed location from the fetal side of the placenta, approximately 1-1.5 cm below the chorio-amniotic membrane, and preserved the biopsies at -80°C. Chorio-amniotic membrane contamination was avoided by careful visual examination and dissection.

DNA methylation analysis

Genomic DNA was isolated from placental tissue ($n = 381$) using the QIAamp DNA mini kit (Qiagen, Inc., Venlo, the Netherlands). We performed DNA methylation analysis by highly quantitative bisulfite-PCR pyrosequencing as previously described in detail.¹²⁵ Briefly, bisulfite conversions were performed using 1 µg of extracted genomic DNA with the EZ-96 DNA methylation Gold kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. We interrogated CpG sites within specific regions of the mitochondrial genome (*MT-RNR1* and *D-loop*) as described by Byun *et al.*¹⁶⁶ and Janssen *et al.*¹²⁵ (**Figure 1**). Detailed information regarding primer sequences is given in **Supplemental Material, Table S1**. PCR amplification of regions of interest prior to pyrosequencing was performed in a total reaction volume of 30

μl, using 15 μl GoTaq Hot Start Green Master Mix (Promega, Madison, WI, USA), 10 pmol forward primer, 10 pmol reverse primer, 1 μl bisulfite-treated genomic DNA, and water. PCR products were purified and sequenced by pyrosequencing using the PyroMark Q96 MD Pyrosequencing System (Qiagen, Inc., Germantown, MD, USA). The degree of methylation was expressed as the percentage of methylated cytosines over the sum of methylated and unmethylated cytosines. Samples were run in duplicate on two different plates from which the average methylation levels were used. The coefficient of variation for *MT-RNR1* was 3.7% and for *D-loop* 5.8%. The efficiency of the bisulfite-conversion process was assessed using non-CpG cytosine residues within the sequence. The between-and within-placenta variability, exemplified by the intra-class correlation coefficient, was evaluated in a subset of nineteen placentas and was 58% vs. 42% ($p = 0.009$) for *MT-RNR1* and 61% vs. 39% ($p = 0.01$) for the *D-loop* region.¹²⁵

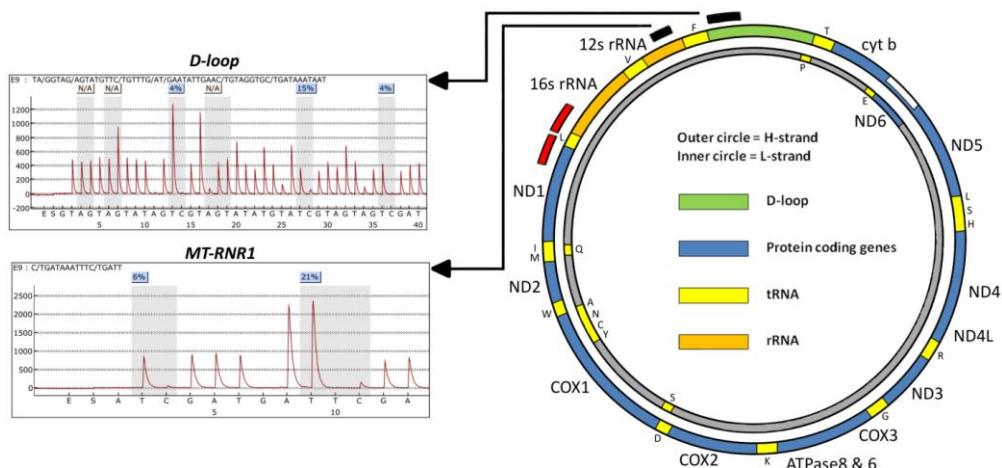


Figure 1. Location of mitochondrial targets in the human mitochondrial genome. The double stranded, circular mtDNA (16,569 bp) contains 37 genes, specifying 13 polypeptides (blue boxes), 2 ribosomal RNAs i.e. 12S rRNA and 16S rRNA (orange boxes), and 22 transfer RNAs (yellow boxes). The single letter codon associated with the yellow box refers to the amino acid. The *D-loop* region (green box) contains the origin of replication and is the place where promoters initiate transcription of the H-strand. The target location for DNA methylation analysis in the *D-loop* and *MT-RNR1* region are denoted by the black arcs with magnified pyrograms. The red arcs depict the targets for mtDNA content analysis (*MTF3212/R3319* and *ND1*). Abbreviations: NADH dehydrogenase (ND); Cytochrome c oxidase (COX); F1FO-ATP synthase (ATPase); Cytochrome b (Cyt b).

mtDNA content analysis

mtDNA content was measured in placental tissue ($n = 381$) by determining the ratio of two mitochondrial gene copy numbers (*MTF3212/R3319* and *ND1*) (**Figure 1**) to two single-copy nuclear control genes (*RPLP0* and *ACTB*) using a quantitative real-time polymerase chain reaction (qPCR) assay as previously described¹³³ but with small modification. Briefly, 2.5 μ l diluted genomic DNA (5 ng/ μ l) was added to 7.5 μ l mastermix consisting of Fast SYBR® Green I dye 2x (5 μ l/reaction), forward and reverse primer (each 0.3 μ l/reaction), and RNase free water (1.9 μ l/reaction), for a final volume of 10 μ l per reaction. Primers (**Supplemental Material, Table S2**) were diluted to a final concentration of 300 nM in the master mix. Samples were run in triplicate in 384-well format. Real-time PCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with following thermal cycling profile: 20 s at 95°C (activation), followed by 40 cycles of 1 s at 95°C (denaturation) and 20 s at 60°C (annealing/extension), ending with melting curve analysis (15 s at 95°C, 15 s at 60°C, 15 s at 95°C). qBase software (Biogazelle, Zwijnaarde, BE) automatically averages triplicate measurements that pass quality control and normalizes the data to nuclear reference genes while correcting for run-to-run differences.¹⁹⁹

Statistical analysis

For database management and statistical analysis, we used the SAS software program (version 9.2; SAS Institute Inc., Cary, NC, USA). mtDNA content was \log_{10} -transformed to improve normality. We measured methylation levels of two and three CpG sites for the *MT-RNR1* and *D-loop* region respectively (**Figure 1**). Pearson correlation coefficients were used to assess correlations between adjacent CpG sites within one region (*MT-RNR1* or *D-loop*) and to assess the correlation between mtDNA content and mtDNA methylation. Within each region, the associations with individual CpG positions were similar in size and direction ($r \geq 0.90$, **Supplemental Material, Figure S1**) and we assumed equal correlation between CpG positions in our models. We used two statistical modeling approaches to interpret mtDNA methylation data: 1/ with mixed-effect models, we took into account each CpG dinucleotide position of both regions (combination of *MT-RNR1* and *D-loop*) to obtain a robust estimate of the

average effect on DNA methylation in the mitochondrial genome, 2/ with mixed-effect models, we took into account each CpG dinucleotide position of either *MT-RNR1* or *D-loop* to obtain an estimate of each region separately. We performed multiple linear regression to determine the effect-size of PM_{2.5} exposure during pregnancy on placental mtDNA content. All adjusted models were controlled for *a priori* chosen variables including gender, maternal age, gestational age, smoking, maternal education, parity, ethnicity, and season at conception. β regression coefficients represent a relative (mtDNA content) or absolute (mtDNA methylation) percentage change for an increment between the IQR (25th-75th percentile) in the independent variable. The Shapiro-Wilk statistic and Q-Q plots of the residuals were used to test the assumptions of all linear models.

We used mediation analysis to investigate potential mechanisms that underlie the association between the exposure variable (PM_{2.5}) and outcome variable (mtDNA content) by examining how they relate to a third intermediate variable, the mediator (mtDNA methylation).²⁰⁰ We accomplished this by decomposing the total effect into direct effects (exposure effect on outcome at a fixed level of the mediator) and indirect effects (exposure effect on outcome that operate through mediator levels). Mediation analysis usually requires a significant relation of the outcome to the exposure, a significant relation of the outcome to the mediator and a significant relation of the mediator to the exposure. This is based on the *a priori* assumption that a mediated effect is biologically plausible. For the mediation analysis, we considered PM_{2.5} exposure during the entire pregnancy and average methylation levels of each mitochondrial region. We used SAS macros provided by Valeri *et al.*²⁰⁰

In a sensitivity analysis, we assessed the association between PM_{2.5} exposure during pregnancy and mtDNA methylation or mtDNA content while excluding preterm births (< 37 weeks) or women who smoked during pregnancy. In addition, we examined whether the PM_{2.5} exposure associations we observed in the main analysis were specific to certain CpG positions within the two mitochondrial regions. Finally, we investigated whether mtDNA methylation was associated with adverse pregnancy outcomes such as low birth weight using linear regression models and small for gestational age using logistic regression models.

RESULTS

Participant characteristics and exposure levels

Demographic and pregnancy-related characteristics of the 381 mother-newborn pairs are reported in **Table 1**. Briefly, mean maternal age was 29.0 years (range: 18–42 yr) and more than 50% of the mothers were high educated. Pre-pregnancy body mass index (BMI) of the participating mothers averaged (SD) 24.3 (4.5) kg/m². Most women (66.9%, $n = 255$) never smoked cigarettes and 65 women (17.1%) stopped smoking before pregnancy (median with 25th-75th percentile: 1.5 (0-5) years), whereas 61 women (16.0%) reported to have smoked during pregnancy (on average 7.8 cigarettes per day). The newborns, among them 193 girls (50.7%), had a mean gestational age of 39.2 weeks (range, 35-42) and comprised 200 (52.5%) primiparous and 141 (37.0%) secundiparous newborns. About 90% ($n = 332$) of the newborns were Europeans of Caucasian ethnicity. Birth weight averaged 3,431 (425) grams and 19 (5.0%) newborns were delivered with a caesarian section.

Table 2 displays the daily outdoor PM_{2.5} exposure levels averaged for the entire pregnancy and each of the three trimesters of pregnancy. Average (interquartile range [IQR]: 25th-75th percentile) trimester-specific PM_{2.5} exposure was 16.0 (11.8-19.6) µg/m³ for the first trimester, 16.9 (12.2-20.4) µg/m³ for the second trimester, 17.3 (11.9-21.7) µg/m³ for the third trimester and 16.7 (15.2-18.2) µg/m³ for the entire pregnancy.

Table 1. Characteristics of mother-newborn pairs ($n = 381$)

Characteristics	Mean \pm SD or range and number (%)
Maternal	
Age, y	29.0 (18–42)
Pre-pregnancy BMI, kg/m ²	24.3 \pm 4.5
Maternal education	
Low	47 (12.7%)
Middle	132 (34.7%)
High	200 (52.6%)
Smoking status	
Never-smoker	255 (66.9%)
Past-smoker	65 (17.1%)
Smoker	61 (16.0%)
Parity	
1	200 (52.5%)
2	141 (37.0%)
≥ 3	40 (10.5%)
Mode of delivery	
Caesarian section	19 (5.0%)
Newborn	
Gender	
Female	193 (50.7%)
Ethnicity	
European	332 (87.1%)
Non-European	49 (12.9%)
Gestational age, w	39.2 (35–42)
Season at conception	
Winter	90 (23.6%)
Spring	87 (22.8%)
Summer	118 (31.0%)
Fall	86 (22.6%)
Apgar score after 5 min	
≤ 8	22 (5.7%)
9	110 (28.9%)
10	249 (65.4%)
Birth weight, g	3,431 \pm 425
MT-RNR1 mtDNA methylation, % [†]	9.54 \pm 4.2
D-loop mtDNA methylation, % [†]	3.60 \pm 1.28 ($n = 356$)
mtDNA content [‡]	1.04 (0.45–2.45)

[†] Measured in placental tissue.[‡] Geometric mean with 10th–90th percentiles.

Table 2. Exposure characteristics ($n = 381$)

Exposure variable	Mean	\pm SD	25 th percentile	75 th percentile
PM_{2.5}, $\mu\text{g}/\text{m}^3$				
1 st trimester	16.0	\pm 5.3	11.8	19.6
2 nd trimester	16.9	\pm 5.0	12.2	20.4
3 rd trimester	17.3	\pm 5.8	11.9	21.7
Entire pregnancy	16.7	\pm 2.3	15.2	18.2

Placental mtDNA methylation and mtDNA content in association with PM_{2.5} exposure during gestation

We found a positive association of placental mtDNA methylation (*MT-RNR1* and *D-loop* separately as well as combined) with PM_{2.5} exposure during the entire gestation, which was independent of gender, age, gestational age, smoking, maternal education, parity, ethnicity, and season at conception (**Table 3**). The association was most pronounced in the first trimester of pregnancy (*MT-RNR1*: 1.27%, 95% CI: 0.23 to 2.32%; *D-loop*: 0.44%, 95% CI: 0.12 to 0.75%, $n = 356$ for an IQR increment of PM_{2.5} levels).

Furthermore, when adjusting for the aforementioned covariates, a lower placental mtDNA content of -15.60% (95% CI: -23.92 to -6.38%) was observed for an IQR increment of PM_{2.5} exposure over the entire pregnancy, and the association was most pronounced in the third trimester (-23.58%, 95% CI: -36.27 to -8.37%) (**Table 3**).

Table 3. PM_{2.5} exposure during the different periods of pregnancy in association with placental mtDNA methylation and placental mtDNA content

Variable	First trimester		Second trimester		Third trimester		Entire pregnancy	
	β	(95% CI)	β	(95% CI)	β	(95% CI)	β	(95% CI)
mtDNA methylation, % [†]								
MT-RNR1, %	1.27	(0.23 to 2.32)*	0.19	(-0.80 to 1.16)	1.04	(-0.20 to 1.86)	0.91	(0.56 to 4.18)*
D-loop, % [§]	0.44	(0.12 to 0.75)*	0.09	(-0.22 to 0.39)	0.04	(-0.29 to 0.36)	0.21	(-0.003 to 1.02)
Combined, %	0.75	(0.16 to 1.34)*	0.10	(-0.47 to 0.65)	0.46	(-0.23 to 0.96)	0.47	(0.20 to 2.23)*
mtDNA content, % [#]	-7.57	(-20.78 to 7.86)	-15.19	(-28.34 to 0.38)	-23.58	(-36.27 to -8.37)*	-15.60	(-23.92 to -6.38)*

[†] β represents an absolute change in percentage methylation for an IQR increment of PM_{2.5} exposure ($\mu\text{g}/\text{m}^3$).

[#] β represents a relative change in placental mtDNA content for an IQR increment of PM_{2.5} exposure ($\mu\text{g}/\text{m}^3$).

[§] Data available for 356 individuals.

* p-value significantly lower than 0.05. All models are adjusted for gender, age, gestational age, smoking, maternal education, parity, ethnicity, and season at conception.

Correlation of placental mtDNA content and mtDNA methylation

To explore the functional significance of the association of mtDNA methylation with exposure to PM_{2.5}, we evaluated the correlation between placental mtDNA content, a measure of damaged mitochondria, and mtDNA methylation. Newborns with a lower mtDNA content in placental tissue exhibited higher levels of methylation in both mitochondrial regions as shown in **Figure 2** and remained significant after adjustment for gender, age, gestational age, smoking, maternal education, parity, ethnicity, and season at conception (*MT-RNR1*: $\beta = -0.04 \pm 0.002$, $p < 0.0001$; *D-loop*: $\beta = -0.10 \pm 0.01$, $p < 0.0001$, $n = 356$).

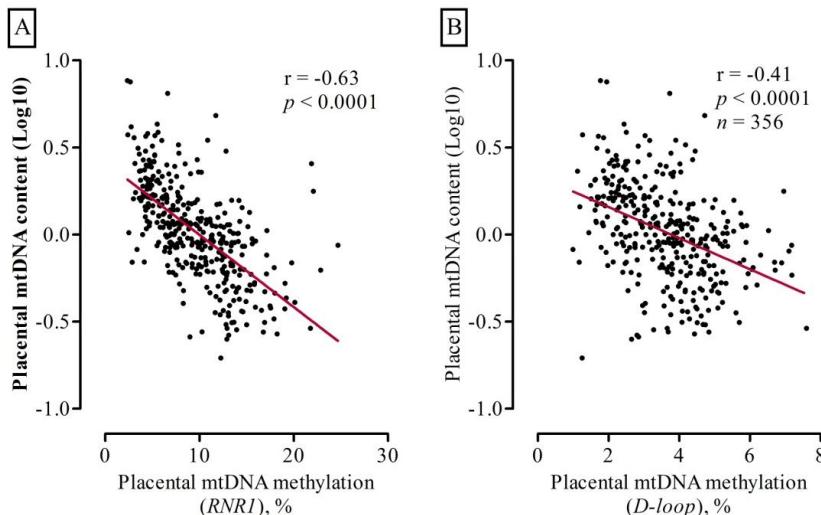


Figure 2. Correlation of mtDNA methylation and mtDNA content in placental tissue. Placental mtDNA content is log₁₀-transformed. Methylation levels are indicated as absolute percentage levels. Panel A: *MT-RNR1* methylation levels. Panel B: *D-loop* methylation levels.

Mediating effects of mtDNA methylation on the association of PM_{2.5} exposure and mtDNA content

We used mediation analysis to test if the negative association between placental mtDNA content and PM_{2.5} exposure during gestation was mediated by increased mtDNA methylation in both mitochondrial regions. All assumptions for mediation analysis were satisfied and we used average methylation levels of *MT-RNR1* and *D-loop* separately. We considered the exposure averaged over the entire pregnancy because of the discrepancy in trimester effect of PM_{2.5} exposure on mtDNA methylation (first trimester) and mtDNA content (third trimester). Estimates of the proportion of mediation indicated that placental *MT-RNR1* and *D-loop* methylation mediated respectively 54% (95% CI: 31 to 60%, $p = 0.01$) and 27% (95% CI: -3 to 36%, $p = 0.06$) of the inverse association between PM_{2.5} exposure and placental mtDNA content (**Figure 3**).

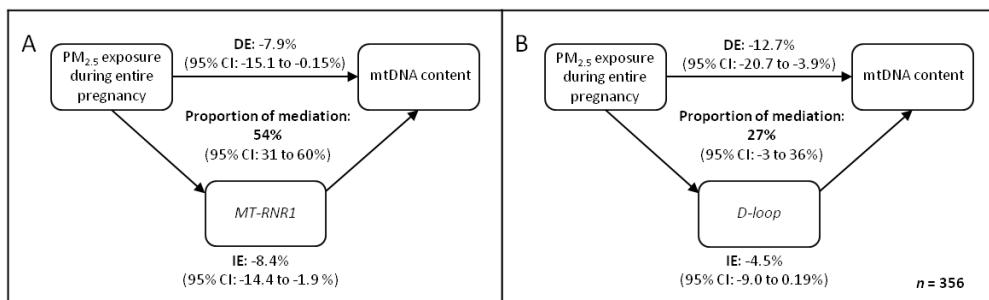


Figure 3. Estimated proportion of effects of PM_{2.5} exposure during gestation on mtDNA content mediated through changes in mtDNA methylation. The figure displays placental mtDNA methylation as mediator (panel A: *MT-RNR1*, panel B: *D-loop*), the estimates of indirect effect (IE), the estimates of the direct effect (DE), and proportion of mediation (IE/DE+IE). The effects are relative changes in placental mtDNA content for an IQR increment of PM_{2.5} exposure. The mediation model was adjusted for gender, age, gestational age, smoking, maternal education, parity, ethnicity, and season at conception.

Sensitivity analysis

In a sensitivity analysis, we repeated the main statistical analysis after additional adjustment of caesarian section but this did not alter the reported association between PM_{2.5} exposure and mtDNA content or mtDNA methylation. In addition, we repeated all statistical analysis while excluding preterm born infants (< 37 weeks, $n = 13$) and also excluding women who smoked during pregnancy ($n = 61$). This did not alter the reported associations between mtDNA content or mtDNA methylation and PM_{2.5} exposures (**Supplemental Material, Table S3**).

The associations in the main analysis between the first trimester PM_{2.5} exposure with the *D-loop* and *MT-RNR1* region differed by CpG position in magnitude and significance. For example, methylation levels at position two and three of the *D-loop* region were strongest associated with the first trimester PM_{2.5} exposure whereas the CpG positions of the *MT-RNR1* region differed in magnitude and significance (**Supplemental Material, Table S4**).

Neither methylation levels of the *MT-RNR1* nor the *D-loop* region were associated with low birth weight (*MT-RNR1*: $p = 0.58$ and *D-loop*: $p = 0.57$) or small for gestational age (*MT-RNR1*: $p = 0.66$ and *D-loop*: $p = 0.72$) (**Supplemental Material, Table S5**).

DISCUSSION

The key finding of our study is that placental mtDNA methylation is positively associated with PM_{2.5} exposure during gestation, especially in the first trimester of pregnancy. Further, these epigenetic modifications in the mitochondria substantially mediate the association between PM_{2.5} exposure during gestation and placental mtDNA content, demonstrating an intermediate mechanism of mtDNA alterations that could reflect mitophagy and mitochondrial death. To date, epigenetic mitochondrial modifications in the context of exposures in the early life environment have not been reported.

Epigenetic changes, of which DNA methylation is the most commonly characterized, can occur throughout the course of life, but much of the epigenome is already established in germ cells and embryos as it appears to be

particularly important for the regulation of embryonic growth and placental development.¹³⁹ Up until recently, it was thought that epigenetic modifications could only occur in nuclear DNA and not in mtDNA. Despite the discovery of the mtDNMT1 enzyme by Shock *et al.*¹⁵³ and studies demonstrating mtDNA methylation in humans,^{166,269,271} mammals,^{269,270} and cultured cells,^{269,272} there are still controversial studies reporting complete absence of mtDNA methylation in human cells lines²⁶⁷ or questioning the clinical usefulness of this biological phenomenon.²⁶⁸ Nevertheless, the rationale of studying mtDNA methylation is underscored by its role in the etiology of a variety of human diseases¹⁰⁰⁻¹⁰³ and aging.¹⁰⁴ Growing evidence suggests mitochondrial epigenetics as a novel mechanism to understand the pathobiology of diseases with a mitochondrial dysfunction involvement.^{102,105} An important hotspot for epigenetic regulation in the mitochondrial genome is the displacement loop or *D-loop*.²⁶⁹ DNA methylation levels in this control region are suggested to play an important role in modulating either replication or transcription of mtDNA since nearly the entire mitochondrial genome transcribes from this region.¹⁵⁴ In tumor and corresponding noncancerous tissues from colorectal cancer patients, Feng *et al.*¹⁰¹ observed a correlation between *D-loop* methylation and expression of *ND2*, a subunit of NADH encoded by mtDNA, which is a rate-limiting enzyme of the oxidative phosphorylation system. In addition, the methylation status of *ND6*, which is another crucial subunit for complex I assembly, significantly impacts the transcriptional regulation of the gene,¹⁰² an effect probably dependent on the enhanced expression of *DNMT1*.¹⁵³ Our other studied gene *MT-RNR1* encodes for the 12S rRNA protein which is critical for normal function and integrity of the mitochondrial ribosome. Methylation of *MT-RNR1* may cause malfunction of mitochondrial ribosomes and abolished translation of mtDNA-encoded RNAs into proteins.²⁷⁰ A preliminary study in human blood showed that the methylation status of mitochondrial *MT-RNR1* gene decreased during aging.²⁷³

Epigenetic modifications represent a potential link between adverse insults and altered fetal development. Previously, we have shown that PM_{2.5} exposure during pregnancy affects global DNA methylation levels in placental tissue,¹³⁰ while in this study, we investigated the impact of PM_{2.5} exposure upon epigenetics of placental mitochondria. Using the bisulfite-pyrosequencing approach, we were able to detect subtle differences in methylation levels of

specific regions in the mitochondrial genome (**Figure 1**). Our observations in newborns are partially in accordance with steel workers. Byun and colleagues¹⁶⁶ found a significant positive association between metal-rich PM₁ and DNA methylation at the *MT-RNR1* sequence, but not at the *D-loop*, in blood leukocytes of steel workers, whereas we found positive associations of PM_{2.5} exposure with methylation at both mitochondrial regions in placental tissue. It is noteworthy to mention that we observed an inverse correlation between placental mtDNA methylation and mtDNA content in contrast to a positive correlation observed in blood leukocytes.¹⁶⁶ Previous investigations have shown that mtDNA content is variable and fluctuates during aging,^{186,274} under the influence of different environmental factors,¹⁰⁶ and the tissue investigated,^{133,255,274} as well as different effects from recent versus long-term exposures. Nevertheless, we found similar results when we compared placental mtDNA content between mothers who continued smoking versus non-smoking pregnant mothers (i.e. lower mtDNA content in smokers: relative difference of -21.98%, 95% CI: -41.10 to -0.86%, $p = 0.01$). Considering smoking as a personal form of intensive exposure to particulate air pollution, this consistency with ambient air pollution adds confidence to the causal interpretation of our association between PM_{2.5} exposure and placental mtDNA content. In addition, a study that employed side-by-side epigenome-wide methylation and gene expression arrays in placentas of smokers, showed a significant correlation between methylation and gene expression of 438 genes that were involved in mitochondrial dysfunction, oxidative phosphorylation, and hypoxia pathways.¹⁹⁷

The mitochondrion is an important environmental biosensor and the findings of our study brings us one step closer to unravel the relevance of mtDNA content and mtDNA methylation to exposure-related human diseases.^{105,275} Our data indicate that mtDNA methylation, especially at the *MT-RNR1* region, might be a method by which mitochondrial biogenesis and function is regulated. We observed that the association between PM_{2.5} exposure and mtDNA methylation is most pronounced in the first trimester and the association between PM_{2.5} exposure and mtDNA content most pronounced in the third trimester of pregnancy. Given the functional relevance of the *D-loop* region and the 12S rRNA, encoded by *MT-RNR1*, in controlling replication, transcription, and translation of the mtDNA, we postulate that aberrant mtDNA methylation at

these hotspots could interfere with mtDNA biogenesis. This premise holds true when we consider the results of our mediation analysis indicating that a substantial proportion of the association between PM_{2.5} exposure and placental mtDNA content was mediated by mtDNA methylation and, therefore, strengthens the evidence of an intermediate mechanism of mtDNA alterations. Our findings of lower mtDNA content in placental tissue in association with prenatal PM exposure might reflect increased mitophagy and mitochondrial death, or it could indicate a decline in mitochondrial biogenesis due to demethylation of the mtDNA-specific polymerase gamma A (*POLGA*)²⁷⁶ or a reduction in levels of *PPARGC1A* (peroxisome proliferator-activated receptor-gamma coactivator1α), the master regulator of mitochondrial biogenesis¹⁴⁹. Furthermore, depletion of mtDNA results in significant changes in methylation pattern of a number of nuclear genes,⁷⁵ indicating a possible effect of mitochondrial dysfunction on the epigenetic landscape of the nuclear genome.⁷⁴

We acknowledge several limitations in the present study. Although we reported effects on two mtDNA regions separately and performed a combined analysis of these two regions, we cannot extrapolate these findings to the entire mitochondrial genome. We selected two regions with potential functional impacts, including a promoter region and a key ribosomal RNA sequence (**Figure 1**). Future work is warranted to determine whether DNA methylation and hydroxymethylation in other regions of the mtDNA, such as *ND2* and *ND6* that have a biological significance for mitochondrial function, are specifically sensitive to air pollutants, and whether these changes are linked to functional alterations in expression of mitochondrial genes. Secondly, we postulate that aberrant mtDNA methylation could interfere with mtDNA biogenesis. However, because our measurements were performed at birth, we cannot ascertain the temporal sequence although our proposed biological model seems plausible. Indeed, in epidemiological settings, even by use of mediation analysis, the biological direction of observed associations cannot be determined. Lastly, we can only speculate about the potential health consequences of altered mtDNA methylation and mtDNA content in placental tissue in response to PM_{2.5} exposure and, therefore, a follow-up study is warranted. Of note, a preliminary study showed differential mtDNA methylation in growth restricted placentas and underscores a possible functional relevance of mtDNA methylation in the

placenta.²⁷⁷ However, in our study we did not find an association between altered placental mtDNA methylation and adverse pregnancy outcomes such as low birth weight or small for gestational age.

CONCLUSION

Our study indicates mtDNA methylation as an early molecular event involved, at least partially, in the inverse association between prenatal exposure to PM_{2.5} and placental mtDNA content, which might reflect mitophagy. Although concise pathological roles of mitochondrial methylation during development need to be further elucidated, our findings open a new area to the molecular epidemiological understanding of mitochondrial alterations in early life.

Acknowledgements

The authors thank the participating women, as well as Anja Moors and Anneleen Staelens of the maternity ward, midwives, and the staff of the clinical laboratory of East-Limburg Hospital in Genk. We thank dr. Linda Valeri for her help with the mediation analysis. The ENVIRONAGE birth cohort is supported by the EU Program 'Ideas' (ERC-2012-StG 310898), by the Flemish Scientific Fund (FWO, N1516112/G.0.873.11.N.10), and Bijzonder Onderzoeks Fonds of Hasselt University (BOF). This work was also supported by funding from the National Institute of Environmental Health Sciences (R01ES021733, R01ES021357, and R21ES022694-01A1).

SUPPLEMENTAL MATERIAL

Table S1. Bisulfite-pyrosequencing primer sequence information based upon Assembly GRCh37/hg19 of the UCSC genome browser

Gene symbol	Chr	Amplicon start-end	Primer set (Forward / Reverse / Sequence)	Amplicon (bp)	Annealing temp (°C)	Target CpGs
<i>MT-RNR1</i>	M	1191-	F: 5'-TTTTAGAGGAGTTGTTTGTAAAT-3'	176	58.3	2
	(+)	1366	R: 5'-ATAACCCATTCTTACCACTCTATA-3'			
			S: 5'-AGTTGTTTGTAAAT-3'			
<i>D-loop</i>	M	6-	F: 5'-TGTGTAGATATTAAATTGTTATTA-3'	254	54.1	3
	(-)	259	R: 5'-CAAATCTATCACCCATTAAACCAC-3'			
			S: 5'-TAATTAATTAAATATATT-3'			

Abbreviations: *MT-RNR1*: 12S ribosomal RNA; *D-loop*: Displacement loop.

Table S2. Mitochondrial and nuclear primer sequence information based upon Assembly GRCh37/hg19 of the UCSC genome browser

Gene symbol	Chr	Amplicon start-end	Primer set (Forward / Reverse)	Amplicon (bp)	Primer efficiency (%)
<i>MTF3212/R3319</i>	M	3213-	F:5'-CACCCAAGAACAGGGTTGT-3'	108	96.3
		3320	R:5'-TTAACACATACCCATGGCCA-3'		
<i>ND1</i>	M	3314-	F:5'-ATGGCCAACCTCCTACTCCT-3'	115	99.3
		3428	R:5'-AAAGGCCCAACGTTGTAG-3'		
<i>RPLP0</i>	12	120636904-	F:5'-CCCAATTGTCCTTACCT-3'	85	100.7
		120636988	R:5'-GAACACAAAGCCCACATTCC-3'		
<i>ACTB</i>	7	5567833-	F:5'-ACTCTCCAGCCTCCTTCC-3'	102	96.8
		5567934	R:5'-TGTGGAAGCTAAGTCCTGCC-3'		

Abbreviations: Mitochondrial forward primer from nucleotide 3212 and reverse primer from nucleotide 3319 (*MTF3212/R3319*); Mitochondrial encoded NADH dehydrogenase 1 (*ND1*); Acidic ribosomal phosphoprotein P0 (*RPLP0*); Beta actin (*ACTB*).

Table S3. PM_{2.5} exposure during the different periods of pregnancy in association with placental mtDNA methylation and placental mtDNA content while excluding preterm births, < 37 weeks of gestation ($n = 13$) or smokers ($n = 61$)

	First trimester		Second trimester		Third trimester		Entire pregnancy	
Variable	β	(95% CI)	β	(95% CI)	β	(95% CI)	β	(95% CI)
Excl. preterm births								
mtDNA methylation, %†								
MT-RNR1, %	1.09	(0.03 to 2.14)*	0.25	(-0.81 to 1.32)	0.99	(-0.31 to 2.30)	0.87	(0.16 to 1.57)*
D-loop, %§	0.40	(0.08 to 0.72)*	0.11	(-0.22 to 0.44)	-0.001	(-0.41 to 0.40)	0.19	(-0.02 to 0.40)
Combined, %	0.66	(0.07 to 1.26)*	0.15	(-0.46 to 0.75)	0.43	(-0.32 to 1.18)	0.45	(0.06 to 0.85)*
mtDNA content, %‡	-5.19	(-18.98 to 10.94)	-14.19	(-27.79 to 1.98)	-22.04	(-35.19 to -6.22)*	-14.21	(-22.88 to -4.58)*
Excl. smokers								
mtDNA methylation, %†								
MT-RNR1, %	1.24	(0.15 to 2.32)*	0.55	(-0.53 to 1.63)	0.90	(-0.49 to 2.29)	0.99	(0.24 to 1.73)*
D-loop, %§	0.42	(0.08 to 0.77)*	0.16	(-0.18 to 0.50)	0.04	(-0.40 to 0.47)	0.22	(-0.001 to 0.44)
Combined, %	0.72	(0.10 to 1.35)*	0.27	(-0.35 to 0.88)	0.41	(-0.40 to 1.21)	0.50	(0.08 to 0.92)*
mtDNA content, %‡	-7.87	(-22.51 to 9.54)	-20.00	(-33.32 to -4.03)*	-19.08	(-33.45 to -1.59)*	-16.27	(-25.31 to -6.14)*

† β represents an absolute change in percentage placental mtDNA methylation for an IQR increment of PM_{2.5} exposure ($\mu\text{g}/\text{m}^3$).‡ β represents a relative change in placental mtDNA content for an IQR increment of PM_{2.5} exposure ($\mu\text{g}/\text{m}^3$).

§ Data available for 356 individuals.

* p -value significantly lower than 0.05. All models are adjusted for gender, age, gestational age, smoking, maternal education, parity, ethnicity, and season at conception.

Table S4. Associations of PM_{2.5} exposure during the first trimester of pregnancy and mtDNA methylation across different CpG positions

Variable	β‡	(95% CI)	p-value
<i>MT-RNR1, %</i>			
Mean	1.27	(0.23 to 2.32)	0.02
Position 1	0.49	(-0.03 to 1.01)	0.07
Position 2	2.06	(0.43 to 3.68)	0.01
<i>D-loop, %†</i>			
Mean	0.44	(0.12 to 0.75)	0.01
Position 1	0.24	(0.05 to 0.43)	0.02
Position 2	0.64	(0.14 to 1.13)	0.01
Position 3	0.43	(0.15 to 0.72)	0.003

† Data available for 356 individuals.

‡ β represents an absolute change in percentage mtDNA methylation for an IQR increment of PM_{2.5} exposure ($\mu\text{g}/\text{m}^3$), adjusted for gender, age, gestational age, smoking, maternal education, parity, ethnicity, and season at conception.

Table S5. Placental mtDNA methylation and pregnancy outcomes

	MT-RNR1	D-loop‡
Variable	β^{\dagger} (95% CI)	β^{\dagger} (95% CI)
Variable	OR (95% CI)	OR (95% CI)
Low birth weight	2.5 (-6.6 to 11.7)	9.2 (-22.9 to 41.3)
SGA	-2.3 (-11.9 to 8.4)	1.8 (-28.0 to 44.1)

† β represents a change in grams for one percentage increase in placental mtDNA methylation (%), adjusted for gender, age, gestational age, smoking, maternal education, parity, ethnicity, and season at conception.

‡ Data available for 356 individuals.

Abbreviations: OR = odds ratio. SGA = small for gestational age (infants born with a birth weight less than the 10th percentile).

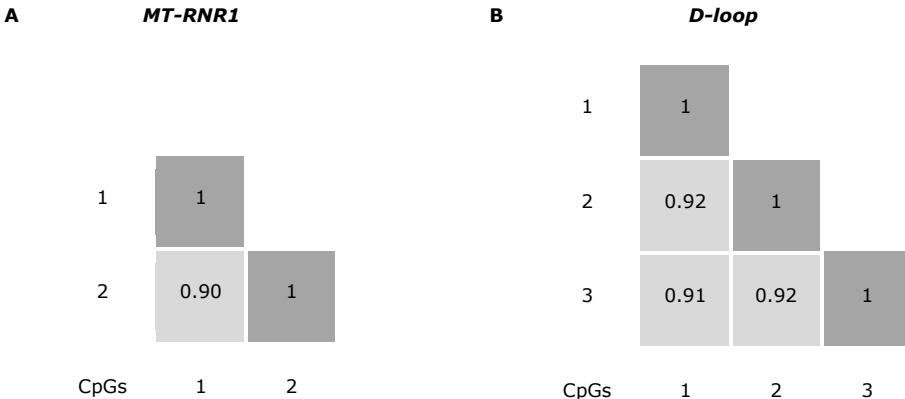


Figure S1. Pearson correlation coefficients between CpG sites of the *MT-RNR1* (A) and *D-loop* (B) region. All *p*-values were < 0.0001.

CHAPTER 7.1

EPIDEMIOLOGICAL EVIDENCE FOR FETAL THYROID-DEPENDENT REGULATION AT THE (EPI)GENOMIC LEVEL IN PLACENTAL MITOCHONDRIA

Bram G. Janssen,¹ Hyang-Min Byun,² Harry A. Roels,^{1,3} Wilfried Gyselaers,^{4,5} Joris Penders,^{5,6} Andrea A. Baccarelli,² Tim S. Nawrot^{1,7}

¹ Centre for Environmental Sciences, Hasselt University, Hasselt, Belgium

² Laboratory of Environmental Epigenetics, Exposure Epidemiology and Risk Program, Harvard School of Public Health, Boston, MA, USA

³ Louvain Centre for Toxicology and Applied Pharmacology (LTAP), Université catholique de Louvain, Brussels, Belgium

⁴ Department of Obstetrics, East-Limburg Hospital, Genk, Belgium

⁵ Biomedical Research Institute, Hasselt University, Diepenbeek, Belgium

⁶ Laboratory of Clinical Biology, East-Limburg Hospital, Genk, Belgium

⁷ Department of Public Health & Primary Care, Occupational & Environmental Medicine, Leuven University, Leuven, Belgium

ABSTRACT

Background: Fetal development largely depends on thyroid hormone availability as well as proper placental function with an important role played by placental mitochondria. The biological mechanisms by which thyroid hormones exert their effects on mitochondrial function are not well understood. We investigated the role of fetal thyroid hormones on placental mitochondrial DNA (mtDNA) content and mtDNA methylation.

Methods: We collected placental tissue and cord blood from 305 mother-child pairs that were enrolled between February 2010 and June 2014 in the ENVIRONAGE birth cohort (province of Limburg, Belgium). Placental mtDNA content was determined by qPCR and placental mtDNA methylation by bisulfite-pyrosequencing in two regions, i.e., the *D-loop* control region and 12S ribosomal RNA (*MT-RNR1*). The levels of free thyroid hormones (FT_3 , FT_4) and thyroid stimulating hormone (TSH) were measured in cord blood.

Results: Cord blood FT_3 and FT_4 were negatively associated with placental mtDNA methylation at the *MT-RNR1* ($p \leq 0.01$) and *D-loop* ($p \leq 0.05$) regions, whereas a positive association was observed for both hormones with placental mtDNA content ($p \leq 0.04$). We estimated that *MT-RNR1* and *D-loop* methylation mediated respectively 77% [indirect effect: +14.61% (95% CI: 2.65 to 27.95%, $p = 0.01$)] and 47% [indirect effect: +8.60% (95% CI: 1.25 to 16.49%, $p = 0.02$)] of the positive association between FT_3 and placental mtDNA content. Mediation models with FT_4 gave similar results but the estimated effect proportions were smaller compared with those of FT_3 (respectively 54% and 24%).

Conclusions: We showed that epigenetic modification of the mitochondrial genome could intervene with thyroid-dependent regulation of mitochondrial biogenesis.

INTRODUCTION

It is well recognized that the (patho)physiological role of mitochondria widely exceeds that of solely being the biochemical power plant of the cells. A new area of research that stretches beyond the nuclear genome called 'mitochondriomics', is dedicated to clarify whether mitochondria are novel sensors and mediators of environmental effects by exploring mitochondrial DNA (mtDNA) abundance or content, mutations and deletions, epigenetics (e.g. DNA methylation), and mtDNA-encoded proteins.⁵⁵

Thyroid hormones, which are essential for fetal development,¹⁷ are known to have profound effects on mitochondrial energetics and biogenesis.^{27,28} Under the control of thyroid-stimulating hormone (TSH), the thyroid gland produces thyroxine (T₄), the major form of thyroid hormone, and triiodothyronine (T₃), the active form. On account of its biological activity, unbound or free T₃ (FT₃) regulates mitochondrial biogenesis most likely in two different cell compartments: i) binding of FT₃ to thyroid receptors (TRs) that consecutively bind to response elements in the nucleus, activating the expression of mitochondria-related genes such as peroxisome proliferator-activated receptor γ-coactivator1α (*PPARGC1A*) or ii) direct binding to specific TRs in the mitochondrial matrix, stimulating transcription of the mitochondrial genome.²⁷⁸ In the placenta, a high affinity of nuclear binding sites for FT₃ has been shown to stimulate the production of factors that control trophoblast growth and development.²² The placenta, in conjunction with the fetal thyroid gland, liver, and kidneys, maintains the optimal concentration of thyroid hormones in the fetal circulation at each stage of development¹⁶ by controlling thyroid hormone metabolism through the expression of iodothyronine deiodinases D2 and D3.²¹

The precise mechanisms by which thyroid hormones exert their effects on mitochondrial function are not well understood. In this mother-newborn study, we aimed to elucidate possible mechanisms involved in the regulation of mitochondrial function by investigating the association between fetal FT₃, FT₄, and TSH with mtDNA content and mtDNA methylation in placental tissue. We hypothesized that methylation at the mitochondrial genome could interfere with thyroid hormone-dependent regulation of mitochondrial biogenesis.

MATERIALS AND METHODS

Study population

Within the on-going ENVIRONAGE birth cohort (ENVIROnmental influence ON early AGEing), we conducted our investigation in a group of 589 singleton pregnancies for which the mothers agreed withdrawal of cord blood, the collection of the placenta after delivery, and the use of information from their medical files. The placenta could not be collected for ten newborns, four placentas had insufficient DNA yield, 12 placentas had missing measurements of mtDNA content, and 16 mothers with thyroid gland complications were excluded. Because mtDNA methylation was measured only in a subset of this large study sample ($n = 547$), we ended with a final sample size of 305 newborns for the main analysis (see flowchart; **Supplemental Material, Figure S1**).

Mother-child pairs were recruited from February 2010 to June 2014 at the East-Limburg Hospital in Genk (Belgium) following procedures approved by the Ethical Committee of Hasselt University and the East-Limburg Hospital, and according to the principles outlined in the Helsinki Declaration for investigation of human subjects as previously described in detail.¹³³ Briefly, written informed consent was obtained from eligible participants before delivery. Questionnaires and medical records consulted after birth provided information on maternal age, maternal education, smoking status, ethnicity, pre-pregnancy body mass index (BMI), gestational age, newborn's gender, Apgar scores, birth weight and length, parity, and ultrasonographic data. Information about maternal tobacco smoke exposure was obtained by asking whether mothers smoked during pregnancy, whether they smoked at any time during their life or if they never smoked in their life. All neonates were assessed for congenital anomalies immediately after birth and were considered healthy with an Apgar score after 5 min ranging between 7 and 10. No neonate was delivered in the Neonatal Intensive Care Unit. The ENVIRONAGE birth cohort generally consists of mothers with normal pregnancies without complications and with healthy neonates.

Cord blood collection and thyroid hormones measurements

Immediately after delivery, the umbilical cord was clamped and cord blood was drawn in plastic BD Vacutainer® Lithium Heparin Tubes (BD, Franklin Lakes, NJ, USA). Blood tubes were centrifuged at 3,200 rpm for 15 min to retrieve plasma which was instantly kept at -80°C. Free T₄ (FT₄, pmol/L), free T₃ (FT₃, pmol/L) and TSH (mU/L) were measured in plasma using an electro-chemiluminescence immunoassay using the Modular E170 automatic analyzer (Roche, Basel, Switzerland) at the clinical lab of East-Limburg Hospital.

Placental collection

Placentas were deep-frozen within ten minutes of delivery and afterwards, placental specimens were taken for DNA extraction after minimally thawing of the placentas. We took villous tissue (1 to 2 cm³) at a fixed location from the fetal side of the placenta, approximately 1-1.5 cm below the chorio-amniotic membrane, and preserved the biopsies at -80°C.¹²⁵ Genomic DNA was isolated from placental tissue using the QIAamp DNA mini kit (Qiagen, Inc., Venlo, the Netherlands) and stored at -80°C until further use.

DNA methylation analyses

In 305 placentas, we performed DNA methylation analysis by highly quantitative bisulfite-PCR pyrosequencing as previously described.¹²⁵ Briefly, bisulfite conversions were performed using 1 µg of extracted genomic DNA with the EZ-96 DNA methylation Gold kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. We interrogated CpG sites within specific regions of the mitochondrial genome (*MT-RNR1* and *D-loop*) and the promoter of *PPARGC1A* as described by Byun *et al.*¹⁶⁶ and Janssen *et al.*¹²⁵ Detailed information regarding primer sequences is given in **Supplemental Material, Table S1**. PCR amplification of regions of interest prior to pyrosequencing was performed in a total reaction volume of 30 µl, using 15 µl GoTaq Hot Start Green Master Mix (Promega, Madison, WI, USA), 10 pmol forward primer, 10 pmol reverse primer, 1 µl bisulfite-treated genomic DNA, and water. PCR products were purified and sequenced by pyrosequencing using the PyroMark Q96 MD Pyrosequencing System (Qiagen, Inc., Germantown, MD, USA). The degree of methylation was expressed as the percentage of methylated cytosines over the

sum of methylated and unmethylated cytosines. Samples were run in duplicate on two different plates from which the average methylation levels were used. The coefficient of variation was 3.7% for *MT-RNR1*, 5.8% for *D-loop*, and 8.1% for *PPARGC1A*. The efficiency of the bisulfite-conversion process was assessed using non-CpG cytosine residues within the sequence. The between- and within-placenta variability, exemplified by the intra-class correlation coefficient, was evaluated in a subset of 19 placentas and was 58% vs. 42% ($p = 0.009$) for *MT-RNR1*, 61% vs. 39% ($p = 0.01$) for the *D-loop* region, and 64% vs. 36% ($p = 0.005$) for *PPARGC1A*.¹²⁵

mtDNA content analysis

mtDNA content was measured in placental tissue by determining the ratio of two mitochondrial gene copy numbers (*MTF3212/R3319* and *MT-ND1*) to two single-copy nuclear control genes (*RPLP0* and *ACTB*) using a quantitative real-time polymerase chain reaction (qPCR) assay as previously described¹³³ but with small modification. Briefly, 2.5 μ l diluted genomic DNA (5 ng/ μ l) was added to 7.5 μ l mastermix consisting of Fast SYBR® Green I dye 2x (5 μ l/reaction), forward and reverse primer (each 0.3 μ l/reaction), and RNase free water (1.9 μ l/reaction). Primer sequences (**Supplemental Material, Table S2**) were diluted to a final concentration of 300 nM in the master mix. Samples were run in triplicate in 384-well format. Real-time PCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following thermal cycling profile: 20 s at 95° (activation), followed by 40 cycles of 1 s at 95°C (denaturation) and 20 s at 60°C (annealing/extension), ending with melting curve analysis (15 s at 95°C, 15 s at 60°C, 15 s at 95°C). qBase software (Biogazelle, Zwijnaarde, BE) was used to normalize data and correct for run-to-run differences.¹⁹⁹

Statistical analysis

For database management and statistical analysis, we used the SAS software program (version 9.2; SAS Institute Inc., Cary, NC, USA). mtDNA content and thyroid hormone levels were \log_{10} -transformed to improve normality. For each subject, we measured in placental tissue methylation levels of CpG sites at two regions of the mitochondrial genome and at the promoter region of *PPARGC1A*.

The pyrosequencing-based DNA methylation analysis produced a methylation value (%) for each CpG of *MT-RNR1* (two CpGs), the *D-loop* region (three CpGs), and *PPARGC1A* (three CpGs). In the main analysis, we used the average methylation levels of the different CpGs. Pearson correlation coefficients were calculated between the different thyroid hormone levels in cord blood (FT_3 , FT_4 , and TSH), mtDNA methylation (*MT-RNR1* and *D-loop*), and mtDNA content in placental tissue using R software packages. We performed multiple linear regression to determine the association between thyroid hormones and placental mtDNA methylation (both *MT-RNR1* and *D-loop*), and between thyroid hormones and placental mtDNA content. Thyroid hormones were fitted as linear variables in the models and effect estimates on mtDNA methylation and mtDNA content were calculated for a 10th-90th percentile increment in thyroid hormones, which corresponds to a 52% change in FT_3 , a 12% change in FT_4 , and a 84% change in TSH. All models were adjusted for maternal age, pre-pregnancy BMI, gestational age, newborn's gender, smoking, parity, maternal education, and ethnicity. The Shapiro-Wilk statistic and Q-Q plots of the residuals were used to test the assumptions of all linear models.

We used mediation analysis to investigate potential associations that may underlie the relation between the exposure variable (FT_3 or FT_4) and the continuous outcome variable (mtDNA content) by examining how they relate to a third variable, the mediator (mtDNA methylation).²⁰⁰ We accomplished this by decomposing the total effect into a direct effect (DE: exposure effect on outcome at a fixed level of the mediator) and an indirect effect (IE: exposure effect on outcome that operates through the mediator).

Since we have complete data on mtDNA content and thyroid hormones for 547 newborns, we reanalyzed our data to investigate the association between thyroid hormones and placental mtDNA content in a sensitivity analysis. Furthermore, using mixed-effect models, we took into account each CpG dinucleotide position and reanalyzed the association between fetal thyroid hormones and mtDNA methylation at the *MT-RNR1* and *D-loop* region. To underscore important CpG sites, we performed linear regression analysis between levels of FT_3 or FT_4 and the methylation levels at the separate CpG sites of *MT-RNR1* and *D-loop*. Lastly, since mitochondrial biogenesis relies on a tightly coordinated process between the nuclear and mitochondrial genome, we used

linear regression to investigate the association between fetal thyroid hormones and placental promoter methylation of the nuclear gene *PPARGC1A*, a central regulator of mitochondrial gene expression and biogenesis that is controlled by FT₃.²⁷⁹

RESULTS

Mother-newborn characteristics and demographics

The study included 305 mother-child pairs (mean maternal age, 29.1 yr; range, 18–42 yr). Demographic and prenatal lifestyle factors are reported in **Table 1**. Briefly, mean (10th-90th percentile) pre-pregnancy BMI of the participating mothers was 24.1 (19.7-29.7) kg/m². 52 mothers (17.1%) reported to have smoked during pregnancy and smoked on average 7.8 cigarettes per day. Most women (64.9%, *n* = 198) never smoked cigarettes. The majority (> 50%) of the mothers were highly educated. Nearly half of the newborn population were boys (*n* = 151; 49.5%). The overall mean gestational age was 39.2 weeks (10th-90th percentile: 18-41) and included a vast majority of primiparous (51.8%, *n* = 158) or secundiparous (36.7%, *n* = 112) newborns.

Table 1. Characteristics of mother-child pairs (*n* = 305)

Characteristics	Mean [10 th -90 th percentile] or number (%)
Mother	
Age, y	29.1 [23-36]
Pre-pregnancy BMI, kg/m ²	24.1 [19.7-29.7]
Net weight gain, kg	14.7 [7.5-22.0]
Maternal education†	
Low	37 (12.1%)
Middle	107 (35.1%)
High	161 (52.8%)
Self-reported smoking habit	
Never smoker	198 (64.9%)
Cessation before pregnancy	55 (18.0%)
Smoker during pregnancy	52 (17.1%)
Parity	
1	158 (51.8%)
2	112 (36.7%)
≥ 3	35 (11.5%)
Newborn	
Gender	
Male	151 (49.5%)
Ethnicity‡	
European-Caucasian	269 (88.2%)
Gestational age, w	39.2 [38-41]
Season at delivery	
Winter (Dec-Mar)	80 (26.2%)
Spring (Mar-Jun)	89 (29.2%)
Summer (Jun-Sep)	60 (19.7%)
Autumn (Sep-Dec)	76 (24.9%)
Apgar score after 5 min	
7 or 8	20 (6.6%)
9	90 (29.5%)
10	195 (63.9%)
Birth weight, g	3,421 [2,880-3,985]

† Mother's education: low (no high school diploma), middle (high school diploma), high (college or university diploma).

‡ Based on the native country of the newborn's grandparents. European-Caucasian when two or more grandparents were European, or non-European when at least three grandparents were of non-European origin.

Levels of fetal thyroid hormones, placental mtDNA methylation, and mtDNA content

The geometric values (10th-90th percentile) of thyroid hormone levels in cord blood were 2.63 (2.15-3.22) pmol/L for FT₃, 15.66 (13.26-18.53) pmol/L for FT₄, and 11.65 (5.16-20.59) mU/L for TSH (**Table 2**). A positive correlation was observed between the FT₃ and FT₄ values ($r = 0.25, p < 0.0001$) and between the FT₃ and TSH values ($r = 0.19, p = 0.0008$) (**Figure 1**).

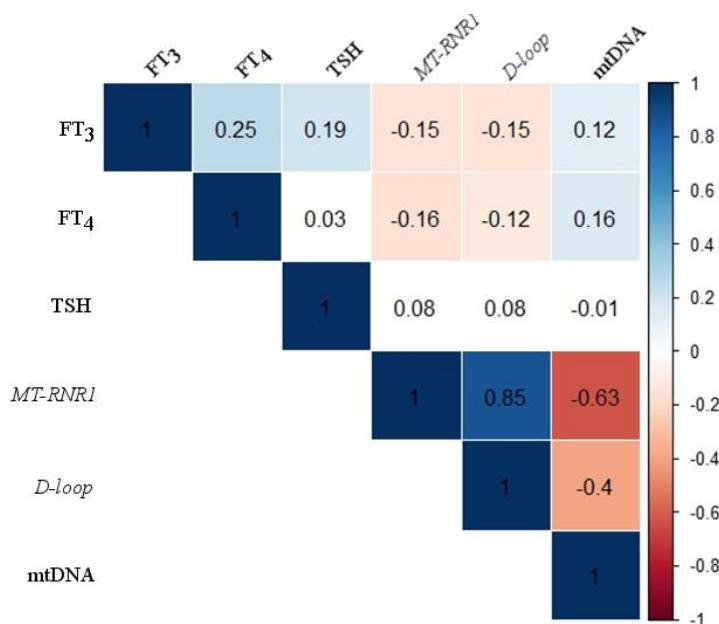


Figure 1. Unadjusted correlation matrix between thyroid hormones (FT₃, FT₄, TSH), mtDNA methylation (MT-RNR1, D-loop), and mtDNA content. Numbers represent Pearson correlation coefficients and colored squares indicate significant correlations at p -level < 0.05 (blue: positive correlation; red: negative correlation).

Table 2. Cord blood thyroid hormone levels and placental mtDNA content and mtDNA methylation

Variable	Mean	SD	10 th percentile	90 th percentile
Thyroid hormones†				
FT ₃ , pmol/L	2.63	0.47	2.15	3.22
FT ₄ , pmol/L	15.66	2.08	13.26	18.53
TSH, mU/L	11.65	7.60	5.16	20.59
mtDNA methylation‡				
MT-RNR1, %	9.51	4.19	4.46	14.67
D-loop, %	3.61	1.31	1.94	5.24
mtDNA content‡				
	1.11	0.96	0.49	2.49

Values are presented as geometric means with 10th-90th percentiles, except for mtDNA methylation for which the arithmetic mean is given.

† Laboratory reference values for adults range from 4.0 to 6.8 pmol/L for FT₃, from 12.0 to 21.9 pmol/L for FT₄, and from 0.3 to 4.2 mIU/L for TSH.

‡ Measured in placental tissue.

Table 2 also shows mtDNA methylation and mtDNA content values measured in placental tissue. Placental mtDNA methylation averaged (10th-90th percentile) 9.51% (4.46-14.67) for MT-RNR1 and 3.61% (1.94-5.24) for D-loop. The methylation levels of MT-RNR1 and D-loop were strongly correlated ($r = 0.85$, $p < 0.0001$) (**Figure 1**). Mean placental mtDNA content was 1.11 (0.49-2.49) (unitless). Previously, Janssen *et al.*¹³⁵ reported a strong negative correlation between placental mtDNA methylation and mtDNA content which has been corroborated in this study ($r = -0.63$, $p < 0.0001$ for MT-RNR1 and $r = -0.40$, $p < 0.0001$ for D-loop) (**Figure 1**).

Association of fetal thyroid hormones with placental mtDNA methylation and mtDNA content

Both fetal FT₃ and FT₄ were negatively correlated with mtDNA methylation at the MT-RNR1 and D-loop region, whereas a positive correlation was observed for both hormones with mtDNA content in placental tissue (see **Figure 1** for correlations). Fetal TSH levels did not correlate with either placental mtDNA methylation or mtDNA content.

The associations remained significant after adjustment for maternal age, pre-pregnancy BMI, gestational age, newborn's gender, smoking, parity, maternal education, and ethnicity. A 10th-90th percentile increment (53%) in cord blood FT₃ (\log_{10} -values) was associated with a lowering in absolute methylation of

-1.50% (95% CI: -2.70 to -0.30%, $p = 0.01$) for *MT-RNR1* and -0.46% (95% CI: -0.83 to -0.09%, $p = 0.01$) for *D-loop*. A similar association, but with smaller estimates, was observed for FT_4 (**Table 3**). On the other hand, a 10th-90th percentile increment in cord blood FT_3 (53%) or FT_4 (13%) was associated with a higher placental mtDNA content (relative change) of +20.26% (95% CI: 0.42 to 44.02%, $p = 0.04$) for FT_3 and +11.08% (95% CI: 4.07 to 18.56%, $p = 0.002$) for FT_4 .

Mediation analysis

We performed mediation analysis to estimate the proportion of the associations between cord blood thyroid hormones and placental mtDNA content that might be mediated by changes in mtDNA methylation if the underlying causal assumptions of the mediation analysis are valid. We selected both *MT-RNR1* and *D-loop* as potential mediators because methylation levels at both regions were significantly associated with FT_3 and FT_4 as well as with placental mtDNA content (**Figure 1**). While adjusting for the aforementioned variables, we estimated that *MT-RNR1* methylation mediated 77% [indirect effect: +14.61% (95% CI: 2.65 to 27.95%, $p = 0.01$)] and *D-loop* methylation mediated 47% [indirect effect: +8.60% (95% CI: 1.25 to 16.49%, $p = 0.02$)] of the positive association between FT_3 and placental mtDNA content (**Figure 2**). Mediation models with FT_4 gave similar results but the estimated effect proportions were smaller compared with those of FT_3 (respectively 54% and 24%) (**Supplemental Material, Figure S2**).

Sensitivity analysis

In a sensitivity analysis, we verified whether the association between fetal thyroid hormones and placental mtDNA content was still present in the initial group of 547 mother-child pairs (flowchart **Supplemental Material, Figure S1**). Adjusting for the aforementioned variables, the association between cord blood FT_3 and placental mtDNA content was stronger compared with the reported association in the main analysis, i.e. relative change of +29.61% (95% CI: 16.61 to 44.06%, $p < 0.0001$) for a 10th-90th percentile increment in cord blood FT_3 , whereas the association for FT_4 was less (+6.67%, 95% CI: 2.26 to 11.28%, $p = 0.003$).

We reanalyzed our data using mixed-effect models to investigate the association between fetal thyroid hormones and mtDNA methylation at the *MT-RNR1* and *D-loop* region. As expected, we observed the same results as in the main analysis but with a significant interaction between CpG site and FT₃ or FT₄. Certain CpGs were more associated with fetal thyroid hormones compared to others, underscoring the importance of methylation levels at specific CpG (**Supplemental Material, Table S3**). For example, FT₃ was associated with methylation levels at all three CpG sites of *D-loop* (the transcription start site of the mitochondrial genome).

Lastly, in an additional analysis, we observed a negative association between fetal FT₃ levels and placental *PPARGC1A* promoter methylation, i.e. -2.31% (95% CI: -4.04 to -0.59, $p = 0.009$) for a 10th-90th percentile increment in FT₃ (**Supplemental Material, Table S3**).

Table 3. Associations of placental mtDNA methylation and mtDNA content with cord blood thyroid hormones

Variable	FT ₃ , pmol/L		FT ₄ , pmol/L		TSH, mU/L	
	β	(95% CI)	β	(95% CI)	β	(95% CI)
mtDNA methylation [†]						
MT-RNR1, %	-1.50	(-2.70 to -0.30)*	-0.60	(-1.04 to -0.16)*	0.38	(-0.21 to 0.97)
D-loop, %	-0.46	(-0.83 to -0.09)*	-0.14	(-0.27 to -0.002)*	0.11	(-0.07 to 0.29)
mtDNA content, % [‡]	20.26	(0.42 to 44.02)*	11.08	(4.07 to 18.56)**	-5.18	(-13.17 to 3.54)

[†] β represents an absolute change in placental mtDNA methylation percentage (%) for a 10th-90th percentile increment of cord blood thyroid hormone.

[‡] β represents a relative change (%) in placental mtDNA content for a 10th-90th percentile increment of cord blood thyroid hormone.

A 10th-90th percentile increment in log₁₀ FT₃, FT₄, and TSH corresponds to a 53%, 12%, and 84% change in FT₃, FT₄, and TSH respectively.

All models are adjusted for maternal age, pre-pregnancy BMI, gestational age, newborn's gender, smoking, parity, maternal education, and ethnicity.

* p-value < 0.05, ** p-value < 0.005.

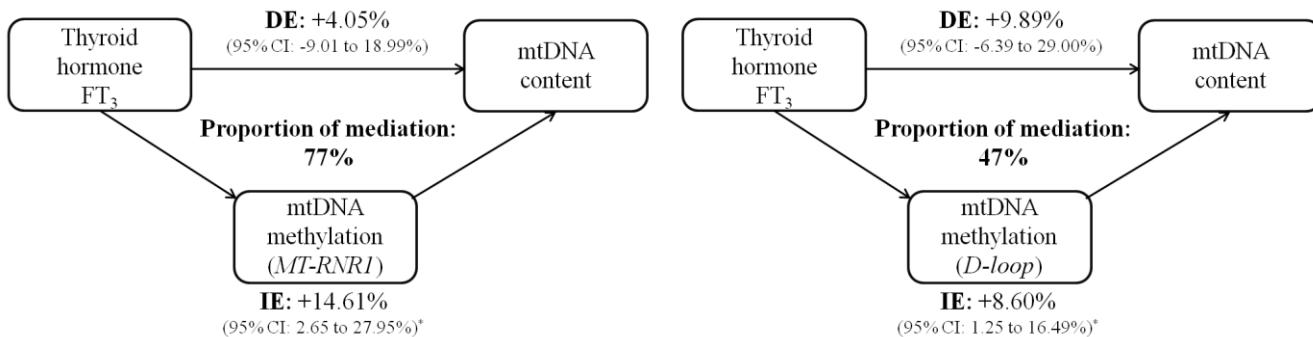


Figure 2. Estimated proportion of effects of FT_3 exposure on mtDNA content mediated by mtDNA methylation. The figure displays placental mtDNA methylation as mediator (left panel: *MT-RNR1*; right panel: *D-loop*), the estimates of indirect effect (IE), the estimates of the direct effect (DE), and proportion of mediation (IE/DE+IE). The effects represent a relative change (%) in placental mtDNA content for an increment between the 10th-90th percentile of FT_3 . All models were adjusted for maternal age, pre-pregnancy BMI, gestational age, newborn's gender, smoking, parity, maternal education, and ethnicity. * p -value < 0.05.

DISCUSSION

In this study, we report for the first time associations between fetal thyroid hormones and epigenetic modification in the mitochondrial genome that could, at least in part, mediate the fetal thyroid-dependent regulation of mitochondrial biogenesis in placental tissue.

The human placenta regulates the passage of thyroid hormones from the maternal to the fetal circulation, ensuring that the required levels are present in the fetus at each stage of development.¹⁶ From mid-gestation onwards, the fetus starts secreting small amounts of thyroid hormone in conjunction with the transplacental supply of maternal thyroid hormones.²⁸⁰ The placenta expresses two types of iodothyronine deiodinases (D2 and D3) that are capable of metabolizing FT₃ and FT₄,²¹ and thus, plays an important role in thyroid hormone homeostasis. Besides regulating fetal development,¹⁷ thyroid hormones have profound effects on mitochondrial energetics and biogenesis.^{27,28}

Despite spectacular progress in the knowledge of the T₄-T₃ nuclear pathway, a clear answer to how it regulates mitochondrial biogenesis is lacking. While it is known that an upregulation of nuclear-encoded respiratory genes including PPARGC1A occurs within hours after injection of T₃ in hypothyroid rats, other non-genomic direct effects are detectable within minutes suggesting another mode of action.^{281,282} T₃ binds directly to specific receptors inside mitochondria. A 43 kDa c-Erb A alpha1 protein (p43), located exclusively in the mitochondrial matrix, acts as a T₃-dependent transcription factor and specifically binds to four mitochondrial DNA sequences with a high similarity to nuclear T₃ response elements.^{283,284} Overexpression of p43 increases mitochondrial genome transcription and protein synthesis, stimulating mitochondrial biogenesis in a T₃-dependent manner.²⁸⁴ Interestingly, of the four mitochondrial DNA sequences two response elements are located in the *D-loop* region and one in the *MT-RNR1* gene. *MT-RNR1* encodes for the 12S rRNA protein which is critical for normal function and integrity of the mitochondrial ribosome,²⁷⁰ whereas the *D-loop*, or displacement loop, is the site from which mitochondrial transcription starts.¹⁵⁴ We hypothesized that DNA methylation at these mitochondrial hotspots could interfere with thyroid hormone-dependent regulation of mitochondrial biogenesis. First, we have shown that fetal thyroid hormones, especially FT₃, are

positively associated with placental mtDNA content and negatively associated with placental methylation levels at the *D-loop* and *MT-RNR1* region. Next, we underscored a substantial mediating role of placental mtDNA methylation between the association of fetal thyroid hormones and placental mtDNA content. Hence, we postulate that high DNA methylation levels in the mitochondrial genome are related to conformational or structural changes making the mtDNA less accessible to proteins and transcription factors such as the T₃-dependent transcription factor p43. Previously, we have shown that exposure to particulate matter (PM_{2.5}) during pregnancy is associated with higher levels of methylation of the *D-loop* as well as the *MT-RNR1* sequence, affecting mtDNA content in placental tissue.¹³⁵ An increase in mtDNA methylation was observed in blood leukocytes of steel workers exposed to air pollution,¹⁶⁶ and in an experimental study of human cultured cells treated with ethidium bromide.²⁷² Cells recovering from mtDNA depletion induced by ethidium bromide treatment, had increased overall methylation levels indicating that mtDNA was less packed with proteins during active mtDNA replication.²⁷²

Our mediation analysis revealed that the effect of FT₃ on mtDNA content is mediated for 77% by *MT-RNR1* and for 54% by *D-loop* methylation levels, meaning that there are alternative routes, most likely nuclear pathways, by which thyroid hormones exert their action on mitochondrial biogenesis. T₃ controls the expression of *PPARGC1A*,²⁸¹ a transcriptional co-activator of several nuclear-encoded transcription factors including mitochondrial transcription factor A (*TFAM*) that regulates mitochondrial biogenesis¹⁵¹ (**Figure 3**). In addition to specific mitochondrial actions of FT₃, we also observed a negative association between placental *PPARGC1A* promoter methylation and fetal FT₃ levels. Most likely this is indicative of up-regulated *PPARGC1A* mRNA levels in the presence of high FT₃ concentrations since promoter hypomethylation is usually associated with increased gene expression due to unwound DNA that is available for transcription factors. Interestingly, the interrogated region is a putative binding site for CREB transcription factors. The transcriptional activity of CREB is critical for the establishment and maintenance of energy homeostasis in mice neonates¹⁶⁹ and appears to be involved in the regulation of *PPARGC1A*. It is noteworthy to mention that the promoter region of *PPARGC1A* was largely unmethylated in cord blood but not in placental tissue,¹²⁵ suggesting differential

regulation of *PPARGC1A* in cord blood and placental tissue. Even though nuclear-encoded genes that are involved in mitochondrial function are regulated by epigenetic mechanisms,²⁸⁵ it would be interesting to determine the direct functional significance at mRNA expression level in response to FT₃ levels. Unfortunately, we did not measure mRNA expression due to lack of suitable RNA samples and consider this a limitation of the study. Another limitation of our study is that we were not able to measure thyroid hormones in placental tissue but we used cord blood levels as a proxy since it circulates through the fetal side of the placenta.

CONCLUSION

Given our epidemiological findings and other experimental research data, it seems that there exist coordinated events between mtDNA methylation, mtDNA content, and thyroid hormones. We summarized our findings in a simplified scheme (**Figure 3**) indicating that epigenetic modification of the mitochondrial genome could intervene with thyroid-dependent regulation of mitochondrial biogenesis. Our findings could contribute to further epidemiological understanding of mitochondrial disorders. Whether alterations in mitochondrial function or newborn's thyroid hormone levels may have health consequences later in life should be elucidated.

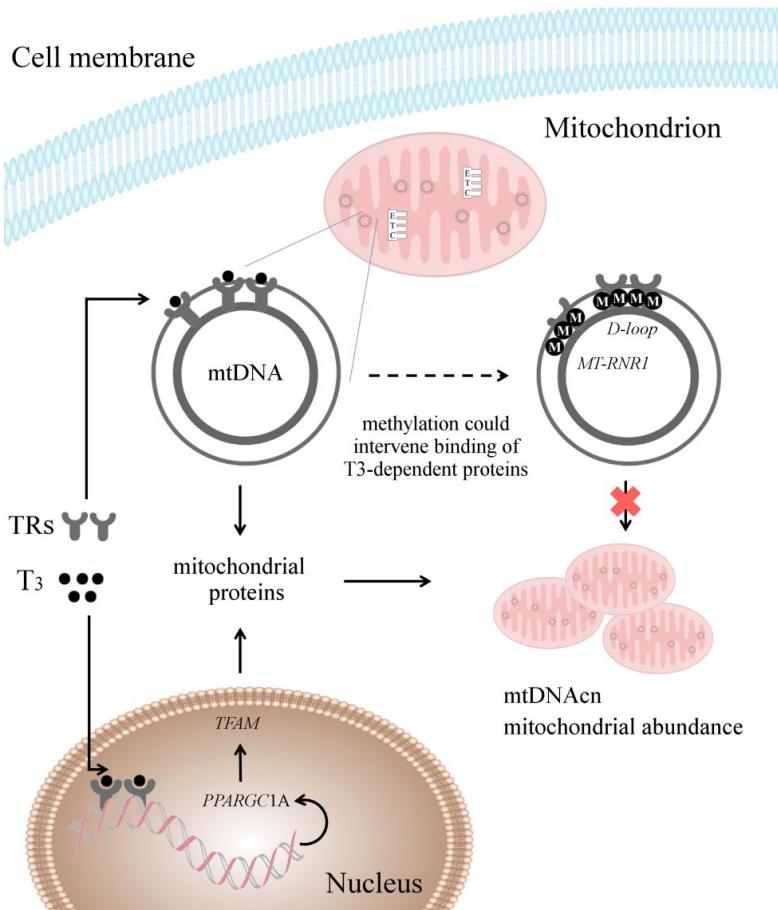


Figure 3. Simplified scheme of T_3 -dependent mitochondrial biogenesis through coordinated regulation of nuclear and mitochondrial gene products. T_3 binds to thyroid receptors (TRs) which consecutively bind to response elements in the nucleus activating expression of mitochondrial-related genes such as *PPARGC1A*. Alternatively, specific TRs are localized in the mitochondrial matrix (p43). The T_3 -p43 complex binds to response elements in the mitochondrial genome, of which two elements are located in the *D-loop* and one in the 12S rRNA (*MT-RNR1*) gene. We suggest that methylation of the mtDNA genome, in particular in the *D-loop* and *MT-RNR1* region, could intervene with T_3 -dependent mitochondrial protein production through conformational or structural changes making the mtDNA less accessible to proteins and transcription factors such as the T_3 -dependent transcription factor p43.

Acknowledgments

The authors thank the participating mothers and neonates, as well as the staff of the maternity ward, midwives, and the staff of the clinical laboratory of East-Limburg Hospital in Genk. The ENVIRONAGE birth cohort is supported by the European Research Council (ERC-2012-StG.310898), by the Flemish Scientific Fund (FWO, N1516112 / G.0.873.11.N.10) and the Special Research Fund (BOF) of Hasselt University.

SUPPLEMENTAL MATERIAL

Table S1. Bisulfite-pyrosequencing primer sequence information based upon Assembly GRCh37/hg19 of the UCSC genome browser

Gene symbol	Chr	Amplicon start-end	Primer set (Forward / Reverse / Sequence)	Amplicon (bp)	Annealing temp (°C)	Target CpGs
<i>MT-RNR1</i>	M	1191-	F: 5'-TTTTTAGAGGAGTTGTTTGTAAAT-3'	176	58.3	2
	(+)	1366	R: 5' <u>ATAACCCATTCTTACCACTCATA</u> -3'			
			S: 5'-AGTTTGTGGTAAAT-3'			
<i>D-loop</i>	M	6-	F: 5'-TGTGAGATATTAAATTGTTATT-3'	254	54.1	3
	(-)	259	R: 5' <u>CAAATCTATCACCTATTAAACCAC</u> -3'			
			S: 5'-TAATTAAATTAAATATATT-3'			
<i>PPARGC1A</i>	4	23891766-	F: 5'-TTTTTGTTGAGTTGTTAA-3'	231	53	3
		23891997	R: 5' <u>TTACAAAAAATTAAATTATTATATAACCA</u> -3'			
			S: 5'-AGTTTGTGGTAAATT-3'			

Abbreviations: *MT-RNR1*: 12S ribosomal RNA; *D-loop*: Displacement loop; *PPARGC1A*: peroxisome proliferator-activated receptor γ-coactivator1α.

Table S2. Mitochondrial and nuclear primer sequence information based upon Assembly GRCh37/hg19 of the UCSC genome browser

Gene symbol	Chr	Amplicon start-end	Primer set (Forward / Reverse)	Amplicon (bp)	Primer efficiency (%)
<i>MTF3212/R3319</i>	M	3213-	F:5'-CACCCAAGAACAGGGTTGT-3'	108	96.3
		3320	R:5'-TTAACAAACATAACCATGGCCA-3'		
<i>ND1</i>	M	3314-	F:5'-ATGGCCAACCTCCTACTCCT-3'	115	99.3
		3428	R:5'-AAAGGCCCAACGTTGTAG-3'		
<i>RPLP0</i>	12	120636904-	F:5'-CCCAATTGTCCTTACCT-3'	85	100.7
		120636988	R:5'-GAACACAAAGCCCACATTCC-3'		
<i>ACTB</i>	7	5567833-	F:5'-ACTCTCCAGCCTCCTTCC-3'	102	96.8
		5567934	R:5'-TGTGGAAGCTAAGTCCTGCC-3'		

Abbreviations: *MTF3212/R3319*: Mitochondrial forward primer from nucleotide 3212 and reverse primer from nucleotide 3319; *ND1*: Mitochondrial encoded NADH dehydrogenase 1; *RPLP0*: Acidic ribosomal phosphoprotein P0; *ACTB*: Beta actin.

Table S3. Associations of cord blood thyroid hormones FT₃ and FT₄ with CpG-specific mtDNA methylation and PPARGC1A promoter methylation

Gene	FT ₃ , pmol/L		FT ₄ , pmol/L	
	β†	(95% CI)	β‡	(95% CI)
MT-RNR1				
CpG1	-0.46	(-1.05 to 0.13)	-0.33	(-0.54 to -0.11)**
CpG2	-2.54	(-4.41 to -0.67)*	-0.88	(-1.56 to -0.19)*
D-loop				
CpG1	-0.24	(-0.46 to -0.02)*	-0.07	(-0.15 to 0.01)
CpG2	-0.73	(-1.32 to -0.14)*	-0.23	(-0.45 to -0.02)*
CpG3	-0.41	(-0.74 to -0.08)*	-0.11	(-0.23 to 0.01)
PPARGC1A§				
CpG1	-1.85	(-3.29 to -0.41)*	-0.02	(-0.55 to 0.52)
CpG2	-2.12	(-3.96 to -0.29)*	0.08	(-0.60 to 0.76)
CpG3	-2.96	(-5.46 to -0.46)*	0.21	(-0.71 to 1.13)
Average CpGs	-2.31	(-4.04 to -0.59)*	0.09	(-0.55 to 0.73)

†β represents an absolute change in mtDNA methylation percentage (%) for a 10th-90th percentile increment of FT₃ cord blood thyroid hormone which corresponds to a 53% change in FT₃.

‡β represents an absolute change in percentage (%) methylation for a 10th-90th percentile increment of FT₄ cord blood thyroid hormone which corresponds to a 12% change in FT₄.

§ Average (10th-90th percentile) methylation levels of PPARGC1A was 7.95% (2.77-16.45).

All models are adjusted for maternal age, pre-pregnancy BMI, gestational age, newborn's gender, smoking, parity, maternal education, and ethnicity.

* p-value < 0.05, ** p-value < 0.005.

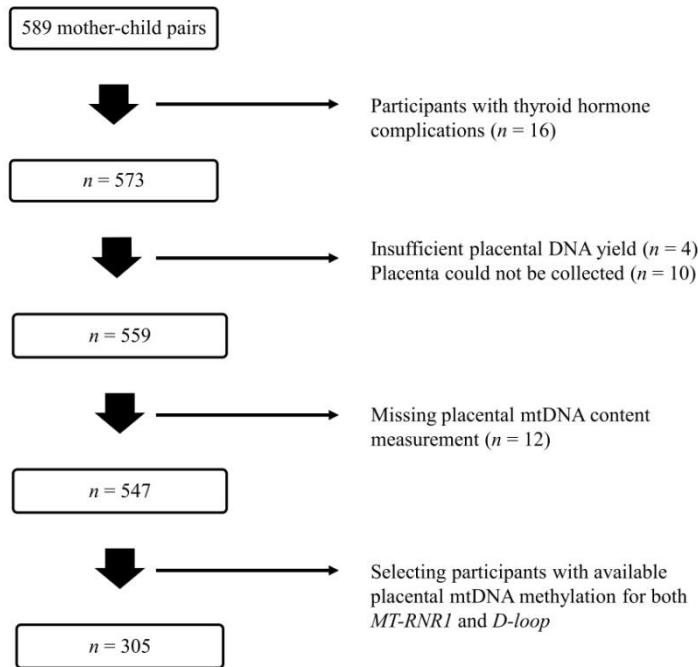


Figure S1. Flowchart depicting the selection for arriving at the final study sample either for placental mtDNA content measurement ($n = 547$) or for mtDNA methylation analysis ($n = 305$).

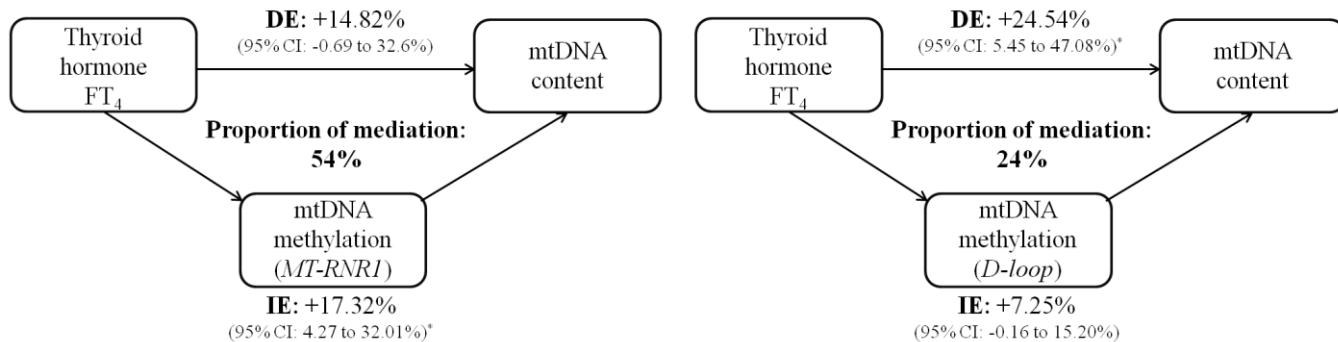


Figure S2. Estimated proportion of effects of FT₄ exposure on mtDNA content mediated by mtDNA methylation. The figure displays placental mtDNA methylation as mediator (left panel: *MT-RNR1*; right panel: *D-loop*), the estimates of indirect effect (IE), the estimates of the direct effect (DE), and proportion of mediation (IE/DE+IE). The effects represent a relative change (%) in placental mtDNA content for an increment between the 10th-90th percentile of FT₄. All models were adjusted for maternal age, pre-pregnancy BMI, gestational age, newborn's gender, smoking, parity, maternal education, and ethnicity.
**p*-value < 0.05.

CHAPTER 7.2

FETAL THYROID FUNCTION, BIRTH WEIGHT, AND *IN UTERO* EXPOSURE TO FINE PARTICLE AIR POLLUTION: A BIRTH COHORT STUDY

Bram G. Janssen,^{1*} Nelly D. Saenen,^{1*} Harry A. Roels,^{1,2} Narjes Madhloum,¹ Wilfried Gyselaers,^{3,4} Wouter Lefebvre,⁵ Joris Penders,^{3,6} Charlotte Vanpoucke,⁷ Karen Vrijens,¹ Tim S. Nawrot^{1,8}

* Authors equally contributed

¹ Centre for Environmental Sciences, Hasselt University, Hasselt, Belgium

² Louvain Centre for Toxicology and Applied Pharmacology (LTAP), Université catholique de Louvain, Brussels, Belgium

³ Biomedical Research Institute, Hasselt University, Hasselt, Belgium

⁴ Department of Obstetrics, East-Limburg Hospital, Genk, Belgium

⁵ Flemish Institute for Technological Research (VITO), Mol, Belgium

⁶ Department of Clinical Biology, East-Limburg Hospital, Genk, Belgium

⁷ Belgian Interregional Environment Agency, Brussels, Belgium

⁸ Department of Public Health & Primary Care, Occupational & Environmental Medicine, Leuven University, Leuven, Belgium

ABSTRACT

Background: Thyroid hormones are critical for fetal development and growth. Currently, there is no information available whether prenatal exposure to fine particle air pollution ($PM_{2.5}$) affects fetal thyroid function and what the impact is on birth weight in normal healthy pregnancies. To determine the impact of third trimester $PM_{2.5}$ exposure on fetal and maternal thyroid hormones and their mediating role on birth weight.

Methods: We measured the levels of free thyroid hormones (FT_3 , FT_4) and thyroid stimulating hormone (TSH) in cord blood ($n = 499$) and maternal blood ($n = 431$) collected after delivery from mother-child pairs enrolled between February 2010 and June 2014 in the ENVIRONAGE birth cohort with catchment area in the province of Limburg, Belgium.

Results: An interquartile range (IQR) increment (+8.2 $\mu\text{g}/\text{m}^3$) in third trimester $PM_{2.5}$ exposure was inversely associated with cord blood TSH levels (-11.6%, 95% CI: -21.8 to -0.1%, $p = 0.05$) and the FT_4/FT_3 ratio (-62.7%, -91.6 to -33.8%, $p < 0.0001$) which was mainly attributed to a reduction in cord FT_4 concentrations. A 10th-90th percentile decrease in cord blood FT_4 levels was associated with a lowering of 56 g birth weight increase (95% CI: -90 to -23 g, $p = 0.001$). The estimated negative effect of an IQR increment in third trimester $PM_{2.5}$ exposure on birth weight was mediated for 21% (-19 g, 95% CI: -37 to -1 g, $p = 0.04$) by decreasing cord blood FT_4 levels. In maternal blood, collected one day after delivery, FT_4 tended to be inversely associated with an IQR increment in third trimester $PM_{2.5}$ exposure (-4.0%, 95% CI: -0.8 to 0.2%, $p = 0.06$).

Conclusions: In normal healthy pregnancies, exposure to $PM_{2.5}$ air pollution negatively affects fetal thyroid function and plays a role in reduced birth weight. Whether $PM_{2.5}$ -induced alterations in fetal thyroid function has bearing on pathological consequences later in life requires further elucidation.

INTRODUCTION

During prenatal life, thyroid hormones are critical for fetal growth and development, especially neurodevelopment.^{17,20} Unbalanced thyroid function influences pregnancy outcomes and adversely affects the fetus. In particular, both maternal hypo- and hyperthyroidism are associated with increased risk of low birth weight,^{23,24} while other studies also suggest an important role of fetal thyroid function in regulating fetal growth.^{25,26}

Thyroxine (T_4), the major form of thyroid hormone, and triiodothyronine (T_3), the active form, are controlled by thyroid-stimulating hormone (TSH) and released by the thyroid gland. Bound to plasma proteins, these thyroid hormones are transported throughout the body and diffuse from maternal blood across the placenta to reach the fetus.¹⁸ However, it is the unbound, free fraction of these hormones (FT_4 and FT_3) that are taken up by different cell types to regulate their function.¹⁹ From the second trimester of gestation onwards, the fetal thyroid gland becomes functional and the fetus is able to produce its own supply of thyroid hormones in addition to the maternal supply.²⁰

Exposure to chemicals such as airborne persistent organic pollutants,²⁸⁶⁻²⁸⁸ cadmium,²⁸⁹ and exposure to active and passive cigarette smoke²⁹⁰ has been found to interfere with thyroid hormone regulation and function in neonates and adults, however epidemiological studies on the impact of exposure to particulate matter (PM) air pollution on thyroid hormones are lacking. In large areas of the world, PM air pollution is an omnipresent environmental risk factor of public health concern, especially the fine particles with an aerodynamic diameter $\leq 2.5 \mu\text{m}$ ($PM_{2.5}$). Exposure to ambient $PM_{2.5}$ pollution during pregnancy has been found to be significantly associated with increased risk of low birth weight at term in mother-child cohorts of 12 European countries³¹ and preterm birth (20-36 weeks of gestation) in a very large cohort of singleton pregnancies from three states of the USA.⁵⁰ However, studies concerning air pollution effects on the child's neurodevelopment are limited. Recently, it has been shown that children (7-10 yrs) exposed to high traffic-related air pollution at school demonstrated lower cognitive development over a one school year period.²⁹¹

In spite of the well-established link between $PM_{2.5}$ air pollution and adverse gestational outcome,³¹ the role of fetal thyroid function in this association has

never been investigated. Therefore, we hypothesizes that airborne PM_{2.5} exposure during gestation affects fetal thyroid hormone function in normal healthy pregnancies and relates to birth weight. We tested this hypothesis in the framework of a mother-child cohort by investigating the impact of third trimester PM_{2.5} exposure on fetal and maternal thyroid hormone function, as reflected by the levels of FT₃, FT₄, and TSH in cord blood and maternal blood, and their mediating role on birth weight.

MATERIALS AND METHODS

Study population

From February 2010 to June 2014, we recruited 640 mother-child pairs after delivery at the East-Limburg Hospital in Genk (Belgium). They were enrolled in the on-going ENVIRONAGE birth cohort study (ENVIRONMENTal influence ON early AGEing) following procedures previously approved by the Ethical Committee of Hasselt University and the East-Limburg Hospital.¹³³ The study was conducted according to the principles outlined in the Helsinki Declaration for investigation of human subjects. The participation rate of eligible mothers in the birth cohort (mothers able to fill out a questionnaire in Dutch and those without planned Caesarean section) was 61% and enrollment was spread equally over all seasons of the year. Midwives recorded the reason of nonparticipation. The main reasons (in descending importance) were failure to ask for participation, communication problems, or complications during labor. Participating mothers provided written informed consent when they arrived at the hospital for delivery. They completed study questionnaires in the post-delivery ward to provide detailed information on maternal age, pre-pregnancy body mass index (BMI), maternal education, occupation, self-reported smoking status, alcohol consumption, place of residence, use of medication, parity, and newborn's ethnicity. Former-smokers were defined as those who had quit smoking before pregnancy. Smokers continued smoking during pregnancy. Based on the native country of the newborn's grandparents we classified his/her ethnicity as European-Caucasian when two or more grandparents were European, or non-European when at least three grandparents were of non-European origin. We asked the mothers whether they consumed alcohol during pregnancy. Maternal

education was coded as 'low' (no diploma or primary school), 'middle' (high school) or 'high' (college or university degree). The ENVIRONAGE birth cohort did not differ from all births in Flanders [data obtained from the Study Centre for Perinatal Epidemiology (SPE)] as to maternal age, education, parity, sex, ethnicity, and birth weight (**Supplemental Material, Table S1**).¹¹¹ After birth, we collected perinatal parameters from the medical files such as birth date, gestational age, newborn's sex, birth weight and length, length of labor, Apgar score, pH of arterial cord blood, and ultrasonographic data.

The main analysis of our investigation was conducted in a cohort of 499 singleton pregnancies (mother-child group) for mothers who agreed withdrawal of maternal and cord blood after delivery and the use of information from their medical files. Mothers with thyroid gland complications, including hyper- or hypothyroidism, were excluded *a priori* ($n = 16$). We excluded 79 mothers from which we had no complete set of thyroid hormone values, 28 Caesarean sections, and 18 preterm births (< 37 weeks). Maternal blood could not be collected from 68 mothers resulting in a study population of 431 for the maternal thyroid hormone analysis (mother group) (see flowchart; **Figure 1**).

Ambient PM_{2.5} exposure assessment

For each mother's residential address, we interpolated the regional background PM_{2.5} ($\mu\text{g}/\text{m}^3$) using a spatial temporal interpolation method (Kriging method)¹²⁰ that uses pollution data collected by the official fixed-site monitoring network and land-cover data obtained from satellite images (CORINE land-cover data set) in combination with a dispersion model.¹²² This model chain provides daily PM_{2.5} values using data from the Belgian telemetric air quality network, and point and line sources which is then interpolated in a high resolution receptor grid. In the Flemish region of Belgium, the interpolation tool explained more than 80% of the temporal and spatial variability.¹²³ We defined the third trimester of pregnancy as from week 27 to delivery and calculated the mean PM_{2.5} values for this trimester. The date of conception was estimated on the basis of the first day of the mother's last menstrual period, combined with the first ultrasound exam. Complete information for the residential address during pregnancy was obtained by questionnaire and checked with hospital records. For those who moved during pregnancy ($n = 54$; 10.8%), we calculated the third

trimester exposure window allowing for the changes in address during this period.

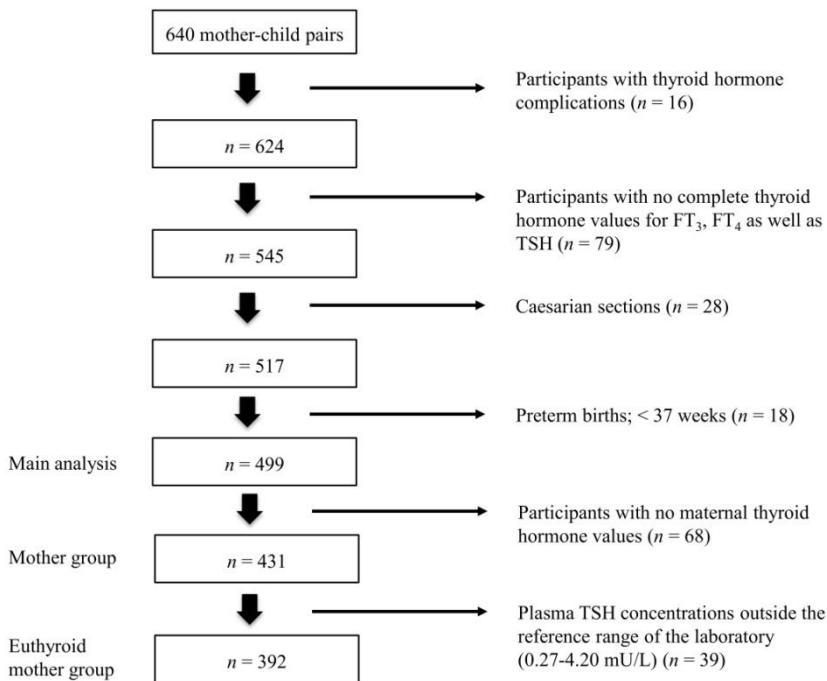


Figure 1. Flowchart depicting the selection procedure of study participants from the ENVIRONAGE birth cohort in Limburg, Belgium

Blood collection and thyroid hormone measurements

Umbilical cord and maternal blood samples (8 mL each) were collected in plastic BD Vacutainer® Lithium Heparin Tubes (BD, Franklin Lakes, NJ, USA) immediately after delivery and one day after delivery respectively. The samples were centrifuged (3,200 rpm for 15 min) to retrieve plasma which was instantly frozen at -80°C . The plasma levels of FT_4 (pmol/L), FT_3 (pmol/L), and TSH (mU/L) were measured with an electro-chemiluminescence immunoassay using the Modular E170 automatic analyzer (Roche, Basel, Switzerland) at the clinical lab of East-Limburg Hospital.

Statistical analysis

For database management and statistical analysis, we used the SAS software program (version 9.2; SAS Institute Inc., Cary, NC, USA). Thyroid hormone levels were \log_{10} -transformed to improve the normality of the distributions and described by geometric mean and 10th-90th percentile. The ratio FT₄/FT₃ was calculated using untransformed values and had a normal distribution. Pearson correlation coefficients were calculated between the different thyroid hormone levels in blood (FT₃, FT₄, and TSH) and between thyroid hormone levels and birth weight. We performed multiple linear regressions to assess the associations between newborn or maternal thyroid hormones and PM_{2.5} exposure during the 3rd trimester of gestation, and between newborn or maternal thyroid hormones and birth weight. Exposures to PM_{2.5} were fitted as linear variables in the models and effect estimates on thyroid hormones were calculated for an interquartile range (IQR) increment in PM_{2.5}. The effect estimates of cord blood FT₄ on birth weight was calculated for a 10th-90th percentile decrease in FT₄, which corresponds to a 11% change in FT₄. All cord blood models were adjusted for sex, gestational age (weeks), season of delivery (Winter [21st of Dec – 20th of Mar] / Spring [21st of Mar – 20th of Jun] / Summer [21st of Jun – 20th of Sep] / Autumn [21st of Sep – 20th of Dec]), Apgar score (< 9 / 9 / 10), maternal age (years), pre-pregnancy BMI (kg/m²), smoking status (never-smoker / cessation before pregnancy / smoker), parity (1 / 2 / ≥ 3), ethnicity (European-Caucasian, yes or no), maternal education (low / middle / high), and apparent temperature (°C), while all models for maternal blood were adjusted for the same covariates except newborn's sex and Apgar score. In an additional analysis, we adjusted the cord blood models for maternal thyroid hormones. The Shapiro-Wilk statistic and Q-Q plots of the residuals were used to test the assumptions of model linearity.

We used mediation analysis to investigate potential associations that may underlie the relation between the exposure variable (PM_{2.5}) and the continuous outcome variable (birth weight, g) by examining how they relate to a third variable, the mediator (cord blood FT₄ levels).²⁰⁰ We accomplished this by decomposing the total effect into a direct effect (DE: exposure effect on outcome at a fixed level of the mediator) and an indirect effect (IE: exposure effect on outcome that operates through the mediator).

In a sensitivity analysis (Supplemental Material), we performed linear regression analysis to examine the associations between newborn or maternal thyroid hormones and smoking, adjusting for the same co-variables as mentioned above except smoking (**Supplemental Material, Table S2**). Additionally, we repeated the analysis between cord blood thyroid hormones and PM_{2.5} exposure while excluding smokers. Secondly, we explored whether other covariates such as seasonal variation,²⁹² alcohol consumption,²⁹³ cord plasma estradiol,²⁹⁴ pH of arterial cord blood,²⁹⁵ passive smoking,²⁹⁰ or length of labor,²⁹⁶ known for their interference with thyroid hormones, may alter the association between cord blood thyroid hormones and 3rd trimester PM_{2.5} exposure (**Supplemental Material, Text S1**). Finally, we examined whether the association between cord blood thyroid hormones and 3rd trimester PM_{2.5} exposure still remained in the subgroup for which maternal thyroid hormones were available (mother group; $n = 431$) and in the euthyroid mother subgroup ($n = 392$), with plasma TSH concentrations within the reference range of the clinical laboratory of the hospital (0.27-4.20 mU/L).

RESULTS

Demographics of participants

Table 1 shows demographic characteristics and perinatal traits of the mother-child pairs ($n = 499$). Mean maternal age was 29.1 years (range: 18-44 yr) and mean (SD) pre-pregnancy BMI was 23.9 (4.3) kg/m². Most women never smoked ($n = 316$), 113 stopped smoking before pregnancy, and 70 mothers reported to continue with smoking during pregnancy (on average 8.6 cigarettes/day). More than 80% of the mothers reported no consumption of alcoholic beverages during pregnancy. The newborns, among them 254 girls (50.8%), had a mean gestational age of 39.4 weeks (range, 35-42) and comprised 275 primiparous and 170 secundiparous newborns. About 90% of the newborns were Europeans of Caucasian ethnicity. The mean (SD) birth weight of the newborns was 3,446 (420) g. Five minutes after delivery, more than 90% of the newborns had an Apgar score ≥ 9 .

Table 1. Characteristics and thyroid hormone levels of mother-newborn pairs

Characteristic	Mother-child group (n = 499)
Mothers	
Age, y	29.1 (23-35)
Pre-pregnancy BMI, kg/m ²	23.9 (19.6-29.8)
Maternal education†	
Low	12.3%
Middle	36.5%
High	51.2%
Self-reported smoking status	
Never smoker	63.2%
Cessation before pregnancy	22.7
Smoker during pregnancy	14.1%
Self-reported passive indoor smoking ^a	8.8%
Alcohol consumption ^b	
None	82.0%
Occasionally	18.0%
Parity	
1	55.0%
2	34.1%
≥ 3	10.9%
Newborns	
Sex	
Female	50.8%
European-Caucasian ethnicity‡	87.2%
Gestational age, w	39.4 (38-41)
Season of delivery	
Winter (Dec - Mar)	28.5%
Spring (Mar - Jun)	22.7%
Summer (Jun - Sep)	21.5%
Autumn (Sep - Dec)	27.3%
Apgar score 5 min after birth	
7 or 8	7.8%
9	27.9%
10	64.3%
pH of arterial cord blood ^c	7.2 (7.2-7.3)
Birth weight, g	3,446 (2,915-3,990)
Minutes of labor ^d	27.3 (8-54)

Table 1. (continued)

Characteristic	Mother-child group (n = 499)
Cord thyroid hormones	
TSH, mU/L	10.3 (5.5-22.3)
FT ₃ , pmol/L	2.5 (2.0-3.2)
FT ₄ , pmol/L	15.7 (13.5-18.5)
Ratio FT ₄ /FT ₃	6.4 (5.0-8.0)

Values are percentages or means (10th-90th percentiles), except for TSH, FT₃, FT₄ for which the geometric mean is given.

† Mother's education: 'low' (no high school diploma), 'middle' (high school diploma), 'high' (college or university diploma).

‡ Based on the native country of the newborn's grandparents. European-Caucasian when two or more grandparents were European, or non-European when at least three grandparents were of non-European origin.

^a Data available for 486 mothers. ^b Data available for 484 mothers. ^c Data available for 431 newborns. ^d Data available for 427 mother-child pairs.

Thyroid hormone levels in cord blood and maternal blood

The geometric means of thyroid hormone levels in cord blood (n = 499) were 10.3 mU/L for TSH, 2.5 pmol/L for FT₃, and 15.7 pmol/L for FT₄ (Table 1), while in maternal blood (n = 431) it was 2.1 mU/L, 4.2 pmol/L, and 12.5 pmol/L respectively (**Supplemental Material, Table S3**). A positive correlation was observed between FT₃ and FT₄ (cord blood: r = 0.30, p < 0.0001, maternal blood: r = 0.27, p < 0.0001) and between FT₃ and TSH (cord blood: r = 0.11, p = 0.01, maternal blood: r = 0.19, p < 0.0001). Maternal FT₄ levels were positively correlated with cord blood FT₄ levels (r = 0.21, p < 0.0001), whereas an inverse correlation was observed with cord blood FT₃ levels (r = -0.11, p = 0.01). Compared to maternal values, the measured cord blood FT₃ levels were approximately two-fold lower and the TSH levels much higher. The thyroid hormone concentrations in cord blood were similar to values published by others.²⁸⁶

Ambient exposure levels

Average (25th-75th percentile) PM_{2.5} exposure and temperature for the third gestational trimester were respectively 16.0 µg/m³ (11.6-19.8) and 8.7°C (3.2-14.7). Mean levels of both parameters remained quite constant throughout the trimesters of pregnancy.

Thyroid hormones and PM_{2.5} exposure during gestation

In cord blood ($n = 499$), TSH levels and FT₄/FT₃ ratios correlated inversely with PM_{2.5} exposure during the third trimester of pregnancy (**Figure 2**). The correlations remained significant after adjustment for sex, gestational age, season of delivery, Apgar score, maternal age, pre-pregnancy BMI, smoking status, parity, ethnicity, maternal education, and apparent temperature. An IQR increment (+8.2 $\mu\text{g}/\text{m}^3$) in PM_{2.5} exposure during the third trimester was associated with a lowering of 11.6% (95% CI: -21.8 to -0.1%, $p = 0.05$) in cord blood TSH levels (**Figure 3, panel A, left**) and a lowering of 62.2% (95% CI: -91.6 to -33.8%, $p < 0.0001$) in cord blood FT₄/FT₃ ratio (**Figure 3, Panel A, right**). Considering the FT₄ and FT₃ levels in cord blood separately (**Figure 3, panel B**), we observed opposite associations for an IQR increment of PM_{2.5} exposure on these two hormones during the third trimester (FT₄: -3.7%, 95% CI: -6.4 to -0.9%, $p = 0.009$, and FT₃: +6.4%, 95% CI: 1.8 to 11.1%, $p = 0.006$). Additional adjustment for maternal thyroid hormones in the cord blood models did not substantially alter our findings for cord blood.

In maternal blood ($n = 431$), TSH and FT₄ levels correlated inversely with third trimester PM_{2.5} exposure ($r = -0.10$, $p = 0.04$ and $r = -0.13$, $p = 0.005$ respectively). After adjustment for gestational age, season of delivery, maternal age, pre-pregnancy BMI, smoking status, parity, ethnicity, maternal education, and apparent temperature, only maternal FT₄ levels tended to be inversely associated with an IQR increment in third trimester PM_{2.5} exposure (-4.0%, 95% CI: -8.0 to 0.2%, $p = 0.06\%$) (**Figure 3, Panel A and B**). Neither TSH nor the FT₄/FT₃ ratio in maternal blood changed significantly with an IQR increment in third trimester PM_{2.5} exposure.

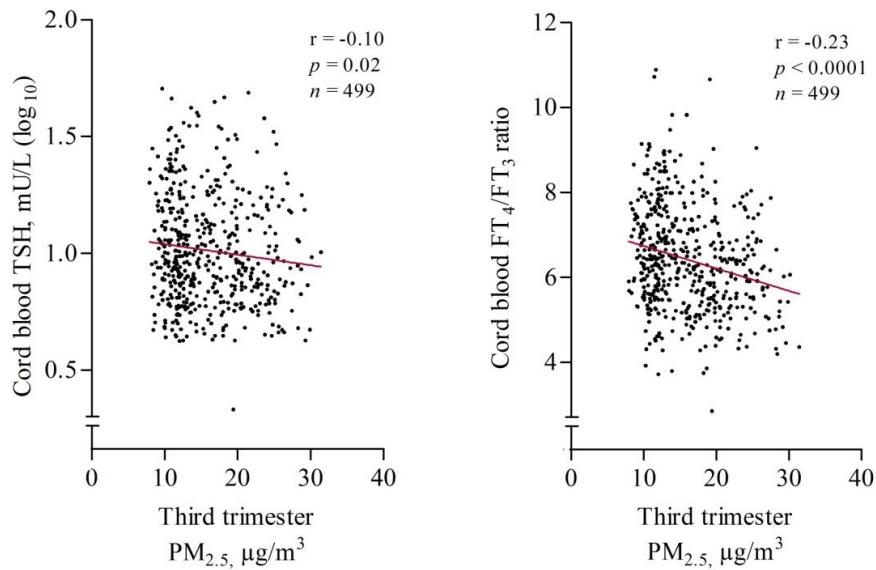


Figure 2. Correlation between third gestational PM_{2.5} exposure ($\mu\text{g}/\text{m}^3$) during pregnancy and cord blood TSH (mU/L , \log_{10}) levels (left) and the FT₄/FT₃ ratio (right).

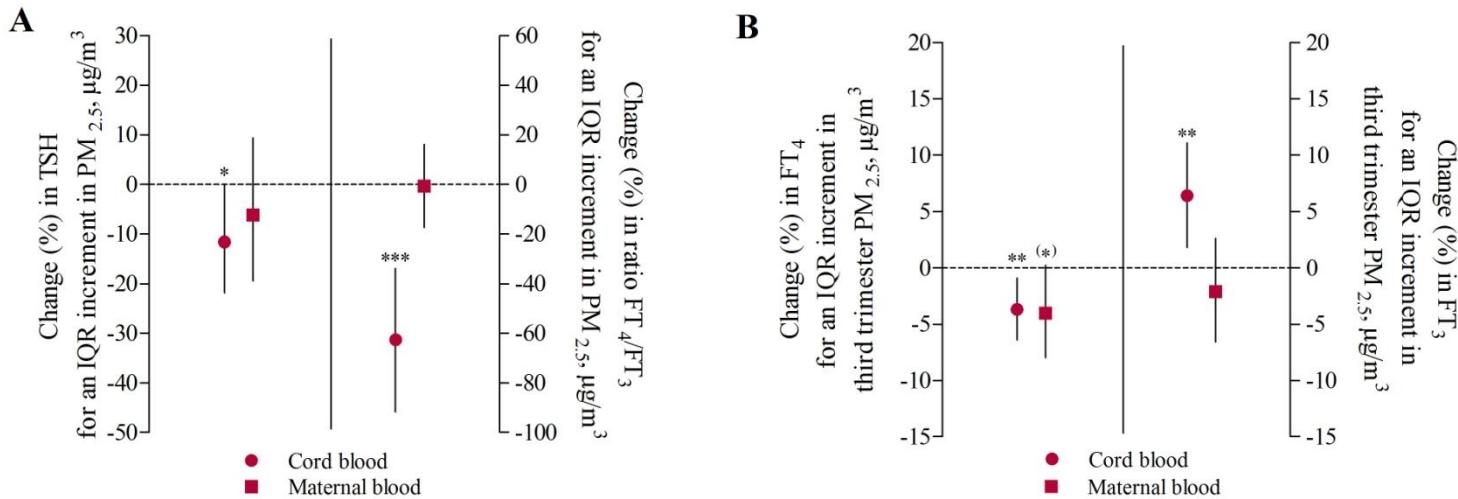


Figure 3. Change in cord (●) and maternal (■) blood thyroid hormones in association with third gestational trimester $\text{PM}_{2.5}$. The estimated relative change in percentage (95% CI) is calculated for an IQR increment ($+8.2 \mu\text{g}/\text{m}^3$) in third trimester $\text{PM}_{2.5}$ exposure. Panel A displays the change in TSH (left) and the change in FT_4/FT_3 ratio (right). Panel B displays the change in FT_4 (left) and FT_3 (right). The cord blood models were adjusted for sex, gestational age, season of delivery, Apgar score, maternal age, pre-pregnancy BMI, smoking status, parity, ethnicity, maternal education, and apparent temperature, whereas for maternal blood sex and Apgar score were excluded. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Thyroid hormones and birth weight

Neither FT₃ nor TSH levels in maternal or cord blood were correlated with birth weight ($p \geq 0.47$). Though, a 10th-90th percentile decrease (11%) in cord blood FT₄ (\log_{10} values) was associated with a lowering in birth weight of 71 g (95% CI: -103 to -38 g, $p < 0.0001$), independently of gestational age and sex. After additional adjustment for maternal age, pre-pregnancy BMI, smoking status, parity, season of delivery, Apgar score, ethnicity, maternal education, and apparent temperature, the association for the cord blood model remained significant (-56 g, 95% CI: -90 to -23 g, $p = 0.001$). On the contrary, a 10th-90th percentile decrease (15%) in maternal FT₄ was associated with an increase in birth weight of +45 g (95% CI: 6 to 83 g, $p = 0.02$), but the statistical significance disappeared after adjustment for the aforementioned variables (+31 g, 95% CI: -7 to 69, $p = 0.11$).

We performed mediation analysis to estimate the proportion of the PM_{2.5} exposure effect on birth weight as mediated by cord blood FT₄. Although we did not observe a significant association between third trimester PM_{2.5} exposure and birth weight ($p = 0.70$), there is consensus among statisticians that the relationship between exposure (e.g. PM_{2.5}) and outcome (e.g. birth weight) does not need to be statistically significant for a variable (e.g. FT₄) to be a mediator.²⁰⁰ While adjusting for the aforementioned variables, estimates of the proportion of mediation indicated that cord blood FT₄ levels explained 21% (indirect effect: -19 g, 95% CI: -37 to -1 g, $p = 0.03$) of the association between third trimester PM_{2.5} exposure and birth weight (**Figure 4**). Since maternal thyroid hormones did not meet the assumptions for mediation, we did not perform a mediation analysis.

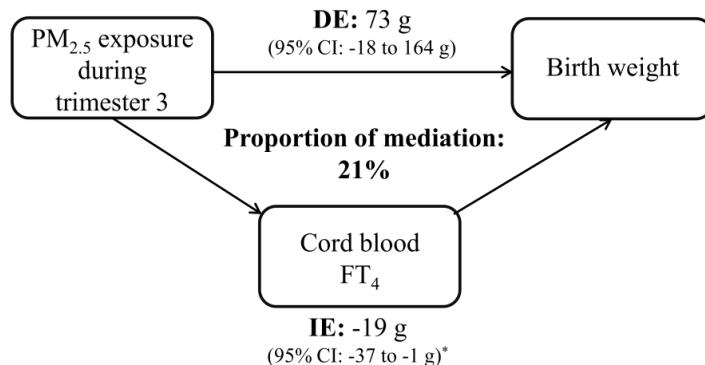


Figure 4. Estimated proportion of the PM_{2.5} exposure effect on birth weight (g) mediated by cord blood FT₄ levels. The figure displays cord blood FT₄ levels as mediator, the estimate of indirect effect (IE), the estimate of the direct effect (DE), and the proportion of mediation (IE/DE+IE). The estimated effect is calculated for an IQR increment (+8.2 µg/m³) in PM_{2.5} exposure during the third trimester. The mediation model was adjusted for sex, gestational age, season of delivery, Apgar score, maternal age, pre-pregnancy BMI, smoking status, parity, ethnicity, maternal education, and apparent temperature.
**p* < 0.05.

DISCUSSION

To the best of our knowledge, our study is the first to show associations between airborne PM_{2.5} exposure and cord blood thyroid hormones. A key finding is that PM_{2.5} exposure during the third trimester of gestation negatively influences TSH levels and the FT₄/FT₃ ratio in cord blood but not in maternal blood. The FT₄/FT₃ ratio in cord blood is a useful indicator of how effectively the body is able to convert T₄ into T₃.²⁹⁷ We also found a mediating role of cord blood FT₄ with regard to the association between last-trimester pregnancy PM_{2.5} exposure and birth weight, assuming the underlying causal assumptions of mediation analysis are valid. Our findings highlight the relevance of a negative impact of early life environmental exposure to PM_{2.5} on fetal thyroid function and the associated fetal growth. In addition, our results remained robust in multiple sensitivity analyses comprising maternal tobacco smoking, passive indoor smoking, seasonal variations, alcohol consumption, fetal hypoxemia, maternal estrogen levels, and length of labor.

Thyroid hormones are primarily responsible for the regulation of metabolism, especially during pregnancy, by stimulating differentiation and growth of the

fetus as well as its neurocognitive development.^{17,20} Despite the fact that the fetus starts secreting small amounts of thyroid hormone from mid-gestation onwards,²⁸⁰ the mother already supplies thyroid hormones to the fetal circulation from the first trimester without compromising her own supply.²⁹⁸ The rise of maternal thyroid hormones in the first trimester of pregnancy is considered critical to ensure normal (neurological) development.²⁰ Maternal T₄ and T₃ diffuse across the placenta to reach concentrations in the fetus which are in the same range as those in adult tissues.¹⁸ Fetal T₃ is generated locally from T₄ by type-2 deiodinase, has a high affinity for nuclear binding sites in the placenta, and stimulates the production of factors that control trophoblast growth and development.²² This suggests that thyroid hormones play an important role in normal placentation and development of the fetus. Shields *et al.*²⁵ showed in women with normal healthy pregnancies that placental weight was positively associated with cord blood FT₄ levels and inferred that thyroid hormones may influence fetal growth indirectly by affecting placental growth. These authors found that lower FT₄ levels in cord blood were associated with reduced birth weight and their results are corroborated by our study and two other studies from the Netherlands.^{26,299} Moreover, our study demonstrated that during the last trimester of pregnancy the estimated effect of PM_{2.5} exposure on birth weight was for 21% (on average -19 g) mediated by cord blood FT₄ levels. As in all observational studies, these estimates should be interpreted with caution because the underlying assumptions of causality between each pair of factors in the mediation analysis cannot be verified. Nevertheless, this finding suggests that the last trimester of pregnancy, when the fetus significantly increases in size, is an important window of susceptibility to PM_{2.5} exposure. Shields *et al.*,²⁵ Medici *et al.*,²⁶ León *et al.*,³⁰⁰ as well as our study, report an inverse association between maternal FT₄ and birth weight, which is opposite to cord blood FT₄. In a study of pregnant women without history of thyroid dysfunction, it has been shown that lower concentrations of FT₄ in maternal blood were related with increased placental growth.³⁰¹ These observations together suggest a functional discrepancy for FT₄ between maternal and fetal blood, especially with regard to fetal growth. In our study, we observed a tendency of an inverse association between maternal FT₄ and third trimester PM_{2.5} exposure in accordance with our findings in cord blood. The attenuated

association could be due to the fact that we sampled maternal blood one day after delivery, after which thyroid hormones tend to return to normal,²⁹⁶ while cord blood samples were collected immediately after delivery. However, the fetal thyroid gland becomes functional from the second trimester of gestation and is able to produce its own supply of thyroid hormones in addition to the maternal supply.²⁰ In this context of a more independent thyroid function of the fetus, differences between maternal and fetal susceptibility to external factors including air pollution, might explain the more pronounced associations in newborns compared to mothers.

Contrary to maternal T₄ and T₃, perfusion experiments with TSH on human term placentas have shown that TSH crosses placental tissue and fetal membranes only sparingly.³⁰² Hence, our finding of an inverse association between cord blood TSH levels and PM_{2.5} exposure during pregnancy is predominantly reflecting changes in fetal thyroid function. Experimental studies showed that PM exposure in healthy rats modulates the hypothalamic-pituitary-thyroid axis and leads to increases in markers of glucocorticoid activity,³⁰³ which are known to suppress TSH release.³⁰⁴ It is likely that inflammatory processes in cells of the fetal thyroid or in the brain may interfere with the feedback mechanism of the hypothalamic-pituitary-thyroid axis. In this regard, it is interesting to note that the ambient PM_{2.5} exposure in our birth cohort induced a systemic oxidative stress response as shown by alterations in placental mitochondrial DNA content¹³³ and increased placental protein-bound 3-nitrotyrosine.³⁰⁵

The FT₄/FT₃ ratio in cord blood, a useful indicator of how effectively the body is able to convert T₄ into T₃ was inversely associated with PM_{2.5} during pregnancy. This finding could be explained by the fact that placental type-2 deiodinase activity increases when the availability of T₄ decreases, thus representing a potential homeostatic mechanism for maintaining T₃ production when T₄ concentrations are reduced.³⁰⁶ Reduced levels of FT₄ and increased levels of FT₃ were found in blood of smoking pregnant mothers³⁰⁷ which is likely to be attributed to constituents of tobacco smoke that stimulate the conversion of T₄ to T₃ in tissues by boosting type-2 deiodinase activity.³⁰⁸ However, whether PM_{2.5} acts via the same mechanism remains uncertain. Low levels of TSH and FT₄ are suggestive of central hypothyroidism.³⁰⁹ Recently, it has been shown that intrauterine exposure to insufficient thyroid hormone levels was associated

with higher scores for attention deficit hyperactivity disorder (ADHD) symptoms in children at 8 years of age.³¹⁰ Whether low prenatal TSH levels and/or insufficient gestational T₄ production associated with PM_{2.5} exposure may contribute to an increased risk for adverse neurological effects or other health consequences in children later in life remains to be clarified. Regarding the neurotoxicity of air pollution, it has been shown that children exposed to high traffic-related air pollution have lower cognitive development over a one school year period.²⁹¹

Our study has some limitations. Firstly, thyroid hormones are responsive to environmental temperature²⁹² and show a seasonal pattern with lower values in the cold period compared to the warm period of the year. Nevertheless, our results were robust for both seasonality differences between subjects as well as adjustment for trimester-specific apparent temperature. Secondly, iodine is required for the synthesis of thyroid hormones, but we did not have information on iodine levels in maternal or cord blood. However, we excluded *a priori* clinically confirmed cases of hypo- and hyperthyroidism. It is unlikely that PM_{2.5} air pollution levels are associated with iodine intake and therefore to be a confounder in our analysis. Whether PM constituents interfere with iodine bioavailability or act directly on the peripheral metabolism of thyroid hormones needs to be elucidated. Lastly, we acknowledge the fact that we cannot fully exclude residual or unmeasured confounding by other factors such as noise, polychlorinated biphenyls, heavy metals, or pesticides that could be associated with both ambient air pollution and thyroid function. However, using residential proximity to major roads as a proxy to traffic-related noise was not associated with fetal thyroid hormones (data not shown). Overall, our findings are generalizable as the ENVIRONAGE birth cohort is representative for the gestational segment of the population at large (**Supplemental Material, Table S1**). We used a standardized fine-scale exposure model for the estimation of residential fine particle air pollution levels of the pregnant mothers (on average 16.0 µg/m³) which are comparable with other European and USA cohorts with mean PM_{2.5} exposure values amounting to 18.5³¹ and 14.5⁵⁰ µg/m³ respectively.

CONCLUSION

Our epidemiological finding of an altered fetal thyroid function in association with PM_{2.5} exposure is in line with the known effects of cigarette smoking on thyroid function during pregnancy. Cord blood FT₄ seems to play a mediating role between PM_{2.5} exposure and birth weight during late pregnancy, however, the underlying mechanisms and the potential later-in-life adverse consequences (especially as to cognitive development) of altered fetal thyroid function is far from elucidated. Our findings on neonatal endocrinologic effects in association with *in utero* exposure to PM_{2.5} air pollution are of critical public health importance because of the ubiquity of fine PM air pollution and the potentially long-term health consequences of early-life alterations in thyroid function. Therefore, to promote a healthier living environment for children our findings support a down-revision of the current EU air pollution limit for PM_{2.5} of 25 µg/m³ (annual average threshold) in the direction of the WHO recommended limit of 10 µg/m³ (annual average).

Acknowledgements

The authors thank the participating women and neonates, as well as the staff of the maternity ward, midwives, and the staff of the clinical laboratory of East-Limburg Hospital in Genk. The ENVIRONAGE birth cohort is supported by the European Research Council (ERC-2012-StG.310898), by the Flemish Scientific Fund (FWO, N1516112 / G.0.873.11.N.10) and the Bijzonder Onderzoeksfonds (BOF) of Hasselt University. Karen Vrijens is a postdoctoral fellow of the FWO.

Conflict of interest disclosures

The authors declare they have no competing financial interests. None of the funding agencies had a role in the design and conduct of the study; in the collection, analysis, and interpretation of the data; or in the preparation, review, or approval of the manuscript.

SUPPLEMENTAL MATERIAL

Table S1. Characteristics of the ENVIRONAGE birth cohort participants and all births from 2002-2011 in Flanders (Northern part of Belgium)

Characteristic	ENVIRONAGE (n = 499)	Flanders [†] (n = 606,877)
Maternal age, y	29.1 (23.0-35.0)	29.5 (23.5-35.8)
<25	16.2%	16.2%
25-35	76.8%	70.7%
>35	7.0%	13.1%
Pre-pregnancy BMI, kg/m ²	23.9 (19.6-29.8)	N/A
Maternal education		
Low	12.3%	13.1%
Middle	36.5%	40.8%
High	51.2%	46.1%
Parity		
1	55.0%	46.9%
2	34.1%	34.7%
≥3	10.9%	18.4%
Sex		
Male	49.2%	51.4%
Ethnicity		
European	87.2%	87.7%
Birth weight, g	3,466 (2,915-3,990)	3,360 (2,740-3,965)

Values are percentages or means (10th-90th percentiles).

These main characteristics of the ENVIRONAGE birth cohort are in line with the birth register of all births between 2002-2011 in the Northern part of Belgium. Therefore our mother-child cohort is representative for the gestational segment of the population in Flanders.

[†]Cox *et al.* 2013.¹¹¹

Text S1: Sensitivity analysis

Tobacco smoking

Tobacco smoke exposure, a form of personalized airborne PM exposure, has been shown to influence maternal and fetal thyroid function through changes in thyroid hormone levels.^{290,307,311,312} Before and after adjustment for newborn's sex, gestational age, season of delivery, Apgar score, maternal age, pre-pregnancy BMI, parity, ethnicity, and apparent temperature, cord blood TSH levels were lower in mothers who continued smoking during pregnancy (-18.7%, 95% CI: -29.1 to -6.7%, $p = 0.003$) and also in those who stopped smoking before pregnancy (-10.3%, 95% CI: -19.6 to 0.1, $p = 0.05$) in comparison with never-smoking mothers (**Supplemental Material, Table S2**). When excluding never-smokers, we observed as expected an inverse association between smoking years and TSH levels in cord blood ($r = -0.21$, $p = 0.004$; $n = 173$). We did not find differences in cord blood levels of FT₃ or FT₄ between smokers compared to non-smokers but newborns from women who stopped smoking before pregnancy had slightly lower FT₃ cord blood levels compared to non-smokers (-3.7%, 95% CI: -7.5 to 0.1%, $p = 0.06$). We did not find an association between maternal thyroid hormones and smoking status during pregnancy. The associations between cord blood thyroid hormones and PM_{2.5} exposures did not alter while excluding women who smoked during pregnancy.

Seasonal variation

Thyroid hormones may show seasonal variations linked to changes in temperature.²⁹² To account for possible seasonality differences between subjects, we calculated for each subject an exposure window covering a 1-year period, i.e. 365 days calculated backwards from the date of delivery. This sensitivity analysis invariably showed for an IQR increment (+3.7 µg/m³) of PM_{2.5} inverse associations with cord blood TSH levels (-8.8%, 95% CI: -14.8 to -2.4%, $p = 0.008$), the FT₄/FT₃ ratio (-32.7%, 95% CI: -48.8 to -16.6%, $p < 0.0001$), the FT₄ levels (-1.7%, 95% CI: -3.2 to -0.1%, $p = 0.03$), and a positive association with FT₃ levels (+3.5%, 95% CI: 1.0 to 6.0%, $p = 0.006$), corroborating the associations found for the third trimester of pregnancy (**Figure 3**).

Additional adjustments

It is known that cord blood thyroid hormone levels are influenced by different external factors including alcohol consumption,²⁹³ fetal hypoxemia,²⁹⁵ estrogen levels,²⁹⁴ and passive smoking.²⁹⁰ Length of labor may influence thyroid hormone levels possibly due to the high energy demand during labor.²⁹⁶ Additional adjustments for alcohol consumption ($n = 484$), pH of arterial cord blood (indicator of hypoxemia) ($n = 431$), cord blood plasma estradiol, or passive indoor tobacco smoke exposure ($n = 486$) did not alter the associations between PM_{2.5} exposure and thyroid hormones shown in the main analysis. Length of labor (minutes) was available for 427 participants. Adjusting the main models for length of labor also did not alter the reported associations except that the association between cord blood TSH levels and third trimester PM_{2.5} exposure was not significant anymore (**Supplemental Material, Figure S1**).

Subgroups of the main study population

We examined whether the association between cord blood thyroid hormones and PM_{2.5} exposure was still present in the group for which maternal thyroid hormones were available (mother group; $n = 431$) and in the euthyroid mother group ($n = 392$). The euthyroid mothers' plasma TSH concentrations were within the reference range of the clinical laboratory of the hospital (0.27-4.20 mU/L). A similar procedure and reference range (0.2-5.5 mU/L) has been used recently in another study.³¹³ The characteristics of the mother group and the euthyroid mother group did not differ from the mother-child group used in the main analysis (**Supplemental Material, Table S3**). The associations between cord blood thyroid hormones and an IQR increment (+8.2 µg/m³) in PM_{2.5} exposure showed for both subgroups results comparable with those reported in the main analysis (**Supplemental Material, Table S4**).

Table S2. Associations between smoking status during pregnancy and thyroid hormones in cord blood and maternal blood

Smoking status [†]	TSH		FT ₃		FT ₄		Ratio FT ₄ /FT ₃	
	β	(95% CI)	β	(95% CI)	β	(95% CI)	β	(95% CI)
Cord blood (n = 499)								
(Ref)	-	-	-	-	-	-	-	-
Self-reported cessation	-10.3	(-19.6, 0.1)(*)	-3.7	(-7.5, 0.1)	-0.5	(-3.1, 2.1)	20.0	(-6.4, 46.4)
Self-reported smoker	-18.7	(-29.1, -6.7)**	3.7	(-1.3, 9.0)	-1.0	(-4.1, 2.2)	-27.4	(-60.3, 56.0)
Maternal blood (n = 431)								
(Ref)	-	-	-	-	-	-	-	-
Self-reported cessation	9.2	(-4.1, 24.4)	3.2	(-0.9, 7.5)	-0.4	(-3.9, 3.3)	-8.4	(-22.6, 5.8)
Self-reported smoker	-1.9	(-16.7, 15.5)	2.7	(-2.5, 8.1)	0.1	(-4.4, 4.8)	-7.8	(-25.8, 10.2)

† β represents the percentage change in thyroid hormone level compared to the reference category (self-reported never smokers). All models were adjusted for sex, gestational age, season of delivery, Apgar score, maternal age, pre-pregnancy BMI, parity, ethnicity, and apparent temperature, except in the models of maternal blood where sex and Apgar score were excluded. (*) $p < 0.05$, * $p < 0.05$, ** $p < 0.01$.

Table S3. Characteristics and thyroid hormone levels of subgroups (mother group and euthyroid mother group) compared with mother-child pairs of the main analysis

Characteristic	Mother-child group (n = 499)	Mother group§ (n = 431)	Euthyroid mother group* (n = 392)
Mothers			
Age, y	29.1 (23-35)	29.0 (23-35)	29.0 (23-35)
Pre-pregnancy BMI, kg/m ²	23.9 (19.6-29.8)	23.9 (19.6-29.3)	23.9 (19.5-29.3)
Maternal education†			
Low	12.3%	12.3%	13.3%
Middle	36.5%	37.3%	36.5%
High	51.2%	50.4%	50.2%
Self-reported smoking habit			
Never smoker	63.2%	62.6%	62.5%
Cessation before pregnancy	22.7	23.0%	22.7%
Smoker during pregnancy	14.1%	14.4%	14.8%
Self-reported passive indoor smoking ^a	8.8%	8.3%	8.4%
Alcohol consumption ^b			
None	82.0%	81.2%	80.8%
Occasionally	18.0%	18.8%	19.2%
Parity			
1	55.0%	53.1%	52.0%
2	34.1%	36.4%	37.5%
≥ 3	10.9%	10.5%	10.5%
Newborns			
Sex			
Female	50.8%	50.8%	51.5%
European-Caucasian ethnicity‡	87.2%	87.2%	86.5%
Gestational age, w	39.4 (38-41)	39.4 (38-41)	39.4 (38-41)
Season of delivery			
Winter (Dec - Mar)	28.5%	26.9%	26.5%
Spring (Mar - Jun)	22.7%	23.4%	24.5%
Summer (Jun - Sep)	21.5%	21.8%	21.7%
Autumn (Sep - Dec)	27.3%	27.9%	27.3%
Apgar score 5 min after birth			
7 or 8	7.8%	7.0%	7.1%
9	27.9%	27.6%	27.8%
10	64.3%	65.4%	65.1%
pH of arterial cord blood ^c	7.2 (7.2-7.3)	7.2 (7.2-7.3)	7.3 (7.2-7.3)
Birth weight, g	3,446 (2,915-3,990)	3441 (2915-3975)	3443 (2915-3965)
Minutes of labor ^d	27.3 (8-54)	26.2 (8-51)	26.4 (8-52)

Table 1. (continued)

Characteristic	Mother-child group (n = 499)	Mother group§ (n = 431)	Euthyroid mother group* (n = 392)
Cord thyroid hormones			
TSH, mU/L	10.3 (5.5-22.3)	10.0 (5.4-20.9)	9.8 (5.4-20.2)
FT ₃ , pmol/L	2.5 (2.0-3.2)	2.5 (2.0-3.2)	2.5 (2.0-3.2)
FT ₄ , pmol/L	15.7 (13.5-18.5)	15.7 (13.5-18.5)	15.8 (13.5-18.5)
Ratio FT ₄ /FT ₃	6.4 (5.0-8.0)	6.4 (5.0-8.0)	6.5 (5.1-8.0)
Maternal thyroid hormones			
TSH, mU/L	N/A	2.1 (1.1-4.0)	2.0 (1.2-3.5)
FT ₃ , pmol/L	N/A	4.2 (3.4-5.1)	4.1 (3.4-5.0)
FT ₄ , pmol/L	N/A	12.5 (10.0-15.2)	12.4 (10.0-15.2)
Ratio FT ₄ /FT ₃	N/A	3.0 (2.4-3.8)	3.1 (2.4-3.8)

Values are percentages or means (10th-90th percentiles), except for TSH, FT₃, and FT₄ for which the geometric mean (10th-90th percentiles) is given.

†Mother's education: 'low' (no high school diploma), 'middle' (high school diploma), 'high' (college or university diploma).

‡Based on the native country of the newborn's grandparents. European-Caucasian when two or more grandparents were European, or non-European when at least three grandparents were of non-European origin.

§Total group minus mothers from whom blood samples were not available.

*Euthyroid women with plasma TSH concentrations between 0.27-4.20 mU/L (reference range of the clinical laboratory of the hospital).

^a Data available for respectively 486, 422 and 383 mothers. ^b Data available for respectively 484, 420 and 381 mothers.

^c Data available for respectively 431, 375 and 339 newborns. ^d Data available for respectively 427, 372, 335 newborns.

Table S4. Percentage change in cord blood thyroid hormones of the mother group and euthyroid mother group in association with third trimester exposure to PM_{2.5}

Exposure window [†]	TSH		FT ₃		FT ₄		Ratio FT ₄ /FT ₃	
	β^{\dagger}	(95% CI)	β^{\dagger}	(95% CI)	β^{\dagger}	(95% CI)	β^{\dagger}	(95% CI)
Mother group (n = 431)								
3 rd trimester	-12.3	(-23.5, 0.4)	6.1	(1.1, 11.3)*	-3.6	(-6.7, -0.5)*	-59.1	(-91.2, -27.1)***
Euthyroid mother group (n = 392)								
3 rd trimester	-14.1	(-25.7, -0.7)*	5.8	0.6, 11.2)*	-3.9	(-7.2, -0.5)**	-58.7	(-92.9, -24.5)***

[†] β represents a percentage change in thyroid hormone levels calculated for an IQR increment (+8.2 µg/m³) in third trimester PM_{2.5} exposure. All models were adjusted for sex, gestational age, season of delivery, Apgar score, maternal age, pre-pregnancy BMI, parity, ethnicity, and apparent temperature. *p < 0.05, **p < 0.01, ***p < 0.001.

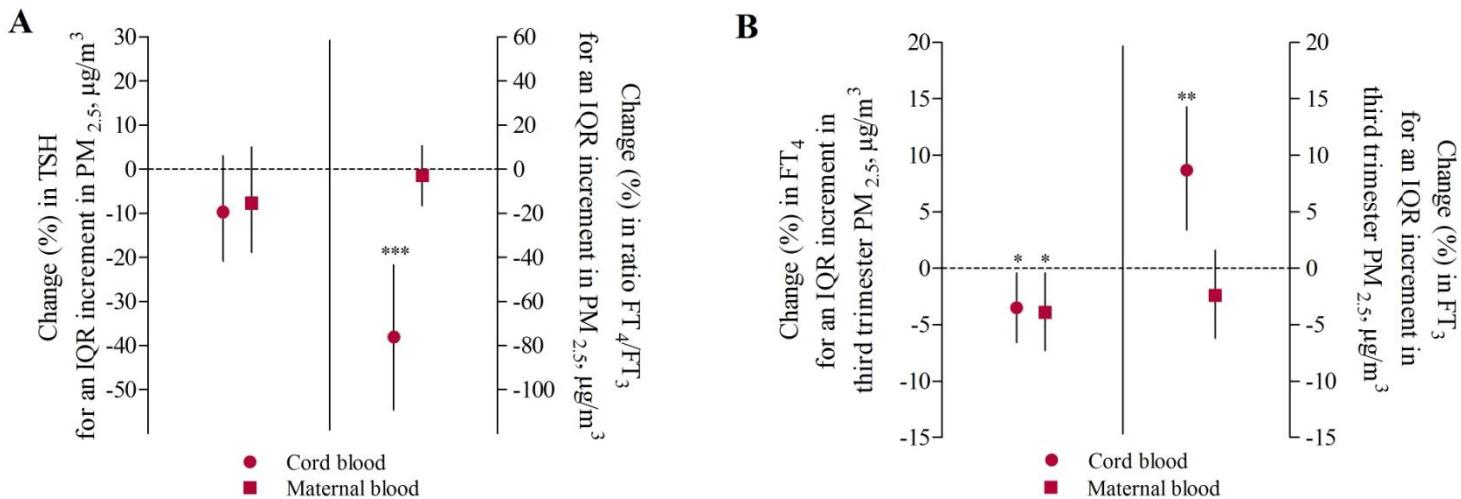


Figure S1. Change in cord (●) and maternal (■) blood thyroid hormones in association with third gestational trimester $\text{PM}_{2.5}$, while adjusting for length of labor ($n = 427$). The estimated relative change in percentage (95% CI) is calculated for an IQR increment ($+8.2 \mu\text{g}/\text{m}^3$) in third trimester $\text{PM}_{2.5}$ exposure. Panel A displays the change in TSH (left) and the change in FT_4/FT_3 ratio (right). Panel B displays the change in FT_4 (left) and FT_3 (right). The cord blood models were adjusted for sex, gestational age, season of delivery, Apgar score, maternal age, pre-pregnancy BMI, smoking status, parity, ethnicity, maternal education, and apparent temperature, whereas for maternal blood sex and Apgar score were excluded. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

CHAPTER 8

EXPRESSION OF MICRONUCLEI-RELATED GENES IN CORD BLOOD IN ASSOCIATION WITH *IN UTERO* PARTICULATE MATTER EXPOSURE: P53 AS A CENTRAL HUB

Bram G. Janssen,^{1*} Karen Vrijens,^{1*} Ellen Winckelmans,¹ Martien Peusens,¹ Harry A. Roels,^{1,2} Charlotte Vanpoucke,³ Wouter Lefebvre,⁴ Wilfried Gyselaers,^{5,6} Ann Cuypers,¹ Tim S. Nawrot^{1,7}

* Authors contributed equally

¹ Centre for Environmental Sciences, Hasselt University, Diepenbeek, Belgium

² Louvain Centre for Toxicology and Applied Pharmacology (LTAP), Université catholique de Louvain, Brussels, Belgium

³ Belgian Interregional Environment Agency, Brussels, Belgium

⁴ Flemish Institute for Technological Research (VITO), Mol, Belgium

⁵ Department of Obstetrics, East-Limburg Hospital, Genk, Belgium

⁶ Biomedical Research Institute, Hasselt University, Diepenbeek, Belgium

⁷ Department of Public Health & Primary Care, Leuven University, Leuven, Belgium

ABSTRACT

Background: Increased micronuclei (MN) frequency, a valid biomarker for genotoxicity, has been associated with particulate matter (PM) exposure-related DNA damage in cord blood. The *P53* gene encodes the tumor suppressor protein P53 and has an important role in many stress responses, including MN formation. We hypothesized that expression of genes involved in the MN formation network and P53 protein levels are altered in association with gestational PM_{2.5} exposure in newborns.

Methods: Gene expression of *P53*, *DNMT1*, *PCNA*, *BAX*, and *P21* was measured by quantitative real-time polymerase chain reaction and *P53* protein expression by ELISA in cord blood retrieved from 170 newborns enrolled in the Belgian birth cohort ENVIRONAGE.

Results: We observed a negative association between expression levels of the MN formation-related genes and PM_{2.5} exposure during pregnancy (relative decrease of -52.0%, 95% CI: -68.7 to -26.2%, $p = 0.0009$ for each 5 µg/m³ increment in PM_{2.5} exposure). The association with PM_{2.5} exposure was most pronounced with expression of *P53*, *BAX*, and *PCNA*. On the other hand, P53 protein levels were positively associated with entire pregnancy PM_{2.5} exposure (relative increase of 56.0%, 95% CI: 23.5 to 97.1%, $p = 0.0003$ for a 5 µg/m³ increment in PM_{2.5}), as with mothers who reported to have smoked during pregnancy (+46.8%, 95% CI: 7.4 to 100.7%, $p = 0.02$ compared to non-smokers).

Conclusions: Based on our transcriptomic analysis, our results are indicative of alterations in expression of MN-related genes in response to PM_{2.5} exposure during pregnancy. Our findings lend support to the development of a reliable reporter gene assay to screen chemically exposed human populations faster and in a less labor-intensive way than using microarrays.

INTRODUCTION

In the last decade, air pollution has been pinpointed as one of the major global health problems affecting people worldwide. In addition to the prominent health effects in the adult population such as cardiovascular events^{5,6} and lung cancer incidence,⁷ growing evidence shows that exposure to ambient air pollution during the most vulnerable stage in life, the *in utero* period, is of critical importance.^{8,9} Changes in the transcriptome biology during fetal development can contribute to disease susceptibility.

Particulate matter (PM) air pollution has been classified as carcinogenic to humans (IARC Group 1)⁴⁵ and is associated with genotoxic biomarkers such as micronuclei (MN) formation or changes in the expression of genes involved in DNA damage and repair.³¹⁴ MN are small extranuclear bodies that are formed when a chromosome or a fragment of a chromosome is not incorporated into the newly formed daughter nucleus after mitosis or meiosis.³¹⁵ MN frequency is a well established biomarker for chromosomal breakage, instability or DNA damage and hence it is often used as a phenotypic anchor for genotoxicity assessment of chemical components.³¹⁶ Despite the clear association between ambient air pollution exposure and MN formation in adults,³¹⁴ few studies have focused on maternal exposure to air pollution during pregnancy and MN formation in cord blood and maternal blood, with less conclusive results.³¹⁷⁻³¹⁹

Network analysis of transcriptomics data in relation to the formation of MN provided a dedicated network of genes, with *P53* as central hub, that could be used to screen populations complementary to the current MN test assay.³²⁰ In this study, we sought to examine whether the expression of genes within the network of MN formation (including *P53*, *BAX*, *DMNT1*, *PCNA*, and *P21*) and P53 protein levels are altered in cord blood in association with *in utero* exposure to PM_{2.5} (particle with an aerodynamic diameter < 2.5 µm).

MATERIALS AND METHODS

Study population

Mother-newborn pairs were enrolled in the ENVIRO/NAGE birth cohort in Belgium (acronym for ENVIROnmental influence ON early AGEing). All procedures were

approved by the Ethical Committee of Hasselt University and East-Limburg Hospital. The study design and procedures were previously described in detail¹³³ and the study was conducted according to the principles outlined in the Helsinki Declaration for investigation of human subjects. Briefly, written informed consent was obtained from eligible participants before delivery. Questionnaires and medical records consulted after birth provided information on maternal age, maternal education, smoking status, ethnicity, pre-pregnancy body mass index (BMI), gestational age, newborn's gender, Apgar scores, birth weight and length, parity, and ultrasonographic data. Information about maternal tobacco smoke exposure was obtained by asking whether mothers smoked during pregnancy, whether they smoked at any time during their life or if they never smoked in their life.

The current study is based on a representative subgroup of the birth cohort including 175 newborns recruited from the East-Limburg Hospital in Genk (Belgium). We excluded three newborns due to missing questionnaires, and of the remaining 172 newborns, we deleted one newborn of which we had no prenatal exposure (living outside the study area), and one newborn of which the mother had a BMI larger than 40 kg/m², resulting in a final sample of 170 mother-child pairs.

Sample collection and processing

Immediately after the baby was born, the umbilical cord was clamped and cord blood was drawn in Tempus Blood RNA Tubes (Applied Biosystems, Foster City, CA, USA) and plastic BD Vacutainer® K2EDTA Tubes (BD, Franklin Lakes, NJ, USA). K2EDTA tubes were immediately centrifuged (3,200 rpm for 15 min) to retrieve plasma which was instantly frozen at -80°C. The RNA stabilizing blood tubes were mixed well and left on the bench for at least two hours at room temperature before freezing at -20°C and afterwards at -80°C. Total RNA was isolated from whole blood with Tempus Spin RNA Isolation kit (Life Technologies) and stored at -80°C until further processing. The RNA concentration was measured using Nanodrop spectrophotometer (ND-1000, Isogen Life Science, De Meern, the Netherlands), and RNA integrity and quality was checked on the Experion™ Automated Electrophoresis System (Bio-Rad). The average RNA Integrity Number (RIN) was 8.17 ± 0.88.

Gene expression analysis

Expression of five genes (*P53*, *BAX*, *DNMT1*, *PCNA*, and *p21*) within the MN formation network was studied (**Table 1**). cDNA was synthesized from 2 µg RNA using the Goscript™ Reverse Transcription System kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. A qPCR reaction was set up by adding 6 ng cDNA together with 5 µl Taqman Fast Advanced Master Mix (Life Technologies, Foster City, CA, USA) and 0.5 µl PrimeTimeTM assay (Integrated DNA Technologies, Coralville, IA, USA) in a final reaction volume of 10 µl. Amplification efficiencies of PrimeTime assays were determined by standard dilution series of a mixed sample, resulting in an efficiency between 90-110% for all assays (**Table 1**) and the amplification specificity was confirmed by visualization of the expected band size on an agarose gel. In each plate, we took along three non-template controls and six inter-run calibrators (IRCs). Samples were measured in triplicate in a MicroAmp Optical 384-Well Reaction Plate compatible with the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Cycling conditions for all transcripts were: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 sec at 95°C, and 1 min at 60°C. After thermal cycling, Cq values were collected and normalized to three reference genes, taking into account run-to-run differences using IRCs with qBase software (Biogazelle, Zwijnaarde, BEL). TATA box binding protein (*TBP*), cytochrome c-1 (*CYC1*), and Importin 8 (*IPO8*) were selected via GeNorm as stable reference genes in cord blood to normalize the data.

P53 protein measurement

After thawing plasma samples from cord blood that we stored at -80°C, 50 µl of plasma was quantified for P53 protein levels using a P53 Human ELISA Kit according to the manufacturer's instructions (ref. ab46067, Abcam, Cambridge, United Kingdom). P53 activity was measured in duplicate in each sample, and its mean value was used for further analysis. Plasma P53 concentrations were expressed in IU/ml. The intra-assay coefficient of variation was 8.0%, the inter-assay coefficient of variation reached 10.9%. Of the 170 cord blood samples, we had 160 plasma samples available and three samples were excluded as outlier (total sample = 157).

Exposure assessment

For each mother's residential address, we interpolated the regional background PM_{2.5} ($\mu\text{g}/\text{m}^3$) using a spatial temporal interpolation method (Kriging)¹²⁰ that uses pollution data collected in the official fixed-site monitoring network and land cover data obtained from satellite images (CORINE land cover data set) in combination with a dispersion model.^{121,122} This model chain provides daily PM_{2.5} values using data from the Belgian telemetric air quality network, and point and line sources which is then interpolated in a high resolution receptor grid. In the Flemish region of Belgium, the interpolation tool explained more than 80% of the temporal and spatial variability.¹²³ Taking into account any residential changes during pregnancy, we calculated entire pregnancy PM_{2.5} exposure (mean of each day of pregnancy) for each individual. The date of conception was estimated based on ultrasound data.

Table 1. Reference and target genes with assay information from IDT (Integrated DNA Technologies, USA)

Gene	Encoded protein	IDT Assay	Ref seq number	Efficiency, %†	Exon Location	Amplicon Length, bp
Reference genes						
<i>TBP</i>	TATA box binding protein	Hs.PT.56a.20792004	NM_003194	103	4-5	113
<i>CYC1</i>	Cytochrome c-1	Hs.PT.56a.20696349.gs	NM_001916	97	5-6	121
<i>IPO8</i>	Importin 8	Hs.PT.56a.40532361	NM_001190995	109	7-8	102
Target genes						
<i>P53</i>	Tumour protein p53	Hs.PT.58.39489752.g	NM_000546	96	11-11	146
<i>BAX</i>	BCL2-associated X protein	Hs.PT.56a.19141193.g	NM_138763	109	2-4	241
<i>CDKN1A (P21)</i>	Cyclin-dependent kinase inhibitor 1A	Hs.PT.58.40874346.g	NM_001220777	98	4-5	143
<i>DNMT1</i>	DNA (cytosine-5-)methyltransferase 1	Hs.PT.58.26259414.gs	NM_001130823	92	33-34	114
<i>PCNA</i>	Proliferating cell nuclear antigen	Hs.PT.58.1446155	NM_182649	97	4-5	126

† Primer efficiency was calculated by a standard dilution series and using the formula: efficiency = $10^{(-1/\text{slope})} - 1$.

Statistical analysis

For database management and statistical analysis, we used the SAS software program (version 9.2; SAS Institute Inc., Cary, NC, USA). All gene expression values and P53 protein concentrations (IU/ml) were \log_{10} -transformed to approximate a normal distribution. Pearson correlation coefficients were calculated between the different gene expression values and between PM_{2.5} exposure and gene expression values or P53 protein levels using R software packages. We applied z-score transformations for each gene individually to put expression values on the same scale. The z-score, or standard score, is calculated according to the following formula so that they share a common underlying distribution:

$$\text{z-score} = \frac{(x_{Gi} - \mu_{Gi})}{SD_{Gi}}$$

where x represents the \log_{10} -transformed individual expression value for Gene i from the MN formation network, μ_{Gi} the \log_{10} population mean of Gene i, and SD the standard deviation of all measured expression values of Gene i. Next, we used multivariate mixed effect models to estimate the associations between the expression values of genes within the MN formation network and PM_{2.5} exposure during gestation. The mixed effect model allows us to treat the gene expression values of the MN formation network as a single outcome while taking into account differences between genes within the pathway. We tested whether the association of PM_{2.5} exposure during gestation was the same across all target genes within the MN formation network by including an interaction term between the gene and exposure. Next, we performed multiple linear regression to determine the association between PM_{2.5} exposure and the original \log_{10} -transformed gene expression levels or cord plasma P53 protein levels. All models were adjusted for maternal age (years), gestational age (weeks), sex, pre-pregnancy BMI (kg/m^2), parity (1 / 2 / ≥ 3), smoking status (self-reported never smoking / self-reported cessation / self-reported smoking), ethnicity (European-Caucasian yes or no), maternal education (low / middle / high), and season of delivery (winter [21st of Dec – 20th of Mar] / spring [21st of Mar – 20th of Jun] / summer [21st of Jun – 20th of Sep] / autumn [21st of Sep – 20th of Dec]). Exposure to PM_{2.5} was fitted as linear variable in all the models and effect

estimates were calculated for a 5 $\mu\text{g}/\text{m}^3$ increment in $\text{PM}_{2.5}$. In addition, we applied a multivariate linear regression (adjusted for the aforementioned covariates except smoking status) to estimate the association between maternal smoking status and *P53* expression or *P53* protein levels. Results are presented as a relative change in percentage of gene expression or protein levels. The Shapiro-Wilk statistic and Q-Q plots of the residuals were used to test the assumptions of model linearity. Reported *p*-values are two-sides and *p*-values < 0.05 were considered significant.

In a sensitivity analysis, we performed all statistical analysis while stratifying for gender, and again while excluding preterm births (< 37 weeks). The stratified models were adjusted for maternal age, gestational age, pre-pregnancy BMI, parity, smoking status, ethnicity, maternal education, and season of delivery. We also repeated our statistical analysis with gene expression data that was normalized to another set of reference genes to test the robustness of our associations.

RESULTS

Participant demographic and lifestyle factors

Demographic characteristics and perinatal factors of 170 mother-newborn pairs are reported in **Table 2**. The newborns, among them 81 boys (47.6%), had a mean gestational age of 39.2 weeks (range, 34-41) and comprised 84 (49.4%) primiparous and 69 (40.6%) secundiparous newborns. 90% (*n* = 153) of the newborns were Europeans of Caucasian ethnicity. The mean (SD) birth weight of the newborns was 3,445 (424) g and mothers had an average pre-pregnancy BMI of 24.0 (4.2) kg/m^2 . Mean maternal age was 29.6 years (range: 21-42 yr) and more than 50% (*n* = 94) was high educated. Most women (60.0%, *n* = 102) never smoked cigarettes and 42 women (24.7%) stopped smoking before pregnancy, whereas 26 mothers (15.3%) reported to have smoked during pregnancy (on average 7.9 cigarettes per day; inter quartile range [IQR]: 5-10). Only 2 individuals (1.2%) underwent a Caesarean section. Gestational exposure to $\text{PM}_{2.5}$ averaged 13.3 (range 7.6-18.8) $\mu\text{g}/\text{m}^3$ for the entire pregnancy.

Table 2. Characteristics of 170 mother-newborn pairs

Characteristic	Mean ± SD or n (%)
Newborn	
Gender	
Male	81 (47.6%)
Ethnicity†	
European-Caucasian	153 (90.0%)
Non-European	17 (10.0%)
Gestational age, w	
	39.2 ± 1.2
Birth weight, g	
	3,445 ± 424
Season of delivery	
Winter	44 (25.9%)
Spring	55 (32.3%)
Summer	29 (17.1%)
Autumn	42 (24.7%)
Mother	
Age, y	
	29.6 ± 4.2
Pre-pregnancy BMI, kg/m ²	
	24.0 ± 4.0
Maternal education‡	
Low	17 (10.1%)
Middle	58 (34.3%)
High	94 (55.6%)
Smoking status	
Self-reported never smoker	102 (60.0%)
Self-reported cessation	42 (24.7%)
Self-reported smoker	26 (15.3%)
Parity	
1	84 (49.4%)
2	69 (40.6%)
≥ 3	17 (10.0%)
Caesarean section	
	2 (1.2%)
Cord plasma P53 protein levels, IU/ml§	
	2.01 (0.82 – 5.33)
PM_{2.5} exposure during pregnancy, µg/m³	
	13.3 (range 7.6–18.8)

† Based on the native country of the newborn's grandparents. European-Caucasian when two or more grandparents were European, or non-European when at least three grandparents were of non-European origin.

‡ Mother's education: 'low' (no high school diploma), 'middle' (high school diploma), 'high' (college or university diploma).

§ Geometric mean with 10th – 90th percentiles, n = 157.

Correlation of genes involved in the micronuclei network

Expression of most of the genes within the MN formation network that we selected in our study correlated well (**Figure 1**). *P53* expression correlated significantly with *BAX* ($r = 0.29, p = 0.0002$), *DNMT1* ($r = 0.23, p = 0.002$), *PCNA* ($r = 0.27, p = 0.0003$), but not with *P21* ($p = 0.97$). Positive correlations were also found between *BAX* expression and *DNMT1* ($r = 0.51, p < 0.0001$), *PCNA* ($r = 0.20, p = 0.008$), and *P21* ($r = 0.18, p = 0.02$). Contrary to these positive correlations, *PCNA* correlated negatively with *P21* ($r = -0.25, p = 0.001$), whereas no correlation was found between *DNMT1* and *PCNA* ($p = 0.74$) or *P21* ($p = 0.48$). The correlations remained the same when we adjusted for maternal age, gestational age, and sex.

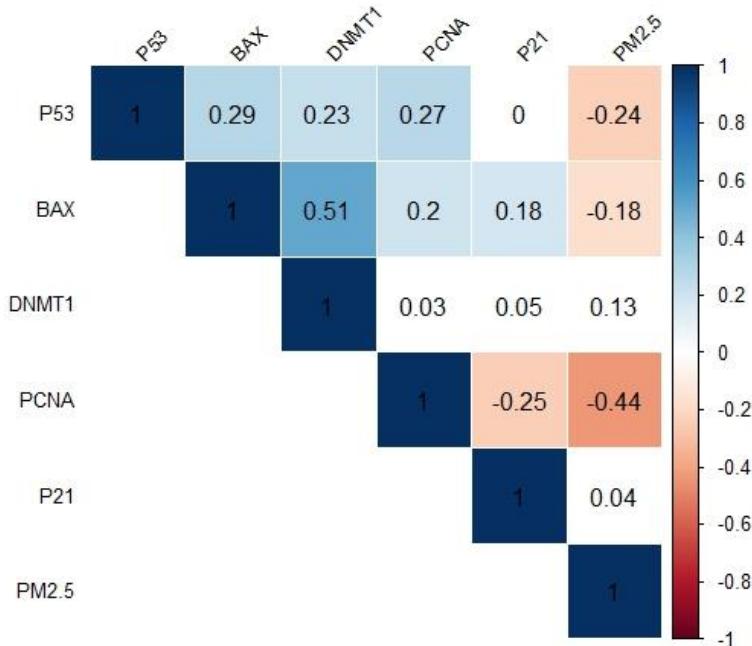


Figure 1. Unadjusted correlation matrix between expression of the selected genes of the MN formation network and $\text{PM}_{2.5}$ exposure during the entire pregnancy. Numbers represent Pearson correlation coefficients and colored squares indicate significant correlations (blue: positive correlation; red: negative correlation) at p -level < 0.05 .

PM_{2.5} exposure and expression of micronuclei-related genes

We determined the association between expression of our selected target genes within the MN formation network (*P53*, *BAX*, *DNMT1*, *PCNA*, and *P21*), and entire pregnancy PM_{2.5} exposure using a mixed effect model. Both univariate (data not shown) and multivariate analysis (adjusted for maternal age, gestational age, sex, pre-pregnancy BMI, parity, smoking status, ethnicity, maternal education, and season of delivery) showed a negative association between expression levels of the MN formation-related genes and PM_{2.5} exposure during pregnancy (relative decrease of -52.0%, 95% CI: -68.7 to -26.2%, $p = 0.0009$ for each 5 $\mu\text{g}/\text{m}^3$ increment in PM_{2.5} exposure). The interaction between the gene and PM_{2.5} exposure that was present in univariate analysis ($p < 0.0001$) lost significance in multivariate analysis ($p = 0.16$).

We further explored the association between individual gene expression and entire pregnancy PM_{2.5} exposure. Unadjusted analysis (**Figure 1**) showed a negative correlation between entire pregnancy PM_{2.5} exposure and expression of *P53* ($r = -0.24$, $p = 0.002$), *BAX* ($r = -0.18$, $p = 0.02$), and *PCNA* ($r = -0.44$, $p < 0.0001$), a marginal positive correlation with *DNMT1* ($r = 0.13$, $p = 0.09$), and no correlation with *P21* ($p = 0.58$) expression, whereas after adjustment for the aforementioned covariates, only the association between PM_{2.5} exposure with *P53* and *PCNA* remained significant (respectively -8.3%, 95%CI: -14.2 to -1.9%, $p = 0.01$ and -22.9%, 95% CI: -30.8 to -14.1%, $p < 0.0001$ for a 5 $\mu\text{g}/\text{m}^3$ increment in PM_{2.5}). (**Table 3**)

Table 3. Unadjusted (Model 1) and adjusted (Model 2) association between PM_{2.5} exposure during the entire pregnancy and expression of genes within the MN formation network

Gene	Model 1			Model 2†		
	Relative %	(95%CI)	p	Relative %	(95%CI)	p
P53	-7.7	(-12.2 to -3.0)	0.002	-8.3	(-14.2 to -1.9)	0.01
BAX	-10.9	(-19.2 to -1.8)	0.02	-9.6	(-21.0 to 3.5)	0.14
DNMT1	7.0	(-1.2 to 15.7)	0.09	1.9	(-8.1 to 12.9)	0.72
PCNA	-22.9	(-29.0 to -16.4)	< 0.0001	-22.9	(-30.8 to -14.1)	< 0.0001
P21	2.4	(-6.0 to 11.6)	0.58	4.6	(-7.3 to 18.1)	0.46

† Model 2 was adjusted for maternal age, gestational age, sex, pre-pregnancy BMI, parity, smoking status, ethnicity, maternal education, and season of delivery.

Verification of alterations in P53 by microarray and protein concentration measurement

Given our prior knowledge that *P53* acts as a central hub for activating other genes involved in the MN formation network,³²⁰ we verified whether *P53* expression was altered using data derived from microarray analysis in cord blood from Winckelmans *et al.* (submitted).³²¹ These authors used participants from the same birth cohort and 139 samples (81.8%) were in overlap with our study. Consistent with our results, they also observed a down-regulation of *P53* gene expression in association with long-term PM_{2.5} exposure. When comparing smokers with non-smokers, we observed lower *P53* expression in smokers but this was not significant (-4.9%, 95% CI: -13.0 to 3.9%, p = 0.26).

Next, we measured protein levels of P53 in plasma cord blood to explore the functional significance of altered gene expression in response to PM_{2.5} exposure during gestation. Protein levels of P53 did not correlate with *P53* expression ($r = -0.11$, $p = 0.16$). However, both before (**Figure 2**) and after adjustment for the aforementioned covariates, we observed a strong positive association between entire pregnancy PM_{2.5} exposure and P53 protein levels (relative increase of 56.0%, 95% CI: 23.5 to 97.1%, $p = 0.0003$ for a 5 µg/m³ increment in PM_{2.5}). In addition, women who reported to have smoked during pregnancy showed higher levels of P53 protein in cord plasma compared with non-smokers (+46.8%, 95% CI: 7.4 to 100.7%, $p = 0.02$).

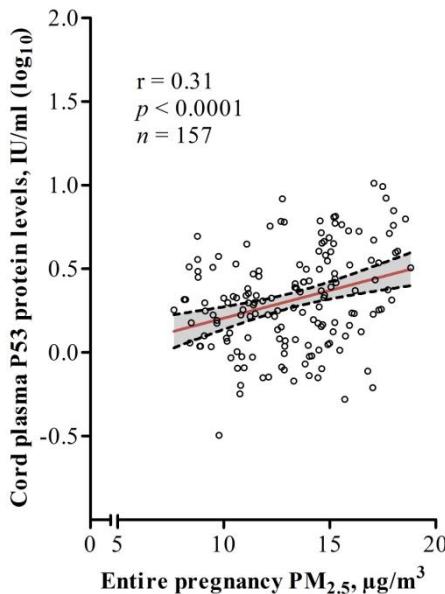


Figure 2. Correlation between protein levels of P53 in plasma cord blood and PM_{2.5} exposure during pregnancy. Protein levels (IU/ml) are log₁₀-transformed and PM_{2.5} concentrations are presented as µg/m³. The correlation plot depicts 95% CI as dashed lines.

Sensitivity analysis

Gene expression patterns in cord blood could show gender-specific differences³²² (Winckelmans *et al.*, personal communication).³²¹ Stratifying our statistical analysis for gender revealed stronger associations for girls than boys (**Supplemental Material, Table S1**), but this could be due to differences in sample size ($n = 75$ and $n = 65$ respectively). In addition, excluding preterm births (gestational age < 37 weeks; $n = 4$) did not change our reported associations. We repeated our statistical analysis with gene expression data that was normalized with another set of reference genes to test the robustness of our associations. Our observed associations between gene expression or protein levels and PM_{2.5} exposure were not changed.

DISCUSSION

The key finding of this study is that PM_{2.5} exposure during pregnancy is associated with a down-regulation in expression of genes within the MN formation network, especially with *P53* and *PCNA*. However, P53 protein levels in

plasma cord blood were positively associated with PM_{2.5} exposure during pregnancy.

Ambient air pollution, especially PM_{2.5} exposure, is a potential risk factor for human health, and detection of MN in cord blood lymphocytes could be an important method for identification of transplacental pollutants in evaluation and protection of children's health. The cytokinesis-block MN test is a standard method for evaluating both genomic instability and genotoxic exposure in human biomonitoring studies,³¹⁶ but gene expression analysis might be a promising new tool for evaluation of MN frequencies. Van Leeuwen and colleagues developed a MN formation network based on *a priori* knowledge retrieved from literature by using the pathway tool MetaCore in combination with data from a genome-wide transcriptomics case study in which MN frequency was measured in children differentially exposed to ambient air pollution in the Czech Republic.^{320,323} The authors concluded that a subset of this network (*BAX*, *DNMT1*, *PCNA*, *HIC1*, *P21*, *CDC20*, *IL-6*, and *P53*) were associated with MN frequencies and PM exposure.

Rationale of gene selection of the MN network

We chose to investigate the transcriptional level of *BAX*, *DNMT1*, *PCNA*, *P21*, and *P53* in cord blood of newborns exposed to PM_{2.5} during pregnancy. The tumor suppressor gene *P53* responds to a variety of environmental and intracellular stresses^{324,325} by increasing P53 protein levels that will interact with sequence-specific sites on its target genes.^{326,327} *P53* is proposed as the central hub of MN formation since there is a direct relationship between P53 protein levels and induction of micronuclei.³²⁸ The protein encoded by *BAX* is a member of the BCL2 protein family which forms a dimer with BCL2, and acts as a pro-apoptotic regulator. Expression of this gene is regulated by P53 and has been shown to be involved in P53-mediated apoptosis. Up-regulation of *BAX* is significantly correlated with increased MN frequency.³²⁰ *DNMT1* is important in the regulation and establishment of tissue-specific patterns of methylated cytosine residues. *DNMT1* levels have been shown to be negatively correlated with MN frequency.³²⁰ A previous study showed a relative decrease of global DNA methylation patterns in placental tissue in association with *in utero* exposure to particulate air pollution.¹³⁰ The encoded protein of *PCNA* helps increase the

processivity of the leading strand synthesis during RNA replication. The protein is ubiquitinated in response to DNA damage and is involved in the RAD6-dependent DNA repair pathway. Findings of Xu and colleagues suggest a complex cellular response to DNA damage in which *P53* transiently activates expression of *PCNA* in order to obtain limited DNA repair.³²⁹ *PCNA* was up-regulated in association with lower MN frequencies.³²⁰ *P21* encodes a universal inhibitor of cyclin-dependent kinases and is in this way a regulator of cell cycle progression at the G1 phase. Up-regulation of *P21* was significantly correlated with increased MN frequencies.³²⁰ In our study we observed positive correlations between *P53* expression and *BAX*, *DNMT1*, *PCNA*, but not with *P21*, which is suggestive of *P53* as a central hub in the expression of MN-related genes.

Expression of MN-related genes in cord blood in association with PM_{2.5} exposure

Exposure to a variety of rural or urban environmental pollutants revealed overall higher MN frequencies in neonates and children compared to referents.^{330,331} Environmental air pollution exposure has been associated with increased MN frequency in cord blood of Serbian,³³² and Danish newborns,³¹⁷ but surprisingly not in Greek newborns.³¹⁹ Contrary to our expectations, our results showed a down-regulation in the expression of the MN-related genes in association with PM_{2.5} exposure during pregnancy, both in the network model as in the individual gene model. The negative association of the MN-related gene expression seemed to be driven predominantly by expression of *P53*, *BAX*, and *PCNA*. We assumed we would observe subtle increases in expression of MN-related genes in response to air pollution exposure, especially with *P53* and *BAX* expression since PM exposure induces *P53* mRNA and P53 protein expression in cells at the bronchoalveolar duct junctions in mouse lungs³³³ and *P53* and *BAX* transcripts in human epithelial lung (L132) cells.³³⁴ Therefore, we validated our expression data with a genome-wide microarray data from the same population and observed the same down-regulation of *P53* in association with PM_{2.5} exposure.³²¹ In addition, these authors observed an up-regulation of *MDM2*, a negative regulator of *P53*,³³⁵ in association with long-term PM_{2.5} exposure. To clarify our puzzling observation, we measured P53 protein levels in plasma cord blood and observed a strong positive association with PM_{2.5} exposure during pregnancy.

In addition, women who smoked during pregnancy had higher P53 protein levels compared to non-smokers, corroborating the findings we observe with PM_{2.5} exposure. Our findings are consistent with what is observed in experimental studies^{333,334} and an observational study of city policemen and bus drivers exposed to PM-absorbed polycyclic aromatic hydrocarbons (PAHs).³³⁶ The biological reason of our discrepancy in direction of association between PM_{2.5} exposure and *P53* mRNA expression and P53 protein expression remains open for discussion. From experimental studies it is known that P53 stabilizes in response to DNA damage,^{337,338} and therefore, a gene's mRNA level does not necessarily predict its protein level. We observed no correlation between *P53* mRNA expression and P53 protein levels. A study comparing breast cancer brain metastases with primary tumors showed that *P53* expression was lower in brain metastases compared with primary tumors but no change was observed at the protein level.³³⁹ Contrary, whereas *BCL-2* mRNA and protein expression were in good agreement, *BAX* mRNA levels were down-regulated in metastases while protein levels were higher.³³⁹ Taken together, these data indicates that the *P53* transcriptional network is extremely complex involving many positive and negative feedback loops and proteins that interact with a large number of other signal transduction pathways in the cell.³²⁵

Our findings need to be interpreted within the context of its strengths and limitations. Unfortunately, we were not able to measure actual MN frequencies in cord blood. It is recommended to measure MN frequencies using the cytokinesis-block micronucleus cytome assay on fresh blood sampled since there are conflicting reports on whether cryopreservation of samples alters the frequency rate of MN in cells.³⁴⁰ Therefore, these measurements are difficult to perform in large-scale epidemiologic studies. However, this does not mean we cannot make a conclusion based on our observed gene expression alterations. Our results of alterations in expression of genes within the MN formation network in response to PM_{2.5} exposure during pregnancy, can serve as potential biomarkers of PM_{2.5} exposure. Our reported associations were robust, even after adjusting for several covariates that could influence the association between PM_{2.5} exposure with gene and protein expression, stratifying our analysis for gender, or using another set of reference genes to normalize our gene expression data. As with all observational studies, we cannot exclude the possibility of residual

confounding by some unknown factor that is associated with both *P53* gene or protein expression and ambient air pollution. Although we used validated exposure models and we took into account address changes throughout pregnancy, there might be some exposure misclassification. We interpolated daily exposure levels at the residence without taking into account exposure levels at the address of employment. However, we assume that most of the time was spent as the home address since a considerable amount of women worked part-time and stopped working with a median of 90 days prior to delivery. Besides the quality of exposure assessment and evaluation of potential confounders, strengths of the study included the validation/replication of our gene expression data with microarray data and the additional measurement of *P53* protein levels in cord blood.

CONCLUSION

In conclusion, we found an association between expression of MN-related genes and PM_{2.5} exposure during pregnancy, which could be indicative of subclinical MN formation in cord blood. *P53*, the major hub of MN formation, showed higher protein levels in cord blood of newborns exposed to high concentrations of PM_{2.5} and in those exposed to *in utero* tobacco smoke. Our findings give support to the development of a reliable reporter gene assay to screen chemically exposed human populations faster and in a less labor-intensive manner than by using microarrays.

Acknowledgments

The authors thank the participating women and neonates, as well as the staff of the maternity ward, midwives, and the staff of the clinical laboratory of East-Limburg Hospital in Genk. The ENVIRONAGE birth cohort is supported by the EU Program 'Ideas' (ERC-2012-StG 310898), and by the Flemish Scientific Fund (FWO, N1516112/G.0.873.11.N.10). Karen Vrijens is a postdoctoral fellow of the FWO.

SUPPLEMENTAL MATERIAL

Table S1. Associations between entire pregnancy PM_{2.5} exposure and expression of MN-related genes or P53 protein levels, stratified by gender

Model†	Boys			Girls		
	Relative %	(95%CI)	p	Relative %	(95%CI)	p
Mixed effect model						
MN network	-45.8	(-72.3 to 6.3)	0.07	-50.4	(-70.3 to -18.6)	0.006
Single regression models						
<i>P53</i>	-5.9	(-15.4 to 4.7)	0.26	-12.7	(-20.7 to -3.9)	0.006
<i>BAX</i>	2.6	(-15.9 to 25.1)	0.80	-17.3	(-32.4 to 1.0)	0.06
<i>DNMT1</i>	5.5	(-12.6 to 27.4)	0.57	0.6	(-11.7 to 14.6)	0.92
<i>PCNA</i>	-21.8	(-34.1 to -7.2)	0.005	-23.5	(-34.7 to -10.4)	0.001
<i>P21</i>	5.6	(-13 to 28.3)	0.58	6.3	(-9.7 to 25.1)	0.46
Protein model (n = 160)						
P53 protein	40.4	(0.8 to 95.6)	0.04	51.6	(4.6 to 120)	0.03

† Stratified models were adjusted for maternal age, gestational age, pre-pregnancy BMI, parity, smoking status, ethnicity, maternal education, and season of delivery.

CHAPTER 9

GENERAL DISCUSSION

Although universal and unavoidable, aging does not occur in a uniform way. Aging is a complex phenotype event responsive to a plethora of environmental stressors, including PM air pollution, from early life onwards. The '*Developmental Origins of Health and Disease*' (DOHaD) hypothesis suggests that small changes in the prenatal and early postnatal environment shape the future probability of the development of age-related diseases.^{107,108} While the mechanisms underlying fetal programming are poorly understood, evidence is mounting that the placenta plays a central role in an adaptive response to environmental perturbations resulting in altered fetal metabolism.

In this doctoral dissertation, we investigated the effect of fine particle exposure on the most important organ in fetal development – the placenta. For this purpose, we focused on three important systems: epigenetics, mitochondria, and thyroid hormones, that could provide a biologically plausible link between *in utero* exposures and disease risks in adulthood. The establishment of the epigenetic landscape coincides with vulnerable phases in development and provides one potential mechanism for long-lasting responses to transient environmental stimuli. On the other hand, besides that mitochondria play an important role in disease processes, growing evidence suggests an interplay of mitochondria with the epigenome.

The novelties of this dissertation include:

- The establishment of a population-based prospective birth cohort 'ENVIRONAGE'.
- The evaluation of DNA methylation variation within the placenta of age- and mitochondrial-related genes.
- Replication of effects of tobacco smoke exposure on birth weight and several molecular signatures in placental tissue.
- The investigation of PM exposure during pregnancy as an environmental determinant for placental mitochondrial function and (mitochondrial) epigenetics.

- The exploration of the role of thyroid hormones on mitochondrial function and epigenetics and whether thyroid hormones are associates with gestational PM exposure.
- The assessment of a reporter gene assay for potential screening of DNA damage in cord blood in response to PM exposure during pregnancy

ESTABLISHMENT OF THE POPULATION-BASED PROSPECTIVE BIRTH COHORT 'ENVIRONAGE'

In the past 20 years, a variety of birth cohort studies have been undertaken worldwide to assess the health risks that the developing child may be confronted with when exposed to harmful chemicals in the air, water, and food. These birth cohort studies usually started during pregnancy and followed children through adolescence or beyond. It is obvious that these cohorts were designed with specific aims focusing on a specific disease or an environmental factor. They started recruitment either at birth or at the beginning of pregnancy, with or without follow-up, and collection of biological material to investigate certain biomarkers of exposure, effect or susceptibility.

One of the spear points in the ENVIRONAGE birth cohort is the life-long health effect of air pollution. Fine particle air pollution is an omnipresent environmental risk factor with major health concerns in adult populations.^{5,42} In this cohort, we underscore the importance of molecular and cellular mechanisms to bridge the gap between environmental exposure in early life and later onset of diseases, by performing detailed repeated physiological and clinical measures. The future of epidemiology depends on innovative strategies and new technologies for in-depth phenotyping of participants, both in measuring environmental agents as the pathophysiology of outcomes.¹³⁷ In the current era of 'omics', the ENVIRONAGE birth cohort study has been designed with a strong integration of these fast-growing 'omics' technologies. The holistic approach of 'exposome' studies, referring to the totality of environmental exposures over time from conception onwards,¹³⁸ is the most complete way to study environmental agents on human health. However, these kinds of studies require a tremendous effort in funding, coordination, and (logistic) organization.

It is becoming increasingly clear that to realize the full potential of birth cohorts, one needs to collaborate.³⁴¹ Collaboration will increase sample sizes to improve causal inference through cross-cohort comparisons and replication, and to study rare but important disease outcomes in infancy and childhood. It will improve methodological approaches including protocols of biological and environmental sample collection to more easily and efficiently perform pooled data analyses. However, pooling of the data is extremely difficult due to differences in general study design, time periods of measurement, disease outcome definitions, or methods for measuring biomarkers and environmental factors. Nevertheless, efforts are made to set up collaborations between birth cohorts. For example, the EU has funded the Environmental Health Risks in European Birth Cohorts (ENRIECO) project to coordinate birth cohort research in Europe in the area of environmental contaminant exposures (ENRIECO 2009).³⁴² Not only cohorts on environmental contaminant but also cohorts interested in specific biological specimens are starting to collaborate. Recently, the Epigenetic Placenta Consortium, of which our cohort is part of, was created as a collaborative platform for researchers who have collected placental tissue from a birth cohort and have done or are planning to do epigenetic work on the placenta.

METHODOLOGICAL CONSIDERATIONS FOR STUDYING PLACENTAL EPIGENETICS

Placental tissue and cord blood are the most frequently used specimens in human reproductive studies due to their functional significance, non-invasive collection, and good accessibility. However, some challenges need to be taken into consideration when studying placental epigenetics. Human reproductive studies provided little information about the within-placenta variation of specific genes. Few studies have investigated within-placenta variation of (global) DNA methylation levels and only focused on a specific set of genes.^{72,143,162,163}

Before studying placental DNA methylation, it is always recommended to evaluate each gene of interest across placental sampling sites, as each gene may display site-specific differences. In **chapter 3**, we explored whether we could use one placental biopsy, taken using a standardized method, to perform

DNA methylation measurements. We took four biopsies in a subset of nineteen placentas to evaluate the within-placenta variation of specific genes within the mitochondrial-telomere axis. For most of the genes, the between-placental variability was considerably larger than the within-placental variability suggesting that these genes were not strongly influenced by sample location. Hence, we used one placental biopsy to represent the placenta. Given the relatively large number of samples or subjects under investigation in an epidemiological context and the related costs for epigenetic measurements, it is not always feasible to analyze multiple samples from one placenta. Future studies on placental methylation may consider pooling biopsies from the same placenta to further reduce sample variability. However, some study protocols do not allow for the collection of multiple biopsies per placenta or the use of multiple biopsies for this purpose.

Our epigenetic associations should be interpreted cautiously within the context of cellular heterogeneity. All tissues are composed of multiple cell types and the degree of methylation measured in a tissue is an average of the methylation in all existing cell types. The placenta is composed of a complex population of cells [mesenchymal cells, mesenchymal derived macrophages (Hofbauer cells), fibroblasts, smooth muscle cells, perivascular cells (pericytes), and endothelial cells] but the majority of cells include cytotrophoblasts, and differentiated invasive syncytiotrophoblasts, that originate from trophoblasts (outer layer of the bastocyst). To our knowledge, there is only one study that explored genome-wide DNA methylation patterns in the two main cell types of the placenta (cytotrophoblasts and fibroblasts).¹⁴³ These cell types did not demonstrate genome-wide differentiation in DNA methylation but some specific genes showed differential promoter methylation. Although these authors demonstrated that the methylation profile of the placenta is mainly driven by cytotrophoblasts, there is a possibility that our epigenetic data is confounded by variation in cell type distributions (due to air pollution, tobacco smoke, inflammation or other causes) and may not reflect true epigenetic differences but only differences in cell type composition. To overcome this caveat, it is necessary to characterize and explore the effects of cellular heterogeneity in heterogeneous tissues such as the placenta.³⁴³ In whole blood, it is possible to correct for differences in blood composition using algorithms that estimate cell

type proportions based on DNA methylation signatures derived from Illumina microarrays.^{343,344} Of importance, statistical adjustments of DNA methylation data for cell distributions require the availability of reference epigenomes for the component cell types created by cell sorting.¹⁷⁰ Currently, there is no algorithm available to estimate the cell type proportions in placental tissue.

Exposure to air pollution and tobacco smoke may initiate inflammatory processes which are accompanied with an increase in a number of markers including white blood cell count and cytokine and plasminogen activator inhibitor-1 (PAI-1) concentrations. Differences in blood cell distribution due to inflammatory processes may confound the association between exposure and outcome of interest. We adjusted our cord blood models for white blood cell count and percentage of neutrophils but statistical correction for cell distribution may not be perfect. We underscore the importance of accounting for cell composition variability wherever possible, especially in epigenetic studies based on heterogeneous tissue sources.

AN EXPLORATIVE ANALYSIS ON THE EFFECTS OF TOBACCO SMOKE

It is well-known that tobacco smoke exposure in the general population affects the heart, lungs, and liver, with an increased risk of the development of cancer and cardiovascular disease. For maternal smoking during pregnancy, there is pooled evidence showing that constituents of cigarette smoke cross the placenta, reduce intrauterine fetal growth, and increase the risk of low birth weight^{180,181} and preterm birth,^{111,182} not only in smoking mothers, but also in non-smoking mothers exposed to secondhand smoke. In addition to perturbations in the fetoplacental circulation,^{183,345} tobacco smoke induces changes at the mitochondrial and epigenetic level. The mtDNA content in placental tissue is extremely adaptive to tobacco-induced oxidative stress.^{132,185} In addition, DNA methylation differences have been demonstrated on the global⁷³ and gene-specific^{191,193,197,346} level in placental cells of neonates from mothers who smoked during pregnancy. In **chapter 4**, we explored whether we could replicate these findings, that are already described in literature. For this purpose, we used a study sample of 382 mother-newborn pairs consisting of 255 non-smokers, 65 former-smokers, and 62 mothers who continued smoking

during pregnancy. As anticipated, babies whose mothers smoked during pregnancy were on average 208 g (95% CI: -318 to -99 g) lighter at birth than babies from non-smoking mother, which is consistent with literature.¹⁸¹ In addition, we observed lower relative mtDNA content and absolute global DNA methylation levels in placental tissue (respectively -21.6%, 95% CI: -35.4 to -4.91% and -0.11%, 95% CI: -0.24 to 0.03%), while absolute mtDNA methylation levels were higher (+0.62%, 95% CI: 0.21 to 1.02%) in smokers compared with non-smokers. Since tobacco smoke exposure is the most personalized form of air pollution, these findings may serve as a positive control to test the robustness of the relation between PM exposure, mitochondria, and (mitochondrial) epigenetics in placental tissue. The novelty of these findings is the epigenetic alterations of the mitochondrial genome in response to tobacco smoke exposure. Taken together, tobacco smoke exposure correlates with a reduction in birth weight and with mitochondrial and epigenetic impairment, suggesting that both could play a role in reducing birth weight in smoking pregnant women.

FINE PARTICLE EXPOSURE AND EPIGENETICS

The last several years have produced an emerging set of literature on the relation between PM air pollution exposure and DNA methylation in children,^{347,348} adults,^{70,166,349-354} and elderly.^{68,69,355} Interestingly, the association between PM air pollution and DNA methylation during the most critical stage of epigenetic programming – the *in utero* period – has never been investigated. In **chapter 5**, we showed in a study of 240 mother-newborn pairs that placental global DNA methylation was inversely associated with first trimester PM_{2.5} exposure, especially during the critical period of implantation (-2.13% per 5 µg/m³ increase in PM_{2.5} exposure, 95% CI: -3.71, -0.54%). Our findings have been replicated by another study showing that pregnant mothers living close to major roadways, as a marker of traffic-related pollution, had lower levels of placental DNA methylation in LINE-1 but not AluYb8, which are surrogate markers of global DNA methylation.³⁵⁶ Additionally, in **chapter 6.2**, we demonstrated in a study sample of 381 mother-newborn pairs, higher absolute methylation levels of the mitochondrial genome in association with prenatal PM_{2.5} exposure, especially during the first trimester (*MT-RNR1*:

+1.27%, 95% CI: 0.23 to 2.32% and *D-loop*: +0.44%, 95% CI: 0.12 to 0.75% for an IQR increment in PM_{2.5} exposure). We corroborated these findings by showing that mitochondrial DNA methylation patterns in placental tissue are altered in the same manner as previously shown in response to maternal tobacco smoke exposure (**chapter 4**). Our findings underscore the sensitivity of the epigenetic system to environmental factors during the early period of developmental plasticity. Methylation patterns are re-established during early pregnancy,⁶⁰ making this a highly sensitive window of susceptibility to the effects of prenatal PM_{2.5} exposure. This may lead to an adaptive response altering placental and fetal development with possibly a long-lasting impact in later life (**Figure 1**). The question remains whether the effects of PM are induced after fertilization – like we assume – or have already been induced in the gametes of parents? Disruption after fertilization may affect the outer blastomeres of the pre-implantation embryo and be reflected only in the placenta. Disruption of the epigenetic profile in the gametes may affect inner and outer blastomeres to the same degree, and hence be reflected in both cord blood and placental tissue.¹⁸⁹ Investigations on the effects of tobacco smoke exposure during pregnancy observed differences in global^{188,190} and gene-specific^{172,192,194–196} methylation levels in cord blood, but failed to study placental tissue in parallel. It can be concluded either that the epigenetic landscape of the gametes is already altered or that cumulative exposure to the intrauterine stressors impacts the methylation profile of both the placenta and cord blood. It would be interesting to perform a study to clarify the timing of epigenetic modification due to environmental stressors in an experimental study or in a human study by recruiting participants before conception.

The proposed mechanisms underlying the relation between PM air pollution exposure and DNA methylation involve oxidative stress and inflammation.²³⁰ Experimental evidence showed that oxidative DNA damage could interfere with the capability of methyltransferases to interact with DNA resulting in lower methylation of cytosine residues at CpG sites.²²⁷ Of importance is that changes in epigenetic signatures need to be interpreted in the context of their biological relevance. Although DNA methylation is usually associated with alterations in gene expression,⁵⁸ it is not known whether small changes in the methylation status of a given promoter necessarily translates an alteration in gene

expression.⁶² However, establishing a cut-off value for differential DNA methylation to be biologically relevant is difficult since this can depend on the type of study, sample size, heterogeneity of the tissue, the method or technique used, or even interpretation of the data. For example, larger differences are desirable between cases and controls for a certain disease phenotype, whereas for epidemiological studies subtle changes in DNA methylation levels can have a functional meaning by revealing biological pathways involved in disease development or to unravel underlying mechanisms of action. For example, in **chapter 6.2**, we showed that relatively small changes of DNA methylation in the mitochondrial genome substantially mediate the association between prenatal PM_{2.5} exposure and placental mtDNA content, demonstrating an intermediate mechanism of mtDNA alterations that could reflect mitophagy and mitochondrial death.

In summary, epigenetic modification may provide a plausible link between PM air pollution and alterations in gene expression that might lead to disease phenotypes related to fetal programming.



Figure 1. Sources of inter-individual epigenetic variation that can contribute to age-related human disease. In addition to genetic and epigenetic inheritance, stochasticity affects the developmental establishment of epigenetic regulation. The epigenetic system is particularly sensitive to environmental factors during the early period of developmental plasticity when the methylation patterns are re-established. Cumulative errors in maintenance of epigenetic information will contribute to interindividual epigenetic variation with age. *Reprinted from Michels; Epigenetic Epidemiology.*⁶²

FINE PARTICLE EXPOSURE AND MITOCHONDRIA

The placenta has a high reliance on mitochondria for its proper development and function. It is well recognized that the (patho)physiological role of mitochondria widely exceeds that of solely being the biochemical power plant of the cells. To date, several reviews support the notion that mutations and oxidative damage to mtDNA increase in an age-dependent manner and is involved in the development of a variety of human diseases.^{76,83,87} There is a new area of research that stretches beyond the nuclear genome called 'mitochondriomics' that is dedicated to examine whether mitochondria are novel biosensors or mediators of environmental effects by exploring mitochondrial DNA abundance, mutations and deletions, epigenetics, and mtDNA-encoded proteins.⁵⁵

Cross-sectional human studies on the relation between PM air pollution and mtDNA content are still limited with inconsistent results.^{247,357-359} In blood of adults and elderly, PM air pollution exposure was associated with an increase,²⁴⁷ a decrease,^{357,359} and no change in mtDNA content.³⁵⁸ Studies investigating the effect of fine particle exposure during the *in utero* period on mtDNA content are limited to maternal tobacco smoke exposure.^{132,185} In **chapter 6.1**, using placental tissue of 174 mother-newborn pairs, we demonstrated for the first time an inverse association between third trimester PM₁₀ (and NO₂) exposure and placental, but not cord blood, mtDNA content (-17.4%, 95% CI: -31.8 to -0.1%, for an increment of 10 µg/m³ in PM₁₀ exposure). The effect estimate was comparable with the effect estimate observed for prenatal tobacco smoke exposure (-21.6%, 95% CI: -35.4 to -4.91%; **chapter 4**). Any observational study has an important limitation as to causal inference because of potential confounding due to socioeconomic factors, cultures, and lifestyles. Therefore, we tried to replicate our findings in an independent European birth cohort, called INMA, located in Spain. Pooled estimates ($n = 926$) indicated that a 10 µg/m³ increment in average NO₂ exposure during pregnancy was associated with a 4.9% (95% CI: -9.3, -0.3%) decrease in placental mtDNA content (PM data not available for INMA).¹³⁴ These results, in addition to the consistent observations that placental mtDNA content was lower in smoking mothers¹³² (**chapter 4**), gives us more confidence in the robustness of our observed association. The previously mentioned discrepancy in the results of mtDNA content, as to direction and effect-size, can be explained by the very dynamic nature of

mtDNA. mtDNA fluctuates under the influence of age, ethnicity, tissue investigated, but most importantly depends on oxidative stress level, cell antioxidant capacity, type of environmental factor, and dose of exposure.^{105,360} The current hypothesis is that mild oxidative stress may stimulate mtDNA copy number synthesis and abundance as a compensatory mechanism, while escalating oxidative stress levels may result in decreased or no synthesis due to severe oxidative damage in cells.¹⁵¹ Taken this hypothesis into account, we performed now an analysis in 641 mother-newborn pairs to examine the shape of association between prenatal PM_{2.5} exposure and placental mtDNA content. Interestingly, we observed a reversed U-shape curve rather than a linear association. We suggest that a cumulative exposure to high concentrations of PM_{2.5} during a nine-month period leads to clearance of placental cells with highly damaged or dysfunctional mitochondria. This biological mechanism seems very plausible if we also take into account our results (i.e. mtDNA depletion) on tobacco smoke exposure, the most severe form of air pollution. Similarly, the relative mtDNA content was increased in the lung tissues of light smokers but significantly decreased in heavy smokers.¹⁸⁶

While the exact mechanisms underlying mitochondrial replication and biogenesis are not fully understood, we suggest that methylation of the mitochondrial genome plays a significant role. Studies on mitochondrial epigenetics are still in its infancy, but given that oxidative stress in the nucleus has been shown to alter nuclear DNA methylation, PM_{2.5} exposure may be a potential candidate with important links to mitochondrial epigenetics.⁵⁵ In a study sample of 381 mother-newborn pairs, we first demonstrated that placental mtDNA methylation is higher in response to prenatal PM_{2.5} exposure (**chapter 6.2**), which is in accordance with a study in blood of steel workers.¹⁶⁶ Furthermore, we showed that mtDNA methylation was estimated to mediate 54% (*MT-RNR1*) and 27% (*D-loop*) of the inverse association between PM_{2.5} exposure and mtDNA content in placental tissue.

Although the exact pathological role(s) of mitochondrial methylation during development needs to be further elucidated, our novel finding provides new insight into the mechanisms of altered mitochondrial function in the early life environment (further discussed in the next section). In summary, mitochondrial

methylation and copy number are sensitive to PM air pollution, highlighting the role of mtDNA as an environmental biosensor.

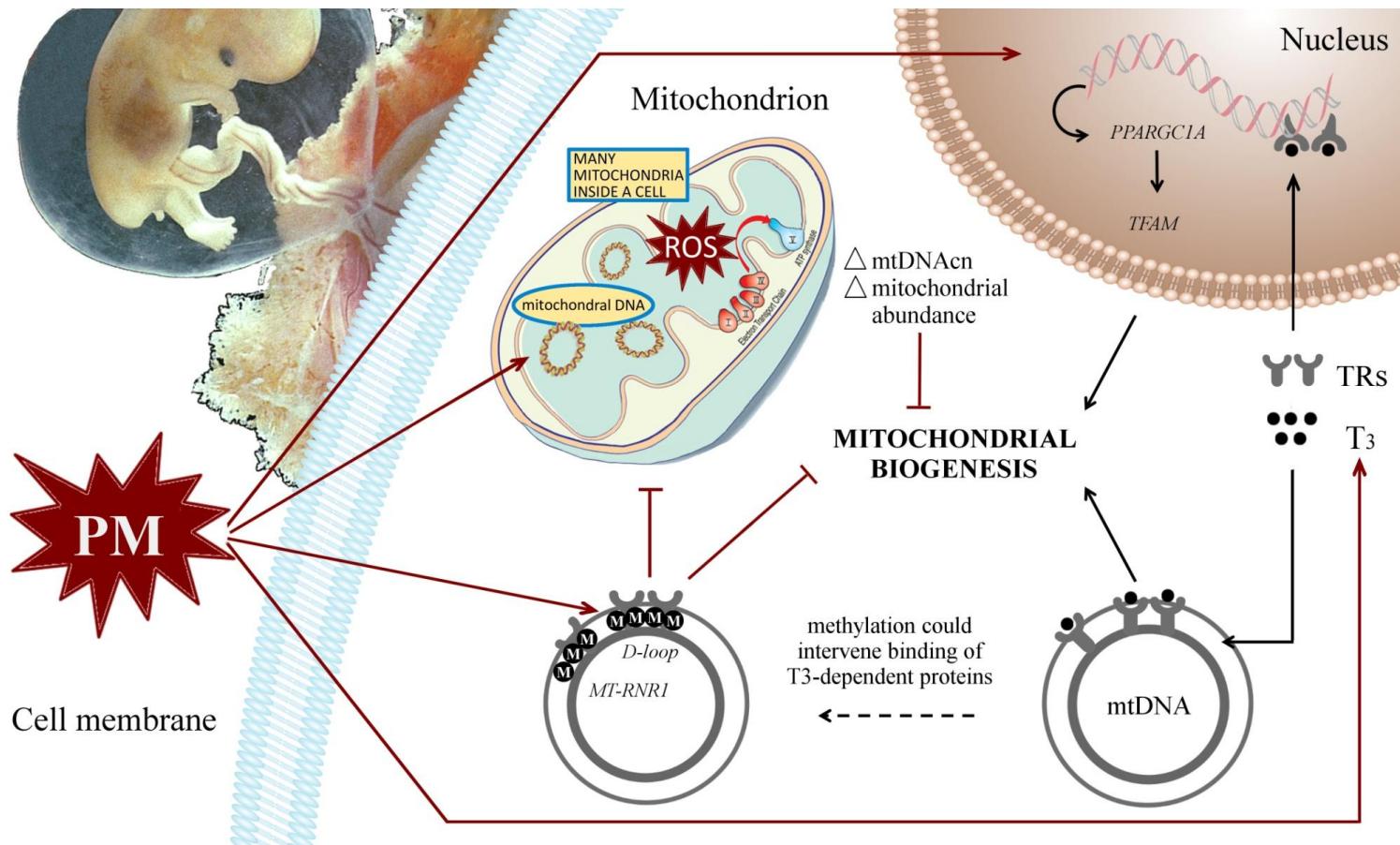
AN INTEGRATED NETWORK OF THYROID HORMONES, MITOCHONDRIA, AND EPIGENETICS

The rationale of studying thyroid hormones originated from their essential role in placental¹⁶ and fetal development,¹⁷ and their profound effects on mitochondrial energetics and biogenesis.^{27,28} The exact mechanisms of how thyroid hormones exert their effects on mitochondrial function are not well understood. Therefore, we explored the role of thyroid hormones on placental mtDNA content and mtDNA methylation in a study sample of 305 mother-newborn pairs (**chapter 7.1**). Both fetal FT₃ and FT₄, but not TSH, were positively associated with placental mtDNA content but negatively associated with placental mtDNA methylation. Next, we underscored a substantial mediating role of placental mtDNA methylation between the association of fetal FT₃ and placental mtDNA content (*MT-RNR1*: 77% and *D-loop*: 47%; results for FT₄ respectively 54% and 24%). We propose a model where high DNA methylation levels in the mitochondrial genome could interfere with thyroid hormone-dependent regulation of mitochondrial biogenesis in placental tissue. In normal circumstances, FT₃ can bind directly to specific receptors (p43) located inside the mitochondrial matrix that subsequently bind to four mitochondrial DNA sequences^{283,284} to stimulate transcription of the mitochondrial genome.²⁷⁸ Interestingly, from the four mitochondrial DNA sequences, two response elements are located in the *D-loop* region and one in the *MT-RNR1* gene, both of which are essential regions for mitochondrial biogenesis. We postulate that high DNA methylation levels in the mitochondrial genome are related to conformational or structural changes making the mtDNA less accessible to proteins and transcription factors such as the T₃-dependent transcription factor p43. This proposed model is particularly interesting knowing that PM_{2.5} exposure results in higher methylation levels at both mtDNA regions in placental tissue, leading to mtDNA depletion (**chapter 6.2**). In addition to the association between prenatal PM_{2.5} exposure and mtDNA methylation, we also observed that PM_{2.5} exposure is associated with alterations in thyroid hormones (**chapter 7.2**), suggesting another mode of action by which PM exposure can affect

mitochondrial biogenesis. In 499 mother-newborns pairs, we demonstrated that prenatal PM_{2.5} exposure was associated with a change in the FT₄/FT₃ ratio (cord blood), which was mainly attributed to a reduction in fetal FT₄ concentrations. The functional significance of this association is emphasized by the fact that the effect of third trimester PM_{2.5} exposure on birth weight was for 21% (on average -19 g) mediated by decreased cord blood FT₄ levels. In accordance with our results, other studies demonstrated the importance of the relation between fetal FT₄ and birth weight,^{25,26,299} underscoring the role of thyroid hormones in normal placentation and fetal growth.

We summarized this integrated network in **Figure 2**. Taken together, cumulative exposure to PM air pollution for nine gestational months induces elevated levels of oxidative stress and inflammation in the placenta. Placental mitochondria respond to this environmental stress by adapting their mtDNA content, either by altered methylation levels in the mitochondrial genome, either in a thyroid-dependent manner or another unknown mode of action. On the other hand, PM exposure may affect mitochondrial biogenesis by inducing changes in DNA methylation of nuclear genes involved in mitochondrial biogenesis including the mtDNA-specific polymerase γ A (POLGA)^{85,276} or PPARGC1A, the master regulator of mitochondrial biogenesis.¹⁴⁹ Conversely, it is also suggested that mtDNA copy number is one of the signals by which mitochondria affect nuclear DNA methylation patterns,⁷⁵ establishing a cross-talk between the mitochondrial and nuclear genome.^{74,360} It is should be emphasized that our proposed model should be validated with experimental studies.

Legend Figure 2 (next page). Proposed model of effects of the cumulative exposure to PM air pollution on placental mitochondria and epigenetics. Mitochondrial biogenesis is a coordinated event of proteins encoded by genes of both the nuclear genome and mitochondrial DNA. Reactive oxygen species (ROS) are generated by PM in addition to the production of ROS as a by-product of oxidative phosphorylation in mitochondria. mtDNA is extremely dynamic and can adapt its copy number in response to environmental stress, either by altered methylation levels in the mitochondrial genome, either in a thyroid-dependent manner, or another unknown mode of action. PM is associated with increased levels of mtDNA methylation at the *D-loop* and *MT-RNR1* region, which could interfere with the binding of the T₃-dependent transcription factor p43 on specific mitochondrial DNA binding sequence, affecting mitochondrial biogenesis. Alternatively, PM is associated with changes in free fetal thyroid hormones that could indirectly change mitochondrial biogenesis by affecting nuclear downstream targets of FT₃ such as PPARGC1A, a coactivator of mitochondrial transcription factor A (*TFAM*). Lastly, since these nuclear-encoded genes are regulated by epigenetic mechanisms,²⁸⁵ PM exposure may affect mitochondrial biogenesis by inducing changes in DNA methylation of these genes.



FINE PARTICLE EXPOSURE AND EFFECTS ON CORD BLOOD

In the previous chapters, we focused on the adaptive response of the placenta to environmental stressors during pregnancy. While it is intuitive that the placenta plays the most important role in fetal development, cord blood is still the most representative tissue for fetal research. Until the 1960s, one believed that the placenta acted as a protective barrier between mother and child, but nowadays it is known that the placental barrier contains placental transporters that can block or facilitate foreign compounds.³⁶¹ Since the placenta can also metabolize some foreign chemicals, it could be argued that the effects of certain environmental toxicants, including PM air pollution, are only observable in the placenta without affecting cord blood. While this is probably not the case for the epigenetic pattern, as shown by the effects of tobacco smoke exposure on global^{188,190} and gene-specific^{172,192,194-196} DNA methylation levels in cord blood, it could be true for mitochondria. In **chapter 6.1** we did observe a relation between prenatal PM exposure and mtDNA content in the placenta, but not in cord blood. One explanation might be that cord blood leukocytes have a higher turnover rate and specifically eliminate cells with high mitochondrial damage. In **chapter 8**, we investigated whether prenatal PM_{2.5} exposure could induce genotoxic effects in cord blood of 170 newborns by exploring the expression of genes involved in micronuclei formation, a validated marker of genotoxicity. Although we observed a discrepancy between expression levels in cord blood and protein levels in cord blood for P53, the central hub for DNA damage responses, our findings lend support to future development of a reliable reporter gene assay to screen chemically exposed human populations faster and in a less labor-intensive way than using microarrays.

OUTLOOK

In this doctoral dissertation, we focused on the role of placental tissue as a molecular footprint that captures the information to which the fetus has been exposed to, especially fine particle air pollution. We emphasized the existence of highly sensitive windows of exposure for prenatal PM air pollution with alterations to the epigenetic system. Secondly, we highlight mitochondria as important environmental biosensors in air pollution research and mtDNA

epigenetics as a new frontier of gene expression modulation in response to environmental stimuli. Findings of this thesis will contribute to the field of epigenetic epidemiology and provide a basis for future studies of early life exposures, epigenetic mechanisms, mitochondria, and the developmental origins of health and disease. In addition, it will promote preventive health care strategies and contribute to a healthier living environment for pregnant mothers and their children.

REFERENCE LIST

REFERENCES

1. Barker DJ, Gluckman PD, Godfrey KM, Harding JE, Owens JA, Robinson JS. Fetal nutrition and cardiovascular disease in adult life. *Lancet* 1993;341:938-41.
2. World Health Organisation. Promoting optimal fetal development: Report of a technical consultation. Geneva, Switzerland 2003 [cited 31 March 2016]. Available from: http://www.who.int/nutrition/topics/fetal_dev_report_EN.pdf.
3. World Health Organisation. Preventing disease through healthy environments: Towards an estimate of the environmental burden of disease. By Prüss-Üstün A and Corvalán C. Geneva, Switzerland 2006 [cited 31 March 2016]. Available from: http://www.who.int/quantifying_ehimpacts/publications/preventingdisease.pdf.
4. World Health Organisation. Health aspects of air pollution with particulate matter, ozone and nitrogen dioxide. Bonn, Germany 2003 [cited 31 March 2016]. Available from: http://www.euro.who.int/__data/assets/pdf_file/0005/112199/E79097.pdf.
5. Cesaroni G, Forastiere F, Stafoggia M, Andersen ZJ, Badaloni C, Beelen R, et al. Long-term exposure to ambient air pollution and incidence of acute coronary events: Prospective cohort study and meta-analysis in 11 European cohorts from the ESCAPE project. *BMJ* 2014;348.
6. Nawrot TS, Perez L, Kunzli N, Munters E, Nemery B. Public health importance of triggers of myocardial infarction: A comparative risk assessment. *Lancet* 2011;377:732-40.
7. Raaschou-Nielsen O, Andersen ZJ, Beelen R, Samoli E, Stafoggia M, Weinmayr G, et al. Air pollution and lung cancer incidence in 17 European cohorts: Prospective analyses from the European study of cohorts for air pollution effects (ESCAPE). *Lancet Oncol* 2013;14:813-22.
8. Winckelmans E, Cox B, Martens E, Fierens F, Nemery B, Nawrot TS. Fetal growth and maternal exposure to particulate air pollution - more marked effects at lower exposure and modification by gestational duration. *Environ Res* 2015;140:611-18.
9. Sram RJ, Binkova B, Dejmek J, Bobak M. Ambient air pollution and pregnancy outcomes: A review of the literature. *Environ Health Perspect* 2005;113:375-82.
10. Longo L. *The rise of fetal and neonatal physiology: Basic science to clinical care*: Springer, 2013; 137-138.
11. Carlson BM. *Human embryology and developmental biology*. Philadelphia, Pa.: Mosby, 2004; 129-149.
12. Newman CG. The thalidomide syndrome: Risks of exposure and spectrum of malformations. *Clin Perinatol* 1986;13:555-73.
13. Myllynen P, Pasanen M, Pelkonen O. Human placenta: A human organ for developmental toxicology research and biomonitoring. *Placenta* 2005;26:361-71.
14. Guttmacher AE, Maddox YT, Spong CY. The human placenta project: Placental structure, development, and function in real time. *Placenta* 2014;35:303-4.
15. Benirschke K, Burton GJ, Baergen R. *Pathology of the human placenta*. Sixth ed: Springer, 2012; 41-46.
16. Chan SY, Vasilopoulou E, Kilby MD. The role of the placenta in thyroid hormone delivery to the fetus. *Nat Clin Pract End Met* 2009;5:45-54.
17. Burrow GN, Fisher DA, Larsen PR. Maternal and fetal thyroid function. *N Engl J Med* 1994;331:1072-8.
18. Calvo RM, Jauniaux E, Gulbis B, Asunción M, Gervy C, Contempré B, et al. Fetal tissues are exposed to biologically relevant free thyroxine concentrations during early phases of development. *J Clin Endocrinol Metab* 2002;87:1768-77.

19. Hennemann G, Docter R, Friesema ECH, Jong Md, Krenning EP, Visser TJ. Plasma membrane transport of thyroid hormones and its role in thyroid hormone metabolism and bioavailability. *Endocr Rev* 2001;22:451-76.
20. Morreale de Escobar G, Obregon MJ, Escobar del Rey F. Role of thyroid hormone during early brain development. *Eur J Endocrinol* 2004;151 Suppl 3:U25-37.
21. Koopdonk-Kool JM, de Vijlder JJ, Veenboer GJ, Ris-Stalpers C, Kok JH, Vulsmma T, et al. Type ii and type iii deiodinase activity in human placenta as a function of gestational age. *J Clin Endocrinol Metab* 1996;81:2154-8.
22. Maruo T, Matsuo H, Mochizuki M. Thyroid hormone as a biological amplifier of differentiated trophoblast function in early pregnancy. *Acta Endocrinol (Copenh)* 1991;125:58-66.
23. Millar LK, Wing DA, Leung AS, Koonings PP, Montoro MN, Mestman JH. Low birth weight and preeclampsia in pregnancies complicated by hyperthyroidism. *Obstet Gynecol* 1994;84:946-9.
24. Blazer S, Moreh-Waterman Y, Miller-Lotan R, Tamir A, Hochberg Z. Maternal hypothyroidism may affect fetal growth and neonatal thyroid function. *Obstet Gynecol* 2003;102:232-41.
25. Shields BM, Knight BA, Hill A, Hattersley AT, Vaidya B. Fetal thyroid hormone level at birth is associated with fetal growth. *J Clin Endocrinol Metab* 2011;96:E934-E38.
26. Medici M, Timmermans S, Visser W, de Muinck Keizer-Schrama SM, Jaddoe VW, Hofman A, et al. Maternal thyroid hormone parameters during early pregnancy and birth weight: The generation R study. *J Clin Endocrinol Metab* 2013;98:59-66.
27. Harper ME, Seifert EL. Thyroid hormone effects on mitochondrial energetics. *Thyroid* 2008;18:145-56.
28. Goglia F, Moreno M, Lanni A. Action of thyroid hormones at the cellular level: The mitochondrial target. *FEBS Lett* 1999;452:115-20.
29. Nemery B, Hoet PH, Nemmar A. The Meuse valley fog of 1930: An air pollution disaster. *Lancet* 2001;357:704-8.
30. Wilkins ET. Air pollution and the London fog of December, 1952. *Journal Royal Sanitary Institute (Great Britain)* 1954;74:1-15; discussion, 15-21.
31. Pedersen M, Giorgis-Allemand L, Bernard C, Aguilera I, Andersen A-MN, Ballester F, et al. Ambient air pollution and low birthweight: A European cohort study (ESCAPE). *Lancet Res Med* 2013;1:695-704.
32. Nawrot TS, Torfs R, Fierens F, De Henauw S, Hoet PH, Van Kersschaever G, et al. Stronger associations between daily mortality and fine particulate air pollution in Summer than in Winter: Evidence from a heavily polluted region in Western Europe. *J Epidemiol Community Health* 2007;61:146-49.
33. Deutsch F, Schrooten L, Broeckx S, Vankerkom J. Milieurapport Vlaanderen, achtergronddocument, verspreiding van zwevend stof. 2007:7-14.
34. World Health Organisation. Health effects of black carbon. By Janssen NAH, Gerlofs-Nijland ME, Lanki T, Salonen RO, Cassee F, Hoek G, Fischer P, Brunekreef B, Krzyzanowski M. 2012. [cited 1 April 2016]. Available from: http://www.euro.who.int/__data/assets/pdf_file/0004/162535/e96541.pdf.
35. Janssen NA, Lanki T, Hoek G, Vallius M, de Hartog JJ, Van Grieken R, et al. Associations between ambient, personal, and indoor exposure to fine particulate matter constituents in dutch and finnish panels of cardiovascular patients. *Occup Environ Med* 2005;62:868-77.

REFERENCES

36. Brook RD, Franklin B, Cascio W, Hong Y, Howard G, Lipsett M, et al. Air pollution and cardiovascular disease: A statement for healthcare professionals from the expert panel on population and prevention science of the american heart association. *Circulation* 2004;109:2655-71.
37. Brauer M, Avila-Casado C, Fortoul TI, Vedral S, Stevens B, Churg A. Air pollution and retained particles in the lung. *Environ Health Perspect* 2001;109:1039-43.
38. Kunihiko H, Jui Chih C, David N, Don S, Stephan FvE. Phagocytosis and clearance of particulate matter by lung macrophages: Effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors (statins). B34 lung macrophage activation in health and disease: American Thoracic Society, 2014:A2764-A64.
39. Oberdorster G, Sharp Z, Atudorei V, Elder A, Gelein R, Lunts A, et al. Extrapulmonary translocation of ultrafine carbon particles following whole-body inhalation exposure of rats. *J Toxicol Environ Health A* 2002;65:1531-43.
40. Nemmar A, Hoet PHM, Vanquickenborne B, Dinsdale D, Thomeer M, Hoylaerts MF, et al. Passage of inhaled particles into the blood circulation in humans. *Circulation* 2002;105:411-14.
41. Fierens F, Vanpoucke C, Adriaenssens S, Trimpeneers E, Peeters O, Dujardin J, et al. Ircel annual report air quality in Belgium (2014).
42. Brunekreef B, Holgate ST. Air pollution and health. *Lancet* 2002;360:1233-42.
43. Amann M, Bertok I, Cofala J, Gyrfas F, Heyes C, Klimont Z, et al. Baseline scenarios for the clean air for Europe (CAFE) programme 2005 [cited 31 March 2016]. Available from: http://ec.europa.eu/environment/archives/cafe/activities/pdf/cafe_scenario_report_1.pdf.
44. European Environment Agency. Air quality in Europe - 2015 report. 2015 [cited 1 April 2016]. Available from: <http://www.eea.europa.eu/publications/air-quality-in-europe-2015>.
45. Loomis D, Grosse Y, Lauby-Secretan B, Ghissassi FE, Bouvard V, Benbrahim-Tallaa L, et al. The carcinogenicity of outdoor air pollution. *Lancet Oncol* 2013;14:1262-63.
46. Li XY, Gilmour PS, Donaldson K, MacNee W. Free radical activity and pro-inflammatory effects of particulate air pollution (PM_{10}) *in vivo* and *in vitro*. *Thorax* 1996;51:1216-22.
47. Donaldson K, Stone V. Current hypotheses on the mechanisms of toxicity of ultrafine particles. *Ann Ist Super Sanita* 2003;39:405-10.
48. Donaldson K, Stone V, Seaton A, MacNee W. Ambient particle inhalation and the cardiovascular system: Potential mechanisms. *Environ Health Perspect* 2001;109:523-27.
49. Scheers H, Mwalili SM, Faes C, Fierens F, Nemery B, Nawrot TS. Does air pollution trigger infant mortality in Western Europe? A case-crossover study. *Environ Health Perspect* 2011;119:1017-22.
50. Rappazzo KM, Daniels JL, Messer LC, Poole C, Lobdell DT. Exposure to fine particulate matter during pregnancy and risk of preterm birth among women in New Jersey, Ohio, and Pennsylvania, 2000-2005. *Environ Health Perspect* 2014;122:992-7.
51. Barker DJP, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet* 1986;327:1077-81.
52. Made KR, Diederick EG, Michiel LB, Manuel Castro C, Lydia EV, Anath O, et al. Lower birth weight predicts metabolic syndrome in young adults: The atherosclerosis risk in young adults (ARYA)-study. *Atherosclerosis* 2006;184:21-27.

53. Barker DJ, Godfrey KM, Fall C, Osmond C, Winter PD, Shaheen SO. Relation of birth weight and childhood respiratory infection to adult lung function and death from chronic obstructive airways disease. *BMJ* 1991;303:671-5.
54. Cooper C, Javaid K, Westlake S, Harvey N, Dennison E. Developmental origins of osteoporotic fracture: The role of maternal vitamin D insufficiency. *J Nutr* 2005;135:2728S-34S.
55. Brunst KJ, Baccarelli AA, Wright RJ. Integrating mitochondriomics in children's environmental health. *J Appl Toxicol* 2015;35:976-91.
56. Waterland RA, Michels KB. Epigenetic epidemiology of the developmental origins hypothesis. *Annu Rev Nutr* 2007;27:363-88.
57. Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. *Science* 2001;293:1089-93.
58. Maunakea AK, Nagarajan RP, Bilenky M, Ballinger TJ, D'Souza C, Fouse SD, et al. Conserved role of intragenic DNA methylation in regulating alternative promoters. *Nature* 2010;466:253-7.
59. Barouki R, Gluckman P, Grandjean P, Hanson M, Heindel J. Developmental origins of non-communicable disease: Implications for research and public health. *Environ Health* 2012;11:42.
60. Jirtle RL, Skinner MK. Environmental epigenomics and disease susceptibility. *Nat Rev Gen* 2007;8:253-62.
61. Santos F, Dean W. Epigenetic reprogramming during early development in mammals. *Reproduction (Cambridge, England)* 2004;127:643-51.
62. Michels KB. *Epigenetic epidemiology*: Springer, 2012;
63. Sinclair KD, Allegrucci C, Singh R, Gardner DS, Sebastian S, Bispham J, et al. DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional b vitamin and methionine status. *Proc Natl Acad Sci USA* 2007;104:19351-56.
64. Dias BG, Ressler KJ. Parental olfactory experience influences behavior and neural structure in subsequent generations. *Nat Neurosci* 2014;17:89-96.
65. Anway MD, Cupp AS, Uzumcu M, Skinner MK. Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science* 2005;308:1466-9.
66. Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci USA* 2008;105:17046-49.
67. Belinsky SA, Snow SS, Nikula KJ, Finch GL, Tellez CS, Palmsano WA. Aberrant CpG island methylation of the p16ink4a and estrogen receptor genes in rat lung tumors induced by particulate carcinogens. *Carcinogenesis* 2002;23:335-39.
68. Madrigano J, Baccarelli A, Mittleman MA, Wright RO, Sparrow D, Vokonas PS, et al. Prolonged exposure to particulate pollution, genes associated with glutathione pathways, and DNA methylation in a cohort of older men. *Environ Health Perspect* 2011;119:977-82.
69. Baccarelli A, Wright RO, Bollati V, Tarantini L, Litonjua AA, Suh HH, et al. Rapid DNA methylation changes after exposure to traffic particles. *Am J Respir Crit Care Med* 2009;179:572-78.

REFERENCES

70. Hou L, Zhang X, Tarantini L, Nordio F, Bonzini M, Angelici L, et al. Ambient PM exposure and DNA methylation in tumor suppressor genes: A cross-sectional study. *Part Fibre Toxicol* 2011;8:25.
71. Fuke C, Shimabukuro M, Petronis A, Sugimoto J, Oda T, Miura K, et al. Age related changes in 5-methylcytosine content in human peripheral leukocytes and placentas: An HPLC-based study. *Ann Hum Genet* 2004;68:196-204.
72. Non AL, Binder AM, Barault L, Rancourt RC, Kubzansky LD, Michels KB. DNA methylation of stress-related genes and line-1 repetitive elements across the healthy human placenta. *Placenta* 2012;33:183-87.
73. Wilhelm-Benartzi CS, Houseman EA, Maccani MA, Poage GM, Koestler DC, Langevin SM, et al. *In utero* exposures, infant growth, and DNA methylation of repetitive elements and developmentally related genes in human placenta. *Environ Health Perspect* 2011;120:296-302.
74. Minocherhomji S, Tollefsbol TO, Singh KK. Mitochondrial regulation of epigenetics and its role in human diseases. *Epigenetics* 2012;7:326-34.
75. Smiraglia DJ, Kulawiec M, Bistulfi GL, Gupta SG, Singh KK. A novel role for mitochondria in regulating epigenetic modification in the nucleus. *Cancer Biol Ther* 2008;7:1182-90.
76. López-Lluch G, Irusta PM, Navas P, de Cabo R. Mitochondrial biogenesis and healthy aging. *Exp Gerontol* 2008;43:813-19.
77. Robin ED, Wong R. Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells. *J Cell Physiol* 1988;136:507-13.
78. Clay Montier LL, Deng JJ, Bai Y. Number matters: Control of mammalian mitochondrial DNA copy number. *J Genet Genom* 2009;36:125-31.
79. Taanman JW. The mitochondrial genome: Structure, transcription, translation and replication. *Biochim Biophys Acta Bioenergetics* 1999;1410:103-23.
80. Scarpulla RC. Nuclear control of respiratory chain expression by nuclear respiratory factors and pgc-1-related coactivator. *Ann N Y Acad Sci* 2008;1147:321-34.
81. Bonawitz ND, Clayton DA, Shadel GS. Initiation and beyond: Multiple functions of the human mitochondrial transcription machinery. *Mol Cell* 2006;24:813-25.
82. Payne BAI, Wilson IJ, Yu-Wai-Man P, Coxhead J, Deehan D, Horvath R, et al. Universal heteroplasmy of human mitochondrial DNA. *Hum Mol Genet* 2013;22:384-90.
83. Lee HC, Wei YH. Mitochondrial role in life and death of the cell. *J Biomed Sci* 2000;7:2-15.
84. Copeland WC. Defects in mitochondrial DNA replication and human disease. *Crit Rev Biochem Mol Biol* 2012;47:64-74.
85. Kelly RDW, Mahmud A, McKenzie M, Trounce IA, St John JC. Mitochondrial DNA copy number is regulated in a tissue specific manner by DNA methylation of the nuclear-encoded DNA polymerase gamma a. *Nucleic Acids Res* 2012;40:10124-38.
86. Sahin E, Colla S, Liesa M, Moslehi J, Muller FL, Guo M, et al. Telomere dysfunction induces metabolic and mitochondrial compromise. *Nature* 2011;470:359-65.
87. Wallace DC. Mitochondrial DNA mutations in disease and aging. *Environ Mol Mutagen* 2010;51:440-50.
88. Choi YS, Kim S, Pak YK. Mitochondrial transcription factor a (MTFA) and diabetes. *Diabetes Res Clin Pract* 2001;54:S3-S9.

89. Wong J, McLennan SV, Molyneaux L, Min D, Twigg SM, Yue DK. Mitochondrial DNA content in peripheral blood monocytes: Relationship with age of diabetes onset and diabetic complications. *Diabetologia* 2009;52:1953-61.
90. Gianotti TF, Sookoian S, Dieuzeide G, Garcia SI, Gemma C, Gonzalez CD, et al. A decreased mitochondrial DNA content is related to insulin resistance in adolescents. *Obesity* 2008;16:1591-5.
91. Xia P, An HX, Dang CX, Radpour R, Kohler C, Fokas E, et al. Decreased mitochondrial DNA content in blood samples of patients with stage I breast cancer. *BMC Cancer* 2009;9:454.
92. Yu M, Zhou Y, Shi Y, Ning L, Yang Y, Wei X, et al. Reduced mitochondrial DNA copy number is correlated with tumor progression and prognosis in Chinese breast cancer patients. *IUBMB Life* 2007;59:450-57.
93. Blokhin A, Vyshkina T, Komoly S, Kalman B. Variations in mitochondrial DNA copy numbers in ms brains. *J Mol Neurosci* 2008;35:283-87.
94. Xing J, Chen M, Wood CG, Lin J, Spitz MR, Ma J, et al. Mitochondrial DNA content: Its genetic heritability and association with renal cell carcinoma. *J Natl Cancer Inst* 2008;100:1104-12.
95. Dasgupta S, Yung RC, Westra WH, Rini DA, Brandes J, Sidransky D. Following mitochondrial footprints through a long mucosal path to lung cancer. *PLoS One* 2009;4:e6533.
96. Lynch SM, Weinstein SJ, Virtamo J, Lan Q, Liu CS, Cheng WL, et al. Mitochondrial DNA copy number and pancreatic cancer in the alpha-tocopherol beta-carotene cancer prevention study. *Cancer Prev Res* 2011;4:1912-19.
97. Lattuada D, Colleoni F, Martinelli A, Garretto A, Magni R, Radaelli T, et al. Higher mitochondrial DNA content in human IUGR placenta. *Placenta* 2008;29:1029-33.
98. Colleoni F, Lattuada D, Garretto A, Massari M, Mandò C, Somigliana E, et al. Maternal blood mitochondrial DNA content during normal and intrauterine growth restricted (IUGR) pregnancy. *Am J Obstet Gynecol* 2010;203:365.e1-65.e6.
99. Lin MT, Beal MF. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature* 2006;443:787-95.
100. Sun C, Reimers LL, Burk RD. Methylation of hpv16 genome CpG sites is associated with cervix precancer and cancer. *Gynecol Oncol* 2012;121:59-63.
101. Feng S, Xiong L, Ji Z, Cheng W, Yang H. Correlation between increased ND2 expression and demethylated displacement loop of mtDNA in colorectal cancer. *Mol Med Report* 2012;6:125-30.
102. Pirola CJ, Gianotti TF, Burgueño AL, Rey Funes M, Loidl CF, Mallardi P, et al. Epigenetic modification of liver mitochondrial DNA is associated with histological severity of nonalcoholic fatty liver disease. *Gut* 2013;62:1356-63.
103. Iacobazzi V, Castegna A, Infantino V, Andria G. Mitochondrial DNA methylation as a next-generation biomarker and diagnostic tool. *Mol Genet Metab* 2013;110:25-34.
104. Dzitoyeva S, Chen H, Manev H. Effect of aging on 5-hydroxymethylcytosine in brain mitochondria. *Neurobiol Aging* 2012;33:2881-91.
105. Shaughnessy DT, McAllister K, Worth L, Haugen AC, Meyer JN, Domann FE, et al. Mitochondria, energetics, epigenetics, and cellular responses to stress. *Environ Health Perspect* 2014;122:1271-78.
106. Meyer JN, Leung MC, Rooney JP, Sendoel A, Hengartner MO, Kisby GE, et al. Mitochondria as a target of environmental toxicants. *Toxicol Sci* 2013;134:1-17.

REFERENCES

107. Barker DJ. Fetal origins of coronary heart disease. *BMJ* 1995;311:171-4.
108. Gluckman PD, Hanson MA, Cooper C, Thornburg KL. Effect of *in utero* and early-life conditions on adult health and disease. *N Engl J Med* 2008;359:61-73.
109. Takayanagi Y, Petersen L, Laursen TM, Casella NG, Sawa A, Mortensen PB, et al. Risk of schizophrenia spectrum and affective disorders associated with small for gestational age birth and height in adulthood. *Schizophr Res* 2014;160:230-2.
110. Nosarti C, Reichenberg A, Murray RM, Cnattingius S, Lambe MP, Yin L, et al. Preterm birth and psychiatric disorders in young adult life. *Arch Gen Psychiatry* 2012;69:E1-8.
111. Cox B, Martens E, Nemery B, Vangronsveld J, Nawrot TS. Impact of a stepwise introduction of smoke-free legislation on the rate of preterm births: Analysis of routinely collected birth data. *BMJ* 2013;346:f441.
112. Cohen S, Kamarck T, Mermelstein R. A global measure of perceived stress. *J Health Soc Behav* 1983;24:385-96.
113. Claeys W, Baert K, Mestdagh F, Vercammen J, Daenens P, De Meulenaer B, et al. Assessment of the acrylamide intake of the belgian population and the effect of mitigation strategies. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* 2010;27:1199-207.
114. Hogervorst JG, Schouten LJ, Konings EJ, Goldbohm RA, van den Brandt PA. A prospective study of dietary acrylamide intake and the risk of endometrial, ovarian, and breast cancer. *Cancer Epidemiol Biomarkers Prev* 2007;16:2304-13.
115. Vannucci RC, Yager JY. Glucose, lactic acid, and perinatal hypoxic-ischemic brain damage. *Pediatr Neurol* 1992;8:3-12.
116. Beunza JJ, Toledo E, Hu FB, Bes-Rastrollo M, Serrano-Martinez M, Sanchez-Villegas A, et al. Adherence to the mediterranean diet, long-term weight change, and incident overweight or obesity: The seguimiento universidad de navarra (sun) cohort. *Am J Clin Nutr* 2010;92:1484-93.
117. Mendez MA, Popkin BM, Jakszyn P, Berenguer A, Tormo MJ, Sanchez MJ, et al. Adherence to a mediterranean diet is associated with reduced 3-year incidence of obesity. *J Nutr* 2006;136:2934-8.
118. Knoops KT, de Groot LC, Kromhout D, Perrin AE, Moreiras-Varela O, Menotti A, et al. Mediterranean diet, lifestyle factors, and 10-year mortality in elderly european men and women: The hale project. *JAMA* 2004;292:1433-9.
119. Martínez-González MA, García-Arellano A, Toledo E, Salas-Salvadó J, Buil-Cosiales P, Corella D, et al. A 14-item mediterranean diet assessment tool and obesity indexes among high-risk subjects: The predimed trial. *PLoS One* 2012;7:e43134.
120. Janssen S, Dumont G, Fierens F, Mensink C. Spatial interpolation of air pollution measurements using corine land cover data. *Atmos Environ* 2008;42:4884-903.
121. Lefebvre W, Degrawe B, Beckx C, Vanhulsel M, Kochan B, Bellemans T, et al. Presentation and evaluation of an integrated model chain to respond to traffic- and health-related policy questions. *Environ Model Softw* 2013;40:160-70.
122. Lefebvre W, Vercauteren J, Schrooten L, Janssen S, Degraeuwe B, Maenhaut W, et al. Validation of the mimosa-aurora-ifdm model chain for policy support: Modeling concentrations of elemental carbon in Flanders. *Atmos Environ* 2011;45:6705-13.

123. Maiheu B, Veldeman B, Viaene P, De Ridder K, Lauwaet D, Smeets N, et al. Identifying the best available large-scale concentration maps for air quality in Belgium. 67-99 2012 accessed on 9 mei 2016. Available from:
http://www.milieurapport.be/Upload/main/0_onderzoeksrapporten/2013/Eindrapport_Concentratiekaarten_29_01_2013_TW.pdf.
124. Vesper HW, Slimani N, Hallmans G, Tjonneland A, Agudo A, Benetou V, et al. Cross-sectional study on acrylamide hemoglobin adducts in subpopulations from the European prospective investigation into cancer and nutrition (EPIC) study. *J Agric Food Chem* 2008;56:6046-53.
125. Janssen BG, Byun HM, Cox B, Gyselaers W, Izzi B, Baccarelli AA, et al. Variation of DNA methylation in candidate age-related targets on the mitochondrial-telomere axis in cord blood and placenta. *Placenta* 2014;35:665-72.
126. Blackburn EH. Structure and function of telomeres. *Nature* 1991;350:569-73.
127. Cawthon RM. Telomere length measurement by a novel monochrome multiplex quantitative PCR method. *Nucleic Acids Res* 2009;37:e21.
128. Noble Y, Boyd R. Neonatal assessments for the preterm infant up to 4 months corrected age: A systematic review. *Dev Med Child Neurol* 2012;54:129-39.
129. Jaenisch R, Bird A. Epigenetic regulation of gene expression: How the genome integrates intrinsic and environmental signals. *Nat Genet* 2003;33:245-54.
130. Janssen BG, Godderis L, Pieters N, Poels K, Kiciński M, Cuypers A, et al. Placental DNA hypomethylation in association with particulate air pollution in early life. *Part Fibre Toxicol* 2013;10:22.
131. Saenen ND, Plusquin M, Bijnens E, Janssen BG, Gyselaers W, Cox B, et al. In utero fine particle air pollution and placental expression of genes in the brain-derived neurotrophic factor signaling pathway: An ENVIRONAGE birth cohort study. *Environ Health Perspect* 2015;123:834-40.
132. Bouhours-Nouet N, May-Panloup P, Coutant R, de Casson FB, Descamps P, Douay O, et al. Maternal smoking is associated with mitochondrial DNA depletion and respiratory chain complex iii deficiency in placenta. *Am J Physiol Endocrinol Metab* 2005;288:E171-7.
133. Janssen BG, Munters E, Pieters N, Smeets K, Cox B, Cuypers A, et al. Placental mitochondrial DNA content and particulate air pollution during *in utero* life. *Environ Health Perspect* 2012;120:1346-52.
134. Clemente DB, Casas M, Vilahur N, Begiristain H, Bustamante M, Carsin AE, et al. Prenatal ambient air pollution, placental mitochondrial DNA content, and birth weight in the INMA (Spain) and ENVIRONAGE (Belgium) birth cohorts. *Environ Health Perspect* 2016; 124:659-65.
135. Janssen BG, Byun H-M, Gyselaers W, Lefebvre W, Baccarelli AA, Nawrot TS. Placental mitochondrial methylation and exposure to airborne particulate matter in the early life environment: An ENVIRONAGE birth cohort study. *Epigenetics* 2015;10:536-44.
136. Grevendonk L, Janssen B, Vanpoucke C, Lefebvre W, Hoxha M, Bollati V, et al. Mitochondrial oxidative DNA damage and exposure to particulate air pollution in mother-newborn pairs. *Environ Health* 2016;15:10.
137. Kuller LH, Bracken MB, Ogino S, Prentice RL, Tracy RP. The role of epidemiology in the era of molecular epidemiology and genomics: Summary of the 2013 aje-sponsored society of epidemiologic research symposium. *Am J Epidemiol* 2013;178:1350-4.

REFERENCES

138. Vrijheid M, Slama R, Robinson O, Chatzi L, Coen M, van den Hazel P, et al. The human early-life exposome (HELIX): Project rationale and design. *Environ Health Perspect* 2014;122:535-44.
139. Constancia M, Hemberger M, Hughes J, Dean W, Ferguson-Smith A, Fundele R, et al. Placental-specific IGF2 is a major modulator of placental and fetal growth. *Nature* 2002;417:945-48.
140. Bourque DK, Avila L, Peñaherrera M, von Dadelszen P, Robinson WP. Decreased placental methylation at the H19/IGF2 imprinting control region is associated with normotensive intrauterine growth restriction but not preeclampsia. *Placenta* 2010;31:197-202.
141. Blair JD, Yuen RKC, Lim BK, McFadden DE, von Dadelszen P, Robinson WP. Widespread DNA hypomethylation at gene enhancer regions in placentas associated with early-onset pre-eclampsia. *Mol Hum Reprod* 2013;19:697-708.
142. Kulkarni A, Dangat K, Kale A, Sable P, Chavan-Gautam P, Joshi S. Effects of altered maternal folic acid, vitamin B12 and docosahexaenoic acid on placental global DNA methylation patterns in wistar rats. *PLoS One* 2011;6:e17706.
143. Grigoriu A, Ferreira JC, Choufani S, Baczyk D, Kingdom J, Weksberg R. Cell specific patterns of methylation in the human placenta. *Epigenetics* 2011;6:368-79.
144. Herzog E, Galvez J, Roks A, Stolk L, Verbiest M, Eilers P, et al. Tissue-specific DNA methylation profiles in newborns. *Clin Epigenetics* 2013;5:8.
145. Ollikainen M, Smith KR, Joo EJ-H, Ng HK, Andronikos R, Novakovic B, et al. DNA methylation analysis of multiple tissues from newborn twins reveals both genetic and intrauterine components to variation in the human neonatal epigenome. *Hum Mol Genet* 2010;19:4176-88.
146. Sahin E, Depinho RA. Linking functional decline of telomeres, mitochondria and stem cells during ageing. *Nature* 2010;464:520-8.
147. Palacios JA, Herranz D, De Bonis ML, Velasco S, Serrano M, Blasco MA. Sirt1 contributes to telomere maintenance and augments global homologous recombination. *J Cell Biol* 2010;191:1299-313.
148. Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, et al. Hsir2(sirt1) functions as an nad-dependent p53 deacetylase. *Cell* 2001;107:149-59.
149. Aquilano K, Vigilanza P, Baldelli S, Pagliei B, Rotilio G, Ciriolo MR. Peroxisome proliferator-activated receptor γ co-activator α (pgc-1α) and sirtuin 1 (sirt1) reside in mitochondria: Possible direct function in mitochondrial biogenesis. *J Biol Chem* 2010;285:21590-99.
150. Sahin E, DePinho RA. Axis of ageing: Telomeres, p53 and mitochondria. *Nat Rev Mol Cell Biol* 2012;13:397-404.
151. Lee HC, Wei YH. Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. *Int J Biochem Cell Biol* 2005;37:822-34.
152. Wieser F, Waite L, Depoix C, Taylor RN. PPAR action in human placental development and pregnancy and its complications. *PPAR Res* 2008;2008:527048.
153. Shock LS, Thakkar PV, Peterson EJ, Moran RG, Taylor SM. DNA methyltransferase 1, cytosine methylation, and cytosine hydroxymethylation in mammalian mitochondria. *Proc Natl Acad Sci USA* 2011;108:3630-5.
154. Aloni Y, Attardi G. Expression of the mitochondrial genome in hela cells. II. Evidence for complete transcription of mitochondrial DNA. *J Mol Biol* 1971;55:251-67.

155. Blasco MA. The epigenetic regulation of mammalian telomeres. *Nat Rev Gen* 2007;8:299-309.
156. Gonzalo S, Jaco I, Fraga MF, Chen T, Li E, Esteller M, et al. DNA methyltransferases control telomere length and telomere recombination in mammalian cells. *Nat Cell Biol* 2006;8:416-24.
157. Benetti R, Garcia-Cao M, Blasco MA. Telomere length regulates the epigenetic status of mammalian telomeres and subtelomeres. *Nat Genet* 2007;39:243-50.
158. Vera E, Canela A, Fraga MF, Esteller M, Blasco MA. Epigenetic regulation of telomeres in human cancer. *Oncogene* 2008;27:6817-33.
159. Gadalla SM, Katki HA, Shebl FM, Giri N, Alter BP, Savage SA. The relationship between DNA methylation and telomere length in dyskeratosis congenita. *Aging Cell* 2012;11:24-28.
160. Boekelheide K, Blumberg B, Chapin RE, Cote I, Graziano JH, Janesick A, et al. Predicting later-life outcomes of early-life exposures. *Environ Health Perspect* 2012;120:1353-61.
161. Okuda K, Bardeguéz A, Gardner JP, Rodriguez P, Ganesh V, Kimura M, et al. Telomere length in the newborn. *Pediatr Res* 2002;52:377-81.
162. Avila L, Yuen RK, Diego-Alvarez D, Peñaherrera MS, Jiang R, Robinson WP. Evaluating DNA methylation and gene expression variability in the human term placenta. *Placenta* 2010;31:1070-77.
163. Vilahur N, Baccarelli AA, Bustamante M, Agramunt S, Byun HM, Fernandez MF, et al. Storage conditions and stability of global DNA methylation in placental tissue. *Epigenomics* 2013;5:341-8.
164. Hogg K, Price EM, Robinson WP. Improved reporting of DNA methylation data derived from studies of the human placenta. *Epigenetics* 2014;9:333 - 37.
165. Adibi JJ, Hauser R, Williams PL, Whyatt RM, Thaker HM, Nelson H, et al. Placental biomarkers of phthalate effects on mRNA transcription: Application in epidemiologic research. *Environ Health* 2009;8:20.
166. Byun HM, Panni T, Motta V, Hou L, Nordio F, Apostoli P, et al. Effects of airborne pollutants on mitochondrial DNA methylation. *Part Fibre Toxicol* 2013;10:18.
167. Choi SH, Worswick S, Byun HM, Shear T, Soussa JC, Wolff EM, et al. Changes in DNA methylation of tandem DNA repeats are different from interspersed repeats in cancer. *Int J Cancer* 2009;125:723-9.
168. Nomura Y, Lambertini L, Rialdi A, Lee M, Mystal EY, Grabie M, et al. Global methylation in the placenta and umbilical cord blood from pregnancies with maternal gestational diabetes, preeclampsia, and obesity. *Reprod Sci* 2013;00:1-7.
169. Wang ND, Finegold MJ, Bradley A, Ou CN, Abdelsayed SV, Wilde MD, et al. Impaired energy homeostasis in c/ebp alpha knockout mice. *Science* 1995;269:1108-12.
170. Michels KB, Binder AM, Dedeurwaerder S, Epstein CB, Greally JM, Gut I, et al. Recommendations for the design and analysis of epigenome-wide association studies. *Nat Meth* 2013;10:949-55.
171. Bollati V, Baccarelli A, Hou L, Bonzini M, Fustinoni S, Cavallo D, et al. Changes in DNA methylation patterns in subjects exposed to low-dose benzene. *Cancer Res* 2007;67:876-80.
172. Joubert BR, Haberg SE, Nilsen RM, Wang X, Vollset SE, Murphy SK, et al. 450k epigenome-wide scan identifies differential DNA methylation in newborns related to maternal smoking during pregnancy. *Environ Health Perspect* 2012;120:1425-31.

REFERENCES

173. Kile ML, Baccarelli A, Hoffman E, Tarantini L, Quamruzzaman Q, Rahman M, et al. Prenatal arsenic exposure and DNA methylation in maternal and umbilical cord blood leukocytes. *Environ Health Perspect* 2012;120:1061-66.
174. Tang WY, Levin L, Talaska G, Cheung YY, Herbstman J, Tang D, et al. Maternal exposure to polycyclic aromatic hydrocarbons and 5'-CpG methylation of interferon-gamma in cord white blood cells. *Environ Health Perspect* 2012;120:1195-200.
175. Izzi B, Francois I, Labarque V, Thys C, Wittevrongel C, Devriendt K, et al. Methylation defect in imprinted genes detected in patients with an Albright's hereditary osteodystrophy like phenotype and platelet gp hypofunction. *PLoS One* 2012;7:e38579.
176. Xu J, Pope SD, Jazirehi AR, Attema JL, Papathanasiou P, Watts JA, et al. Pioneer factor interactions and unmethylated CpG dinucleotides mark silent tissue-specific enhancers in embryonic stem cells. *Proc Natl Acad Sci USA* 2007;104:12377-82.
177. Armstrong DA, Lesseur C, Conradt E, Lester BM, Marsit CJ. Global and gene-specific DNA methylation across multiple tissues in early infancy: Implications for children's health research. *The FASEB Journal* 2014;28:2088-97.
178. Youngren K, Jeanclos E, Aviv H, Kimura M, Stock J, Hanna M, et al. Synchrony in telomere length of the human fetus. *Hum Genet* 1998;102:640-3.
179. Yuen RK, Avila L, Peñaherrera MS, von Dadelszen P, Lefebvre L, Kobor MS, et al. Human placental-specific epipolymorphism and its association with adverse pregnancy outcomes. *PLoS One* 2009;4:e7389.
180. Windham GC, Hopkins B, Fenster L, Swan SH. Prenatal active or passive tobacco smoke exposure and the risk of preterm delivery or low birth weight. *Epidemiology* 2000;11:427-33.
181. Jaddoe VWV, Troe E-JWM, Hofman A, Mackenbach JP, Moll HA, Steegers EAP, et al. Active and passive maternal smoking during pregnancy and the risks of low birthweight and preterm birth: The generation R study. *Paediatr Perinat Epidemiol* 2008;22:162-71.
182. Fantuzzi G, Aggazzotti G, Righi E, Facchinetto F, Bertucci E, Kanitz S, et al. Preterm delivery and exposure to active and passive smoking during pregnancy: A case-control study from Italy. *Paediatr Perinat Epidemiol* 2007;21:194-200.
183. Jauniaux E, Burton GJ. Morphological and biological effects of maternal exposure to tobacco smoke on the feto-placental unit. *Early Hum Dev* 2007;83:699-706.
184. U.S. Department of Health and Human Service. How tobacco smoke causes disease—the biology and behavioral basis for tobacco-attributable disease: A report of the surgeon general. 2010. Available from: http://www.surgeongeneral.gov/library/reports/tobaccosmoke/full_report.pdf.
185. Garrabou G, Hernandez AS, Catalan Garcia M, Moren C, Tobias E, Cordoba S, et al. Molecular basis of reduced birth weight in smoking pregnant women: Mitochondrial dysfunction and apoptosis. *Addict Biol* 2014.
186. Lee HC, Lu CY, Fahn HJ, Wei YH. Aging- and smoking-associated alteration in the relative content of mitochondrial DNA in human lung. *FEBS Lett* 1998;441:292-96.
187. Vaiserman A. Epidemiologic evidence for association between adverse environmental exposures in early life and epigenetic variation: A potential link to disease susceptibility? *Clin Epigenetics* 2015;7:96.
188. Ivorra C, Fraga M, Bayon G, Fernandez A, Garcia-Vicent C, Chaves F, et al. DNA methylation patterns in newborns exposed to tobacco in utero. *J Transl Med* 2015;13:25.

189. Michels KB, Harris HR, Barault L. Birthweight, maternal weight trajectories and global DNA methylation of line-1 repetitive elements. *PLoS One* 2011;6:e25254.
190. Guerrero-Preston R, Goldman LR, Brebi-Mieville P, Ili-Gangas C, Lebron C, Witter FR, et al. Global DNA hypomethylation is associated with *in utero* exposure to cotinine and perfluorinated alkyl compounds. *Epigenetics* 2010;5:539-46.
191. Suter M, Abramovici A, Showalter L, Hu M, Shope CD, Varner M, et al. *In utero* tobacco exposure epigenetically modifies placental CYP1A1 expression. *Metabolism* 2010;59:1481-90.
192. Bouwland-Both M, van Mil N, Tolhoek C, Stolk L, Eilers P, Verbiest M, et al. Prenatal parental tobacco smoking, gene specific DNA methylation, and newborns size: The generation R study. *Clin Epigenetics* 2015;7:83.
193. Novakovic B, Ryan J, Pereira N, Boughton B, Craig JM, Saffery R. Postnatal stability, tissue, and time specific effects of ahrr methylation change in response to maternal smoking in pregnancy. *Epigenetics* 2014;9:377-86.
194. Murphy SK, Adigun A, Huang Z, Overcash F, Wang F, Jirtle RL, et al. Gender-specific methylation differences in relation to prenatal exposure to cigarette smoke. *Gene* 2012;494:36-43.
195. Küpers LK, Xu X, Jankipersadsing SA, Vaez A, la Bastide-van Gemert S, Scholtens S, et al. DNA methylation mediates the effect of maternal smoking during pregnancy on birthweight of the offspring. *Int J Epidemiol* 2015.
196. Markunas CA, Xu Z, Harlid S, Wade PA, Lie RT, Taylor JA, et al. Identification of DNA methylation changes in newborns related to maternal smoking during pregnancy. *Environ Health Perspect* 2014;122:1147-53.
197. Suter M, Ma J, Harris AS, Patterson L, Brown KA, Shope C, et al. Maternal tobacco use modestly alters correlated epigenome-wide placental DNA methylation and gene expression. *Epigenetics* 2011;6:1284-94.
198. Januar V, Desoye G, Novakovic B, Cvitic S, Saffery R. Epigenetic regulation of human placental function and pregnancy outcome: Considerations for causal inference. *Am J Obstet Gynecol* 2015;213:S182-S96.
199. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. Qbase relative quantification framework and software for management and automated analysis of real-time quantitative pcr data. *Genome Biol* 2007;8:R19.
200. Valeri L, Vanderweele TJ. Mediation analysis allowing for exposure-mediator interactions and causal interpretation: Theoretical assumptions and implementation with SAS and SPSS macros. *Psychol Methods* 2013;18:137-50.
201. Linnane AW, Marzuki S, Ozawa T, Tanaka M. Mitochondrial DNA mutations as an important contributor to ageing and degenerative diseases. *Lancet* 1989;1:642-5.
202. Ding WX, Yin XM. Mitophagy: Mechanisms, pathophysiological roles, and analysis. *Biol Chem* 2012;393:547-64.
203. Wadzinski TL, Geromini K, McKinley Brewer J, Bansal R, Abdelouahab N, Langlois MF, et al. Endocrine disruption in human placenta: Expression of the dioxin-inducible enzyme, CYP1A1, is correlated with that of thyroid hormone-regulated genes. *J Clin Endocrinol Metab* 2014;99:E2735-43.

REFERENCES

204. Sbrana E, Suter MA, Abramovici AR, Hawkins HK, Moss JE, Patterson L, et al. Maternal tobacco use is associated with increased markers of oxidative stress in the placenta. *Am J Obstet Gynecol* 2011;205:246.e1-7.
205. Harper PA, Riddick DS, Okey AB. Regulating the regulator: Factors that control levels and activity of the aryl hydrocarbon receptor. *Biochem Pharmacol* 2006;72:267-79.
206. Ballester F, Estarlich M, Iniguez C, Llop S, Ramon R, Esplugues A, et al. Air pollution exposure during pregnancy and reduced birth size: A prospective birth cohort study in Valencia, Spain. *Environ Health* 2010;9:6.
207. Dadvand P, Parker J, Bell ML, Bonzini M, Brauer M, Darrow LA, et al. Maternal exposure to particulate air pollution and term birth weight: A multi-country evaluation of effect and heterogeneity. *Environ Health Perspect* 2013;121:267-373.
208. Ritz B, Wilhelm M, Zhao Y. Air pollution and infant death in Southern California, 1989-2000. *Pediatrics* 2006;118:493-502.
209. Brook RD, Rajagopalan S, Pope CA, Brook JR, Bhatnagar A, Diez-Roux AV, et al. Particulate matter air pollution and cardiovascular disease: An update to the scientific statement from the american heart association. *Circulation* 2010;121:2331-78.
210. Grahame T, Schlesinger R. Oxidative stress-induced telomeric erosion as a mechanism underlying airborne particulate matter-related cardiovascular disease. *Part Fibre Toxicol* 2012;9:21.
211. Koukoura O, Sifakis S, Spandidos DA. DNA methylation in the human placenta and fetal growth (review). *Mol Med Report* 2012;5:883-9.
212. Terry MB, Delgado-Cruzata L, Vin-Raviv N, Wu HC, Santella RM. DNA methylation in white blood cells: Association with risk factors in epidemiologic studies. *Epigenetics* 2011;6:828-37.
213. Jacobs L, Emmerechts J, Mathieu C, Hoylaerts MF, Fierens F, Hoet PH, et al. Air pollution related prothrombotic changes in persons with diabetes. *Environ Health Perspect* 2010;118:191-96.
214. Kalkstein LS, Valimont KM. An evaluation of summer discomfort in the united states using a relative climatological index. *Bulletin of the American Meteorological Society* 1986;7:842-48.
215. Steadman RG. The assessment of sultriness. Part i: A temperature-humidity index based on human physiology and clothing science. *J Clim Appl Meteor* 1979;18:861-73.
216. Tabish AM, Poels K, Hoet P, Godderis L. Epigenetic factors in cancer risk: Effect of chemical carcinogens on global DNA methylation pattern in human TK6 cells. *PLoS One* 2012;7:e34674.
217. Godderis L, De Raedt K, Tabish AM, Poels K, Maertens N, De Ruyck K, et al. Epigenetic changes in lymphocytes of solvent-exposed individuals. *Epigenomics* 2012;4:269-77.
218. Quinlivan EP, Gregory III JF. DNA digestion to deoxyribonucleoside: A simplified one-step procedure. *Anal Biochem* 2008;373:383-85.
219. Harrell FE. Restricted cubic splines. Regression modeling strategies: With applications to linear models, logistic regression, and survival analysis. New York: Springer, 2001:16-26.
220. Perera F, Tang WY, Herbstman J, Tang D, Levin L, Miller R, et al. Relation of DNA methylation of 5'-CpG island of ASCL3 to transplacental exposure to airborne polycyclic aromatic hydrocarbons and childhood asthma. *PLoS One* 2009;4:4488.
221. Li S, Hursting SD, Davis BJ, McLachlan JA, Barrett JC. Environmental exposure, DNA methylation, and gene regulation. *Ann NY Acad Sci* 2003;983:161-69.

222. Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, et al. Induction of tumors in mice by genomic hypomethylation. *Science* 2003;300:489-92.
223. Lengauer C, Kinzler KW, Vogelstein B. DNA methylation and genetic instability in colorectal cancer cells. *Proc Natl Acad Sci USA* 1977;94:2545-50.
224. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R. DNA hypomethylation leads to elevated mutation rates. *Nature* 1998;395:89-93.
225. Yin LJ, Zhang Y, Lv PP, He WH, Wu YT, Liu AX, et al. Insufficient maintenance DNA methylation is associated with abnormal embryonic development. *BMC Med* 2012;10:26.
226. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases dnmt3a and dnmt3b are essential for de novo methylation and mammalian development. *Cell* 1999;99:247-57.
227. Valinluck V, Tsai HH, Rogstad DK, Burdzy A, Bird A, Sowers LC. Oxidative damage to methyl-CpG sequences inhibits the binding of the methyl-CpG binding domain (mbd) of methyl-CpG binding protein 2 (meCP2). *Nucleic Acids Res* 2004;32:4100-08.
228. Adibi JJ, Whyatt RM, Hauser R, Bhat HK, Davis BJ, Calafat AM, et al. Transcriptional biomarkers of steroidogenesis and trophoblast differentiation in the placenta in relation to prenatal phthalate exposure. *Environ Health Perspect* 2010;118:291-6.
229. van den Hooven EH, Pierik FH, de Kluizenaar Y, Hofman A, van Ratingen SW, Zandveld PY, et al. Air pollution exposure and markers of placental growth and function: The generation R study. *Environ Health Perspect* 2012;120:1753-9.
230. Lodovici M, Bigagli E. Oxidative stress and air pollution exposure. *J Toxicol* 2011;2011:487074.
231. Xu Z, Xu X, Zhong M, Hotchkiss I, Lewandowski R, Wagner J, et al. Ambient particulate air pollution induces oxidative stress and alterations of mitochondria and gene expression in brown and white adipose tissues. *Part Fibre Toxicol* 2011;8:20.
232. Nemmar A, Hoylaerts MF, Hoet PHM, Nemery B. Possible mechanisms of the cardiovascular effects of inhaled particles: Systemic translocation and prothrombotic effects. *Toxicol Lett* 2004;149:243-53.
233. Ventolini G. Conditions associated with placental dysfunction. *Minerva Ginecol* 2011;63:459-64.
234. Pinto R, Ivaldi C, Reyes M, Doyen Cc, Mietton F, Mongelard F, et al. Seasonal environmental changes regulate the expression of the histone variant macroH2a in an eurythermal fish. *FEBS Lett* 2005;579:5553-58.
235. Song L, James SR, Kazim L, Karpf AR. Specific method for the determination of genomic DNA methylation by liquid chromatography-electrospray ionization tandem mass spectrometry. *Anal Chem* 2005;77:504-10.
236. Yuen RK, Peñaherrera MS, von Dadelszen P, McFadden DE, Robinson WP. DNA methylation profiling of human placentas reveals promoter hypomethylation of multiple genes in early-onset preeclampsia. *Eur J Hum Genet* 2010;18:1006-12.
237. Frank D, Fortino W, Clark L, Musalo R, Wang W, Saxena A, et al. Placental overgrowth in mice lacking the imprinted gene ipl. *Proc Natl Acad Sci USA* 2002;99:7490-5.
238. Godfrey KM. The role of the placenta in fetal programming: A review. *Placenta* 2002;23:S20-S27.
239. Dejmek J, Selevan SG, Benes I, Solansky I, Sram RJ. Fetal growth and maternal exposure to particulate matter during pregnancy. *Environ Health Perspect* 1999;107:475-80.

REFERENCES

240. Gemma C, Sookoian S, Alvarinas J, Garcia SI, Quintana L, Kanevsky D, et al. Mitochondrial DNA depletion in small- and large-for-gestational-age newborns. *Obesity* 2006;14:2193-99.
241. Glinianaia SV, Rankin J, Bell R, Pless-Mulloli T, Howel D. Particulate air pollution and fetal health: A systematic review of the epidemiologic evidence. *Epidemiology* 2004;15:36-45.
242. Kannan S, Misra DP, Dvonch JT, Krishnakumar A. Exposures to airborne particulate matter and adverse perinatal outcomes: A biologically plausible mechanistic framework for exploring potential effect modification by nutrition. *Environ Health Perspect* 2006;114:1636-42.
243. Morello-Frosch R, Jesdale BM, Sadd JL, Pastor M. Ambient air pollution exposure and full-term birth weight in California. *Environ Health* 2010;9:44.
244. Veras MM, Damaceno-Rodrigues NR, Caldini EG, Maciel Ribeiro AA, Mayhew TM, Saldiva PH, et al. Particulate urban air pollution affects the functional morphology of mouse placenta. *Biol Reprod* 2008;79:578-84.
245. Chahine T, Baccarelli A, Litonjua A, Wright RO, Suh H, Gold DR, et al. Particulate air pollution, oxidative stress genes, and heart rate variability in an elderly cohort. *Environ Health Perspect* 2007;115:1617-22.
246. Salvi S, Blomberg A, Rudell B, Kelly F, Sandstrom T, Holgate ST, et al. Acute inflammatory responses in the airways and peripheral blood after short-term exposure to diesel exhaust in healthy human volunteers. *Am J Respir Crit Care Med* 1999;159:702-09.
247. Hou L, Zhu Z-Z, Zhang X, Nordio F, Bonzini M, Schwartz J, et al. Airborne particulate matter and mitochondrial damage: A cross-sectional study. *Environ Health* 2010;9:48.
248. Geelhoed JJ, Jaddoe VW. Early influences on cardiovascular and renal development. *Eur J Epidemiol* 2010;25:677-92.
249. Hoek G, Brunekreef B, Goldbohm S, Fischer P, van den Brandt PA. Association between mortality and indicators of traffic-related air pollution in the Netherlands: A cohort study. *Lancet* 2002;360:1203-9.
250. Li N, Sioutas C, Cho A, Schmitz D, Misra C, Sempf J, et al. Ultrafine particulate pollutants induce oxidative stress and mitochondrial damage. *Environ Health Perspect* 2003;111.
251. Li Z, Hyseni X, Carter JD, Soukup JM, Dailey LA, Huang YC. Pollutant particles enhanced H₂O₂ production from NAD(P)H oxidase and mitochondria in human pulmonary artery endothelial cells. *Am J Physiol Cell Physiol* 2006;291:C357-65.
252. Peretz A, Sullivan JH, Leotta DF, Trenga CA, Sands FN, Allen J, et al. Diesel exhaust inhalation elicits acute vasoconstriction in vivo. *Environ Health Perspect* 2008;116:937-42.
253. Larsen LG, Clausen HV, Jonsson L. Stereologic examination of placentas from mothers who smoke during pregnancy. *Am J Obstet Gynecol* 2002;186:531-7.
254. Geelhoed JJ, El Marroun H, Verburg BO, van Osch-Gevers L, Hofman A, Huizink AC, et al. Maternal smoking during pregnancy, fetal arterial resistance adaptations and cardiovascular function in childhood. *BJOG* 2011;118:755-62.
255. Andreu AL, Martinez R, Marti R, Garcia-Arumi E. Quantification of mitochondrial DNA copy number: Pre-analytical factors. *Mitochondrion* 2009;9:242-6.
256. Gehring U, Wijga AH, Fischer P, de Jongste JC, Kerkhof M, Koppelman GH, et al. Traffic-related air pollution, preterm birth and term birth weight in the piama birth cohort study. *Environ Res* 2011;111:125-35.

257. Parker JD, Rich DQ, Glinianaia SV, Leem JH, Wartenberg D, Bell ML, et al. The international collaboration on air pollution and pregnancy outcomes: Initial results. *Environ Health Perspect* 2011;119:1023-8.
258. Carugno M, Pesatori AC, Dionis L, Hoxha M, Bollati V, Aliberti B, et al. Increased mitochondrial DNA copy number in occupations associated with low-dose benzene exposure. *Environ Health Perspect* 2012;120.
259. Banas B, Kost BP, Goebel FD. Platelets, a typical source of error in real-time pcr quantification of mitochondrial DNA content in human peripheral blood cells. *Eur J Med Res* 2004;9:371-7.
260. Cossarizza A, Riva A, Pinti M, Ammannato S, Fedeli P, Mussini C, et al. Increased mitochondrial DNA content in peripheral blood lymphocytes from hiv-infected patients with lipodystrophy. *Antivir Ther* 2003;8:315-21.
261. Jalaludin B, Mannes T, Morgan G, Lincoln D, Sheppard V, Corbett S. Impact of ambient air pollution on gestational age is modified by season in Sydney, Australia. *Environ Health* 2007;6:16.
262. Collins ML, Eng S, Hoh R, Hellerstein MK. Measurement of mitochondrial DNA synthesis in vivo using a stable isotope-mass spectrometric technique. *J Appl Physiol* 2003;94:2203-11.
263. Gross NJ, Getz GS, Rabinowitz M. Apparent turnover of mitochondrial deoxyribonucleic acid and mitochondrial phospholipids in the tissues of the rat. *J Biol Chem* 1969;244:1552-62.
264. Wenz T. Mitochondria and pgc-1 α in aging and age-associated diseases. *J Aging Res* 2011;2011:810619.
265. Chistiakov DA, Sobenin IA, Revin VV, Orekhov AN, Bobryshev YV. Mitochondrial aging and age-related dysfunction of mitochondria. *BioMed Research International* 2014;2014:Article ID 238463.
266. Stern S, Adiseshaiah P, Crist R. Autophagy and lysosomal dysfunction as emerging mechanisms of nanomaterial toxicity. Part *Fibre Toxicol* 2012;9:20.
267. Hong EE, Okitsu CY, Smith AD, Hsieh CL. Regionally specific and genome-wide analyses conclusively demonstrate the absence of cpg methylation in human mitochondrial DNA. *Mol Cell Biol* 2013;33:2683-90.
268. Maekawa M, Taniguchi T, Higashi H, Sugimura H, Sugano K, Kanno T. Methylation of mitochondrial DNA is not a useful marker for cancer detection. *Clin Chem* 2004;50:1480-81.
269. Bellizzi D, D'Aquila P, Scafone T, Giordano M, Riso V, Riccio A, et al. The control region of mitochondrial DNA shows an unusual cpg and non-cpg methylation pattern. *DNA Res* 2013;20:537-47.
270. Metodiev MD, Lesko N, Park CB, Cámarra Y, Shi Y, Wibom R, et al. Methylation of 12s rRNA is necessary for in vivo stability of the small subunit of the mammalian mitochondrial ribosome. *Cell Metab* 2009;9:386-97.
271. O'Sullivan M, Rutland P, Lucas D, Ashton E, Hendricks S, Rahman S, et al. Mitochondrial m.1584a 12s m62a rRNA methylation in families with m.1555a>g associated hearing loss. *Hum Mol Genet* 2015;24:1036-44.
272. Rebleo AP, Williams SL, Moraes CT. In vivo methylation of mtDNA reveals the dynamics of protein-mtDNA interactions. *Nucleic Acids Res* 2009;37:6701-15.
273. Methylation of the human mitochondrial 12s rRNA gene is correlated with aging. 12th FISV Congress; 2012; Rome, Italy.

REFERENCES

274. Cassano P, Sciancalepore AG, Lezza AM, Leeuwenburgh C, Cantatore P, Gadaleta MN. Tissue-specific effect of age and caloric restriction diet on mitochondrial DNA content. *Rejuvenation Res* 2006;9:211-4.
275. Chinnery PF, Elliott HR, Hudson G, Samuels DC, Relton CL. Epigenetics, epidemiology and mitochondrial DNA diseases. *Int J Epidemiol* 2012;41:177-87.
276. Lee W, Johnson J, Gough DJ, Donoghue J, Cagnone GLM, Vaghjiani V, et al. Mitochondrial DNA copy number is regulated by DNA methylation and demethylation of POLGA in stem and cancer cells and their differentiated progeny. *Cell Death Dis* 2015;6:e1664.
277. Strong N, Lambertini L, Ma Y, Stone J. Differential mitochondrial DNA methylation in growth restricted placentas. *Am J Obstet Gynecol* 2013;208:S192.
278. Cheng SY, Leonard JL, Davis PJ. Molecular aspects of thyroid hormone actions. *Endocr Rev* 2010;31:139-70.
279. Ventura-Clapier R, Garnier A, Veksler V. Transcriptional control of mitochondrial biogenesis: The central role of pgc-1α. *Cardiovasc Res* 2008;79:208-17.
280. Thorpe-Beeston JG, Nicolaides KH, Felton CV, Butler J, McGregor AM. Maturation of the secretion of thyroid hormone and thyroid-stimulating hormone in the fetus. *N Engl J Med* 1991;324:532-36.
281. Weitzel JM, Radtke C, Seitz HJ. Two thyroid hormone-mediated gene expression patterns in vivo identified by cdna expression arrays in rat. *Nucleic Acids Res* 2001;29:5148-55.
282. Hiroi Y, Kim HH, Ying H, Furuya F, Huang Z, Simoncini T, et al. Rapid nongenomic actions of thyroid hormone. *Proc Natl Acad Sci USA* 2006;103:14104-9.
283. Wrutniak-Cabello C, Casas F, Cabello G. Thyroid hormone action in mitochondria. *J Mol Endocrinol* 2001;26:67-77.
284. Casas F, Rochard P, Rodier A, Cassar-Malek I, Marchal-Victorion S, Wiesner RJ, et al. A variant form of the nuclear triiodothyronine receptor c-erbaalpha1 plays a direct role in regulation of mitochondrial RNA synthesis. *Mol Cell Biol* 1999;19:7913-24.
285. Barrès R, Osler ME, Yan J, Rune A, Fritz T, Caidahl K, et al. Non-CpG methylation of the pgc-1α promoter through DNMT3b controls mitochondrial density. *Cell Metab* 2009;10:189-98.
286. Abdelouahab N, Langlois MF, Lavoie L, Corbin F, Pasquier JC, Takser L. Maternal and cord blood thyroid hormone levels and exposure to polybrominated diphenyl ethers and polychlorinated biphenyls during early pregnancy. *Am J Epidemiol* 2013;178:701-13.
287. Maervoet J, Vermeir G, Covaci A, Van Larebeke N, Koppen G, Schoeters G, et al. Association of thyroid hormone concentrations with levels of organochlorine compounds in cord blood of neonates. *Environ Health Perspect* 2007;115:1780-6.
288. Baccarelli A, Giacomini SM, Corbetta C, Landi MT, Bonzini M, Consonni D, et al. Neonatal thyroid function in Seveso 25 years after maternal exposure to dioxin. *PLoS Med* 2008;5:e161.
289. Iijima K, Otake T, Yoshinaga J, Ikegami M, Suzuki E, Naruse H, et al. Cadmium, lead, and selenium in cord blood and thyroid hormone status of newborns. *Biol Trace Elem Res* 2007;119:10-18.
290. Soldin OP, Goughenour BE, Gilbert SZ, Landy HJ, Soldin SJ. Thyroid hormone levels associated with active and passive cigarette smoking. *Thyroid* 2009;19:817-23.

291. Sunyer J, Esnaola M, Alvarez-Pedrerol M, Forns J, Rivas I, Lopez-Vicente M, et al. Association between traffic-related air pollution in schools and cognitive development in primary school children: A prospective cohort study. *PLoS Med* 2015;12:e1001792.
292. Reed HL. Circannual changes in thyroid hormone physiology: The role of cold environmental temperatures. *Arctic Med Res* 1995;54 Suppl 2:9-15.
293. Herbstman J, Apelberg BJ, Witter FR, Panny S, Goldman LR. Maternal, infant, and delivery factors associated with neonatal thyroid hormone status. *Thyroid* 2008;18:67-76.
294. Lv PP, Meng Y, Lv M, Feng C, Liu Y, Li JY, et al. Altered thyroid hormone profile in offspring after exposure to high estradiol environment during the first trimester of pregnancy: A cross-sectional study. *BMC Med* 2014;12:240.
295. Chan LYS, Leung TN, Lau TK. Influences of perinatal factors on cord blood thyroid-stimulating hormone level. *Acta Obstet Gynecol Scand* 2001;80:1014-18.
296. Parate V, Rode M, Pande S, Ansari T, Kamble P. Thyroid function in mothers during the process of normal delivery. *Int J Endocrinol Metab* 2010;8:39-45.
297. Yoshimura Noh J, Momotani N, Fukada S, Ito K, Miyauchi A, Amino N. Ratio of serum free triiodothyronine to free thyroxine in Graves' hyperthyroidism and thyrotoxicosis caused by painless thyroiditis. *Endocr J* 2005;52:537-42.
298. Vulsmo T, Gons MH, de Vijlder JJM. Maternal-fetal transfer of thyroxine in congenital hypothyroidism due to a total organification defect or thyroid agenesis. *N Engl J Med* 1989;321:13-16.
299. Korevaar TI, Chaker L, Jaddoe VW, Visser TJ, Medici M, Peeters RP. Maternal and birth characteristics are determinants of offspring thyroid function. *J Clin Endocrinol Metab* 2015;jc20153559.
300. León G, Murcia M, Rebagliato M, Álvarez-Pedrerol M, Castilla AM, Basterrechea M, et al. Maternal thyroid dysfunction during gestation, preterm delivery, and birthweight. The INFancia y Medio Ambiente cohort, Spain. *Paediatr Perinat Epidemiol* 2015;29:113-22.
301. Bassols J, Prats-Puig A, Soriano-Rodríguez P, García-González MM, Reid J, Martínez-Pascual M, et al. Lower free thyroxin associates with a less favorable metabolic phenotype in healthy pregnant women. *J Clin Endocrinol Metab* 2011;96:3717-23.
302. Bajoria R, Fisk NM. Permeability of human placenta and fetal membranes to thyrotropin-stimulating hormone in vitro. *Pediatric research* 1998;43:621-8.
303. Thomson EM, Vladisavljevic D, Mohottalage S, Kumarathasan P, Vincent R. Mapping acute systemic effects of inhaled particulate matter and ozone: Multiorgan gene expression and glucocorticoid activity. *Toxicol Sci* 2013;135:169-81.
304. Wilber JF, Utiger RD. The effect of glucocorticoids on thyrotropin secretion. *J Clin Invest* 1969;48:2096-103.
305. Saenen ND, Vrijens K, Janssen BG, Madhloum N, Peusens M, Gyselaers W, et al. Placental nitrosative stress and exposure to ambient air pollution during gestation: A population study. *Am J Epidemiol* 2016, in press.
306. Glinoer D. The regulation of thyroid function during normal pregnancy: Importance of the iodine nutrition status. *Best Pract Res Clin Endocrinol Metab* 2004;18:133-52.
307. Männistö T, Hartikainen AL, Vääräsmäki M, Bloigu A, Surcel HM, Pouta A, et al. Smoking and early pregnancy thyroid hormone and anti-thyroid antibody levels in euthyroid mothers of the Northern Finland birth cohort 1986. *Thyroid* 2012;22:944-50.

REFERENCES

308. Gondou A, Toyoda N, Nishikawa M, Yonemoto T, Sakaguchi N, Tokoro T, et al. Effect of nicotine on type 2 deiodinase activity in cultured rat glial cells. *Endocr J* 1999;46:107-12.
309. Persani L. Central hypothyroidism: Pathogenic, diagnostic, and therapeutic challenges. *J Clin Endocrinol Metab* 2012;97:3068-78.
310. Modesto T, Tiemeier H, Peeters RP, Jaddoe VW, Hofman A, Verhulst FC, et al. Maternal mild thyroid hormone insufficiency in early pregnancy and attention-deficit/hyperactivity disorder symptoms in children. *JAMA Pediatrics* 2015;169:838-45.
311. McDonald SD, Walker MC, Ohlsson A, Murphy KE, Beyene J, Perkins SL. The effect of tobacco exposure on maternal and fetal thyroid function. *Eur J Obstet Gyn R B* 2008;140:38-42.
312. Shields B, Hill A, Bilous M, Knight B, Hattersley AT, Bilous RW, et al. Cigarette smoking during pregnancy is associated with alterations in maternal and fetal thyroid function. *J Clin Endocrinol Metab* 2009;94:570-4.
313. Shine B, McKnight RF, Leaver L, Geddes JR. Long-term effects of lithium on renal, thyroid, and parathyroid function: A retrospective analysis of laboratory data. *The Lancet* 2015;386:461-68.
314. DeMarini DM. Genotoxicity biomarkers associated with exposure to traffic and near-road atmospheres: A review. *Mutagenesis* 2013;28:485-505.
315. Fenech M, Kirsch-Volders M, Natarajan AT, Surralles J, Crott JW, Parry J, et al. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. *Mutagenesis* 2011;26:125-32.
316. Fenech M. The cytokinesis-block micronucleus technique and its application to genotoxicity studies in human populations. *Environmental health perspectives* 1993;101:101.
317. Pedersen M, Wichmann J, Autrup H, Dang DA, Decordier I, Hvidberg M, et al. Increased micronuclei and bulky DNA adducts in cord blood after maternal exposures to traffic-related air pollution. *Environ Res* 2009;109:1012-20.
318. Rossnerova A, Spatova M, Pastorkova A, Tabashidze N, Veleminsky Jr M, Balascak I, et al. Micronuclei levels in mothers and their newborns from regions with different types of air pollution. *Mutat Res Fundam Mol Mech Mutagen* 2011;715:72-78.
319. O'Callaghan-Gordo C, Fthenou E, Pedersen M, Espinosa A, Chatzi L, Beelen R, et al. Outdoor air pollution exposures and micronuclei frequencies in lymphocytes from pregnant women and newborns in Crete, Greece (rhea cohort). *Environ Res* 2015;143, Part A:170-76.
320. van Leeuwen DM, Pedersen M, Knudsen LE, Bonassi S, Fenech M, Kleinjans JC, et al. Transcriptomic network analysis of micronuclei-related genes: A case study. *Mutagenesis* 2011;26:27-32.
321. Winckelmans E, Vrijens K, Tsamou M, Janssen BG, Saenen N, Roels HA, et al. Newborn sex-specific transcriptome signatures and gestational exposure to fine particles: Findings from the environage birth cohort, *submitted*.
322. Hochstenbach K, van Leeuwen DM, Gmuender H, Gottschalk RW, Løvik M, Granum B, et al. Global gene expression analysis in cord blood reveals gender-specific differences in response to carcinogenic exposure *in utero*. *Cancer Epidemiol Biomarkers Prev* 2012;21:1756-67.
323. van Leeuwen DM, van Herwijnen MHM, Pedersen M, Knudsen LE, Kirsch-Volders M, Sram RJ, et al. Genome-wide differential gene expression in children exposed to air pollution in the czech republic. *Mutat Res Fundam Mol Mech Mutagen* 2006;600:12-22.

324. Ryan KM, Phillips AC, Vousden KH. Regulation and function of the p53 tumor suppressor protein. *Current opinion in cell biology* 2001;13:332-7.
325. Harris SL, Levine AJ. The p53 pathway: Positive and negative feedback loops. *Oncogene* 2005;24:2899-908.
326. Hess R, Plaumann B, Lutum AS, Haessler C, Heinz B, Fritzsche M, et al. Nuclear accumulation of p53 in response to treatment with DNA-damaging agents. *Toxicology letters* 1994;72:43-52.
327. Appella E, Anderson CW. Post-translational modifications and activation of p53 by genotoxic stresses. *European Journal of Biochemistry* 2001;268:2764-72.
328. Salazar AM, Sordo M, Ostrosky-Wegman P. Relationship between micronuclei formation and p53 induction. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 2009;672:124-28.
329. Xu J, Morris GF. P53-mediated regulation of proliferating cell nuclear antigen expression in cells exposed to ionizing radiation. *Molecular and cellular biology* 1999;19:12-20.
330. Holland N, Fucic A, Merlo DF, Sram R, Kirsch-Volders M. Micronuclei in neonates and children: Effects of environmental, genetic, demographic and disease variables. *Mutagenesis* 2011;26:51-56.
331. Neri M, Ugolini D, Bonassi S, Fucic A, Holland N, Knudsen LE, et al. Children's exposure to environmental pollutants and biomarkers of genetic damage: II. Results of a comprehensive literature search and meta-analysis. *Mutation Research/Reviews in Mutation Research* 2006;612:14-39.
332. Milošević-Djordjević O, Grujić D, Arsenijević S, Brkić M, Ugrinović S, Marinković D. Micronuclei in cord blood lymphocytes as a biomarker of transplacental exposure to environmental pollutants. *Tohoku Journal of Experimental Medicine* 2007;213.
333. Soberanes S, Panduri V, Mutlu GM, Ghio A, Bundinger GR, Kamp DW. P53 mediates particulate matter-induced alveolar epithelial cell mitochondria-regulated apoptosis. *Am J Respir Crit Care Med* 2006;174:1229-38.
334. Dagher Z, Garçon G, Billet S, Gosset P, Ledoux F, Courcet D, et al. Activation of different pathways of apoptosis by air pollution particulate matter (PM_{2.5}) in human epithelial lung cells (I132) in culture. *Toxicology* 2006;225:12-24.
335. Moll UM, Petrenko O. The mdm2-p53 interaction. *Mol Cancer Res* 2003;1:1001-08.
336. Rossner Jr P, Binkova B, Milcova A, Solansky I, Zidzik J, Lyubomirova KD, et al. Air pollution by carcinogenic pahs and plasma levels of p53 and p21waf1 proteins. *Mutat Res Fundam Mol Mech Mutagen* 2007;620:34-40.
337. Chehab NH, Malikzay A, Stavridi ES, Halazonetis TD. Phosphorylation of ser-20 mediates stabilization of human p53 in response to DNA damage. *Proc Natl Acad Sci USA* 1999;96:13777-82.
338. Khan QA, Vousden KH, Dipple A. Cellular response to DNA damage from a potent carcinogen involves stabilization of p53 without induction of p21(waf1/cip1). *Carcinogenesis* 1997;18:2313-18.
339. Stark AM, Pfannenschmidt S, Tscheslog H, Maass N, Rösel F, Mehdorn HM, et al. Reduced mRNA and protein expression of bcl-2 versus decreased mrna and increased protein expression of bax in breast cancer brain metastases: A real-time PCR and immunohistochemical evaluation. *Neurol Res* 2006;28:787-93.

REFERENCES

340. Fenech M. Cytokinesis-block micronucleus cytome assay. *Nat Protocols* 2007;2:1084-104.
341. Kogevinas M, Andersen A-MN, Olsen J. Collaboration is needed to coordinate European birth cohort studies. *Int J Epidemiol* 2004;33:1172-73.
342. Vrijheid M, Casas M, Bergström A, Carmichael A, Cordier S, Eggesbø M, et al. European birth cohorts for environmental health research. *Environ Health Perspect* 2012;120:29-37.
343. Jaffe AE, Irizarry RA. Accounting for cellular heterogeneity is critical in epigenome-wide association studies. *Genome Biol* 2014;15:1-9.
344. Houseman EA, Accomando WP, Koestler DC, Christensen BC, Marsit CJ, Nelson HH, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. *BMC Bioinformatics* 2012;13:86.
345. Rath G, Dhuria R, Salhan S, Jain AK. Morphology and morphometric analysis of stromal capillaries in full term human placental villi of smoking mothers: An electron microscopic study. *Clin Ter* 2011;162:301-5.
346. Maccani JZ, Koestler DC, Houseman EA, Marsit CJ, Kelsey KT. Placental DNA methylation alterations associated with maternal tobacco smoking at the runx3 gene are also associated with gestational age. *Epigenomics* 2013;5:619-30.
347. Breton CV, Salam MT, Wang X, Byun HM, Siegmund KD, Gilliland FD. Particulate matter, DNA methylation in nitric oxide synthase, and childhood respiratory disease. *Environ Health Perspect* 2012;120:1320-6.
348. Salam MT, Byun HM, Lurmann F, Breton CV, Wang X, Eckel SP, et al. Genetic and epigenetic variations in inducible nitric oxide synthase promoter, particulate pollution, and exhaled nitric oxide levels in children. *J Allergy Clin Immunol* 2012;129:232-9.e1-7.
349. Sanchez-Guerra M, Zheng Y, Osorio-Yanez C, Zhong J, Chervona Y, Wang S, et al. Effects of particulate matter exposure on blood 5-hydroxymethylation: Results from the Beijing truck driver air pollution study. *Epigenetics* 2015;10:633-42.
350. Tarantini L, Bonzini M, Apostoli P, Pegoraro V, Bollati V, Marinelli B, et al. Effects of particulate matter on genomic DNA methylation content and iNOS promoter methylation. *Environ Health Perspect* 2009;117:217-22.
351. Hou L, Zhang X, Zheng Y, Wang S, Dou C, Guo L, et al. Altered methylation in tandem repeat element and elemental component levels in inhalable air particles. *Environ Mol Mutagen* 2013;55:256-65.
352. Dioni L, Hoxha M, Nordio F, Bonzini M, Tarantini L, Albetti B, et al. Effects of short-term exposure to inhalable particulate matter on telomere length, telomerase expression, and telomerase methylation in steel workers. *Environ Health Perspect* 2011;119:622-7.
353. Bellavia A, Urch B, Speck M, Brook RD, Scott JA, Albetti B, et al. DNA hypomethylation, ambient particulate matter, and increased blood pressure: Findings from controlled human exposure experiments. *J Am Heart Assoc* 2013;2:e000212.
354. De Prins S, Koppen G, Jacobs G, Dons E, Van de Mieroop E, Nelen V, et al. Influence of ambient air pollution on global DNA methylation in healthy adults: A seasonal follow-up. *Environ Int* 2013;59:418-24.
355. Madrigano J, Baccarelli A, Mittleman MA, Sparrow D, Spiro A, Vokonas PS, et al. Air pollution and DNA methylation: Interaction by psychological factors in the va normative aging study. *Am J Epidemiol* 2012;176:224-32.

356. Kingsley SL, Eliot MN, Whitsel EA, Huang Y-T, Kelsey KT, Marsit CJ, et al. Maternal residential proximity to major roadways, birth weight, and placental DNA methylation. *Environ Int* 2016;92-93:43-49.
357. Hou L, Zhang X, Dioni L, Barretta F, Dou C, Zheng Y, et al. Inhalable particulate matter and mitochondrial DNA copy number in highly exposed individuals in Beijing, China: A repeated-measure study. *Part Fibre Toxicol* 2013;10:17.
358. Xia Y, Chen R, Wang C, Cai J, Wang L, Zhao Z, et al. Ambient air pollution, blood mitochondrial DNA copy number and telomere length in a panel of diabetes patients. *Inhal Toxicol* 2015;27:481-87.
359. Pieters N, Janssen BG, Dewitte H, Cox B, Cuypers A, Lefebvre W, et al. Biomolecular markers within the core axis of aging and particulate air pollution exposure in the elderly: A cross-sectional study. *Environ Health Perspect* 2015.
360. Castegna A, Iacobazzi V, Infantino V. The mitochondrial side of epigenetics. *Physiol Genomics* 2015;47:299-307.
361. Wick P, Malek A, Manser P, Meili D, Maeder-Althaus X, Diener L, et al. Barrier capacity of human placenta for nanosized materials. *Environ Health Perspect* 2010;118:432-6.

CURRICULUM VITAE

Bram Janssen was born in Neerpelt (Belgium) on June 25th 1988. After he graduated from secondary school at the Don Bosco college Hechtel in 2006, he started his bachelor study in Biomedical Sciences at Hasselt University and got his master's degree Environmental Health Sciences in 2011 (*magna cum laude*). In the same year, he started his PhD in molecular epidemiology at the Centre for Environmental Sciences of Hasselt University, under supervision of Prof. dr. Tim Nawrot. The aim of his research was to establish epidemiological evidence for particulate- and traffic-related air pollution on aging pathways occurring early in life, that may modify health trajectories and affect disease risk in later life (e.g. cardio-respiratory disease). Besides teaching activities, Bram helped to lay the groundwork of the ongoing birth cohort ENVIRONAGE. During his PhD, he went six months to Harvard University, School of Public Health in Boston to study epigenetic mechanisms in the placenta and two months to the Institute of Molecular Toxicology and Pharmacology in Munich to characterize mitochondrial function in placental tissue. He presented his results at several conferences including PPOTX III in Paris, DOHaD in Rotterdam, ISEE in Basel and Barcelona, Targeting mitochondria in Berlin, and IFPA in Brisbane. In 2012, he received the 'Pfizer Young Investigator Award' for his pioneering work on mitochondrial function in placental tissue.

BIBLIOGRAPHY

INTERNATIONAL PEER-REVIEWED PUBLICATIONS

1. **Janssen BG**, Munters E, Pieters N, Smeets K, Cox B, Cuypers A, Fierens F, Penders J, Vangronsveld J, Gyselaers W, Nawrot TS. Placental mitochondrial DNA content and particulate air pollution during *in utero* life. *Environ Health Perspect* 2012;120:1346-1352.
2. **Janssen BG**, Godderis L, Pieters N, Poels K, Kiciński M, Cuypers A, Fierens F, Penders J, Plusquin M, Gyselaers W, Nawrot TS. Placental DNA hypomethylation in association with particulate air pollution in early life. *Part Fibre Toxicol* 2013;10:22.
3. Bijnens E, Pieters N, Dewitte H, Cox B, **Janssen BG**, Saenen N, Dons E, Zeegers MP, Int Panis L, Nawrot TS. Host and environmental predictors of exhaled breath temperature in the elderly. *BMC Public Health* 2013;13:126.
4. **Janssen BG**, Byun HM, Cox B, Gyselaers W, Izzi B, Baccarelli AA, Nawrot TS. Variation of DNA methylation in candidate age-related targets on the mitochondrial-telomere axis in cord blood and placenta. *Placenta* 2014;35:665-72.
5. Pieters N, **Janssen BG**, Valeri L, Cox B, Cuypers A, Dewitte H, Plusquin M, Smeets K, Nawrot TS. Molecular responses in the telomere-mitochondrial axis of ageing in the elderly: A candidate gene approach. *Mech Ageing Dev* 2015;145:51-57.
6. **Janssen BG**, Byun H-M, Gyselaers W, Lefebvre W, Baccarelli AA, Nawrot TS. Placental mitochondrial methylation and exposure to airborne particulate matter in the early life environment: An ENVIRONAGE birth cohort study. *Epigenetics* 2015;10:536-544.
7. Saenen ND, Plusquin M, Bijnens E, **Janssen BG**, Gyselaers W, Cox B, Fierens F, Molenberghs G, Penders J, Vrijens K, De Boever P and Nawrot TS. *In utero* fine particle air pollution and placental expression of genes in the brain-derived neurotrophic factor signaling pathway: An ENVIRONAGE birth cohort study. *Environ Health Perspect* 2015;123:834-40.
8. De Vusser K, Pieters N, **Janssen BG**, Lerut E, Kuypers D, Jochmans I, Monbaliu D, Pirenne J, Nawrot TS, Naessens M. Telomere length, cardiovascular risk and arteriosclerosis in human kidneys: an observational cohort study. *Aging (Albany NY)* 2015;7:766-75.
9. Grevendonk L, **Janssen BG**, Vanpoucke C, Lefebvre W, Hoxha M, Bollati V, Nawrot T. Mitochondrial oxidative DNA damage and exposure to particulate air pollution in mother-newborn pairs. *Environ Health*. 2016;15:10.
10. Clemente DB, Casas M, Vilahur N, Begiristain H, Bustamante M, Carsin AE, Fernandez MF, Fierens F, Gyselaers W, Iniguez C, **Janssen BG**, Lefebvre W, Llop S, Olea N, Pedersen M, Pieters N, Santa Marina L, Souto A, Tardon A, Vanpoucke C, Vrijheid M, Sunyer J, Nawrot TS. Prenatal ambient air pollution, placental mitochondrial DNA content, and birth weight in the INMA (Spain) and ENVIRONAGE (Belgium) birth cohorts. *Environ Health Perspect* 2016; 124:659-65.
11. Pieters N*, **Janssen BG***, Dewitte H, Cox B, Cuypers A, Lefebvre W, Smeets K, Vanpoucke C, Plusquin M, Nawrot TS. Biomolecular markers within the core axis of aging and particulate air pollution exposure in the elderly: A cross-sectional study. *Environ Health Perspect* 2016; *in press*.
* Authors equally contributed.
12. Saenen ND, Vrijens K, **Janssen BG**, Madhloum N, Peusens M, Gyselaers W, Vanpoucke C, Lefebvre W, Roels HA, Nawrot TS. Placental nitrosative stress and *in utero* exposure to airborne particulate matter. *American Journal of Epidemiology*, *in press*.
13. **Janssen BG***, Saenen ND*, Roels HA, Madhloum N, Gyselaers W, Lefebvre W, Penders J, Vanpoucke C, Vrijens K, Nawrot TS. Fetal thyroid function and *in utero* exposure to fine particle air pollution: a birth cohort study. *Environmental Health Perspectives, revised version submitted*.
* Authors equally contributed.
14. Martens DS, Gouveia-Figueira S, Madhloum N, **Janssen BG**, Plusquin M, Vanpoucke C, Lefebvre W, Forsberg B, Nording ML, Nawrot TS. Altered neonatal cord blood oxylipidome in association with exposure to particulate matter in the early life environment. *Environmental Health Perspectives, revised version submitted*.
15. Winckelmans E, Vrijens K, Tsamou M, **Janssen BG**, Saenen ND, Roels HA, Kleinjans J, Lefebvre W, Vanpoucke C, de Kok T, Nawrot TS. Newborn sex-specific transcriptome signatures and gestational exposure to fine particles: Findings from the ENVIRONAGE birth cohort. *Submitted*.

16. Bijnens EM, Nawrot TS, Derom C, **Janssen BG**, Vrijens K, Vlietinck R, Gielen M, Zeegers MP. Placental mitochondrial DNA content is associated with maternal residential traffic and greenness indicators: A twin study. *Submitted*.
17. Madhloum N, **Janssen BG**, Bijnens E, Gyselaers W, Penders J, Plusquin M, Nawrot TS. Cord plasma insulin and early life exposure to particulate air pollution. *Submitted*.
18. Saenen ND, Vrijens K, **Janssen BG**, Roels HA, Neven Kristof, Vandenberghe W, Gyselaers W, Vanpoucke C, De Boever P, Nawrot TS. Lower placental leptin DNA methylation through gestational exposure to particulate matter air pollution. *Submitted*.

BOOKS

1. Nawrot TS, Cox B, **Janssen BG**, Plusquin M. Prevention-passive smoking and pregnancy. In: *European Journal of Cancer Supplement* 2013, Supplement 11 (2): 242-247.

REPORTS

1. Plusquin M, Pieters N, **Janssen BG**, Nawrot TS. Validatiedossier 2013: mitochondriale DNA bepaling. *Steunpunt Milieu en Gezondheid*.

ABSTRACTS

1. **Janssen BG**, Munters E, Pieters N, Smeets K, Cox B, Cuypers A, Fierens F, Penders J, Vangronsveld J, Gyselaers W, Nawrot TS. Placental mitochondrial DNA content and particulate air pollution during *in utero* life. Prenatal Programming and Toxicity III (PPTOXIII), Paris, France, 14-16 May 2012 (**poster presentation**).
2. **Janssen BG**, Munters E, Pieters N, Smeets K, Cox B, Cuypers A, Fierens F, Penders J, Vangronsveld J, Gyselaers W, Nawrot TS. Placental mitochondrial DNA content and particulate air pollution during *in utero* life. Developmental Origins of Health and Disease (DOHaD) 2012 satellite meeting: 'New developments in developmental epidemiology', Rotterdam, the Netherlands, 6-7 Dec 2012 (**oral presentation**).
3. **Janssen BG**, Godderis L, Pieters N, Poels K, Kiciński M, Cuypers A, Fierens F, Penders J, Plusquin M, Gyselaers W, Nawrot TS. Placental DNA hypomethylation in association with particulate air pollution in early life. Developmental Origins of Health and Disease (DOHaD) 2012 satellite meeting: 'New developments in developmental epidemiology', Rotterdam, the Netherlands, 6-7 Dec 2012 (**poster presentation**).
4. **Janssen BG**, Munters E, Pieters N, Smeets K, Cox B, Cuypers A, Fierens F, Penders J, Vangronsveld J, Gyselaers W, Nawrot TS. Placental mitochondrial DNA content and particulate air pollution during *in utero* life. 2nd Symposium of the 'Cluster Milieu', Diepenbeek, Belgium, 2012 (**invited speaker**).
5. **Janssen BG**, Godderis L, Pieters N, Poels K, Kiciński M, Cuypers A, Fierens F, Penders J, Plusquin M, Gyselaers W, Nawrot TS. Placental DNA hypomethylation in association with particulate air pollution in early life. The Epigenetics Revolution: development and disease biology revisited, Leuven, Belgium, 1-4 Jul 2013 (**poster presentation**).
6. **Janssen BG**, Nawrot TS. Mitochondrial DNA content and DNA methylation in association with exposure to particulate air pollution during critical periods of *in utero* life. International Society Environmental Epidemiology (ISEE): 'Environment and Health – Bridging South, North, East and West', Basel, Switzerland, 19-23 Aug 2013 (**invited speaker, oral presentation by Nawrot TS**).

7. **Janssen BG**, Godderis L, Pieters N, Poels K, Kiciński M, Cuypers A, Fierens F, Penders J, Plusquin M, Gyselaers W, Nawrot TS. Placental DNA hypomethylation in association with particulate air pollution in early life. International Society Environmental Epidemiology (ISEE): 'Environment and Health – Bridging South, North, East and West', Basel, Switzerland, 19-23 Aug 2013 (**oral presentation**).
8. **Janssen BG**, Godderis L, Pieters N, Poels K, Kiciński M, Cuypers A, Fierens F, Penders J, Plusquin M, Gyselaers W, Nawrot TS. Placental DNA hypomethylation in association with particulate air pollution in early life. International Society Environmental Epidemiology (ISEE): 'Environment and Health – Bridging South, North, East and West', Basel, Switzerland, 19-23 Aug 2013 (**poster presentation**).
9. **Janssen BG**, Nawrot TS. Impact van blootstelling aan fijn stof op DNA-methylatie in de placenta. Sectie Arbeidstoxicologie en Contactgroep Gezondheid & Chemie (CGC): 'Studiedag 'occupational epigenetics'; Gezondheidseffecten over generaties door (beroepsmatige) blootstelling?', 's Hertogenbosch, the Netherlands, 13 Mar 2014 (**invited speaker**).
10. **Janssen BG**, Nawrot TS. The relevance of a birth cohort: molecular research applications in gynecology; Can we identify the molecular base of adverse pregnancy outcomes? 4th Euregional Meeting departments of Gynecology and Obstetrics, of the Universities of Aachen, Genk, and Maastricht, Aachen, Germany, 14 Mar 2014 (**invited speaker**).
11. **Janssen BG**, Byun H-M, Gyselaers W, Penders J, Baccarelli AA, Nawrot TS. Placental mitochondria as a target of *in utero* particulate air pollution and the modulating role of thyroid hormones. Young Researchers Conference on Environmental Epidemiology (ISEE-EUROPE), CREAL, Barcelona, Spain, 20-21 Oct 2014 (**oral presentation**).
12. **Janssen BG**, Vrijens K, Vanderheijden L, Gyselaers W, Nawrot TS. Cellular DNA damage-related genes in association with *in utero* particulate matter exposure: p53 as a central hub. Young Researchers Conference on Environmental Epidemiology (ISEE-EUROPE), CREAL, Barcelona, Spain, 20-21 Oct 2014 (**oral presentation**).
13. **Janssen BG**, Byun H-M, Gyselaers W, Penders J, Baccarelli AA, Nawrot TS. Placental mitochondria as a target of *in utero* particulate air pollution and the modulating role of thyroid hormones. 5th World Congress on Targeting Mitochondria: Strategies, Innovations & Clinical Applications, Ritz Carlton, Berlin, Germany, 31 Oct 2014 (**oral presentation**).
14. **Janssen BG**, Byun H-M, Gyselaers W, Lefebvre W, Baccarelli AA, Nawrot TS. Placental mitochondrial methylation and exposure to airborne particulate matter in the early life environment: An ENVIRONAGE birth cohort study. International Federation of Placenta Associations (IFPA); The Placenta: Influence and Impact, Brisbane, Australia, 8-11 Sept 2015 (**poster presentation**).
15. **Janssen BG**, Saenen ND, Roels HA, Madhloum N, Gyselaers W, Lefebvre W, Penders J, Vanpoucke C, Vrijens K, Nawrot TS. Fetal thyroid function and *in utero* exposure to fine particulate air pollution. Young Researchers Conference on Environmental Epidemiology (ISEE-EUROPE), Utrecht, the Netherlands, 2 Nov 2015 (**oral presentation**).

AWARDS

1. Pfizer Young Investigator Award,
bestowed in December 2012 at DOHaD Satellite Meeting- New Developments in Developmental Epidemiology in Rotterdam, the Netherlands.

GRANTS

1. FWO travel grant (V4.015.13N) Visiting student researcher at Harvard School of Public Health, Boston, MA, USA. *January – June 2013*.
2. FWO travel grant (K2.141.15) Conference IFPA, Brisbane, QLD, Australia. *September 2015*.

DANKWOORD

PROLOOG

Het jaar 2001 in “het referentiegebied” Eksel. Mijn moeder werkte als één van de veldmedewerkers in een grootschalig bevolkingsonderzoek. Als (lichtjes gedwongen) deelnemer van deze studie kwam ik voor het eerst in aanraking met de term epidemiologie en namen als “Jan Staessen” (onderzoeksleider) en zijn huidige doctoraatsstudent “Tim Nawrot”. Nooit had ik gedacht dat ik deze laatste naam ooit nog zou tegenkomen...

Een bijzondere dank gaat uit naar mijn promotor, prof. dr. Tim Nawrot, en co-promotor prof. dr. Ann Cuypers. Tim, ik kende enkel jouw naam maar na jouw eerste lessen aan de UHasselt wist ik meteen dat ik mijn master thesis bij jou wou doen. Bedankt om me daarna tijdens mijn doctoraat verder op sleeptouw te nemen in de wereld van de (moleculaire) epidemiologie. Je hebt me enorm veel geleerd en mij onnoemelijk veel kansen gegeven om mij te laten groeien als onderzoeker. Ik ben zeer erkentelijk dat ik de mogelijkheid kreeg de wereld te verkennen om het geboortecohort op de kaart te brengen en was dan ook met plezier je “ambassadeur van dienst”. Elke goede relatie kent hoogte- en dieptepunten en we hebben dan ook met tijd en stond onze discussies en aanvaringen gehad maar die hebben we altijd kunnen wegspoelen met een goed glas rode wijn ☺! Het spijt me dat ik de Bed & Breakfast in Hasselt heb moeten laten voor wat het was maar ik heb je altijd graag als gast gehad!

Ann, ook ik ben wel degelijk “gekneed” door jouw aanstekelijke woorden tijdens je wonderbaarlijk interessante lessen. Bedankt om mijn interesse in milieu & gezondheid op te wekken. Ondanks je drukke agenda was je altijd bereid tot wat “small talk” op de gang van de universiteit.

I would like to thank all members of the jury for their critical evaluation of my PhD thesis: prof. dr. Marcel Ameloot, prof. dr. Wilfried Gyselaers, prof. dr. Karen Smeets, prof. dr. Karin Michels, and dr. Benedetta Izzi. Karin, thank you for the constructive suggestions to my PhD thesis. Your work on environmental epigenetics inspired me a lot while writing this doctoral dissertation. I wish you all the best with the expansion of the placental consortium. Wilfried, naast een

zeer aangename samenwerking, wil ik je bedanken voor alle raad en tijd die je wou vrij maken in je drukke agenda om me te helpen met mijn onderzoek. Zonder jou inzet en toewijding voor het geboortecohort stonden we nu niet waar we nu staan. Karen, bedankt voor alle tips die me geholpen hebben om mijn doctoraat af te werken. Je kon me altijd gerust stellen door steeds opecht te vragen hoe het met mijn doctoraat ging.

During my PhD I got the opportunity to perform some of my work abroad. First of all, dr. Benedetta Izzi, I want to thank you for your reassuring words and helping me out during my time in Boston. Victoria, I loved staying at your place, you made me feel at home instantly! My gratitude goes out to prof. dr. Andrea Baccarelli and dr. Hyang-Min Byun. Andrea, thank you for the inspiring classes and the opportunity to work in you lab. Hyang-Min, I cannot describe in words how thankful I am for your help in the lab! Dr. Marco Sanchez Guerra, dr. Rodosthenis Rososthenous, dr. Octavio Jiménez, and dr. Tim Barrow, I will never forget our time in The Crossing! If a career in science does not work out, we can always form a boy band ☺ (*The outfield – Your love!*)! Thank you all for making my stay one of the best periods of my life. Also a big thanks to prof. dr. Hans Zischka and dr. Sabine Schmitt from the Helmholtz Zentrum München for helping me out with my mitochondrial work.

Dit werk was niet mogelijk geweest zonder de hulp van alle coauteurs en verschillende instanties. Veel dank aan dr. Charlotte Vanpoucke en dr. Frans Fierens (Intergewestelijke Cel voor het Leefmilieu), dr. Wouter Lefebvre (Vlaams Instituut voor Technologie Onderzoek), dr. Andy Delcloo (Koninklijk Meteorologisch Instituut) voor het aanleveren van de luchtverontreiniging en meteorologische gegevens. Ook bedankt aan prof. dr. Lode Godderis en dr. Katrien Poels van de KULeuven voor de hulp met de globale methylatiemetingen. Zonder studieelnemers kan je geen onderzoek doen en ik zou dan ook alle moeders en hun kindjes willen bedanken om deel te nemen aan onze studie. Dank aan alle vroedkundigen om de moeders te rekruteren en om stalen af te nemen (al die chocolaatjes hebben toch hun effect gehad ☺). Verder wil ik de mensen van het klinisch labo in het ZOL (prof dr. Joris Penders, Lut Vastmans, Carmen Reynders) bedanken voor hun hulp met de staalverwerking en natuurlijk ook de laboranten van het CMK: Ann Wijaerts en Carine Put. Bedankt aan de Universiteit Hasselt, het Fonds voor

Wetenschappelijk Onderzoek, en de European Research Council voor de financiële ondersteuning van mijn onderzoek.

En dan nu... de collega's. Ik ga beginnen met de "oude garde". Nicky, ik spreek voor heel de groep als ik zeg dat we onze "moederkloek" missen! Bedankt voor de lange koffiepauzes die we nodig hadden om te "ventileren". Ik wens je super veel succes op je nieuwe job! Bianca, door jouw statistische hulp en kritisch naleeswerk (lees: muggenziften ☺) is de kwaliteit van mijn werk er enorm op vooruitgegaan. Je bent een geliefde collega die altijd voor me klaarstaat. Hetzelfde geldt voor Michal, bedankt om me wegwijs te maken in de statistiek. Je was een geduchte tegenstander met pingpongen maar met squashen heb je nog wat werk ☺. Nelly, ze zeggen altijd dat vrouwen meerdere dingen tegelijk kunnen maar wat jij soms op je hooivork neemt is bewonderenswaardig! Esmée, ik appreccieer je gedrevenheid in onderzoek en hoop dat je nog lang op tweelingen onderzoek kan doen! Eline, jou organisatorisch en lay-out talent is opmerkelijk! Bedankt om het follow-up onderzoek mee in goede banen te leiden! Dank aan alle leden van de "nieuwe lichting" Diana, Dries, Ellen, Maria, Narjes, Martien, Annette en Leen om de laatste jaren van mijn doctoraat met kleur te vullen! Diana, jou opgewektheid en goedgezindheid brengt op ieder gezicht, inclusief het mijne, een lach! Dries, met jou voelde ik me niet meer de enige haan in het kippenhok. Bedankt voor de nodige ontspanning op vrijdag met squash en achteraf een duvelke natuurlijk ☺. Ellen, bedankt om me conditioneel een beetje op peil te houden door me wat sneller te laten trappen tijdens het fietsen van/naar het werk ☺. Maria, I admire your perseverance in the lab and how you can keep your calmness. Narjes, hoe jij alles geregeld en gecombineerd krijgt snapt niemand, chapeau! Je optimisme en positieve "vibes" stralen over naar de groep. Annette, je staat altijd voor iedereen klaar, zelfs al is het voor een last minute lift naar de bushalte ☺! Martien, zonder jou zouden "ice queens Mathilda, Fabiola, en Paola" nog altijd een puinhoop zijn, iets waar ik wel van kan janken ☺. Bedankt voor je inzet om alles tip top in orde te krijgen. Leen, hoewel de samenwerking relatief kort was ben ik er van overtuigd dat je mooi werk gaat leveren in Namen (sorry de figuur is nog altijd niet af...☺). Dank aan alle post-docs van "Group Nawrot" (Karen, Michelle, Janneke, Kevin) voor hun begeleiding en inzet om ons, de doctoraatsstudenten, aan het werk te houden ☺. Prof. dr. emeritus Harry Roels, ik ben zeer erkentelijk dat u in de eindfase

van mijn doctoraat mijn thesis (en manuscripten) zo grondig heeft nagelezen. Hoewel ik soms een moeilijke leerling was, heb ik veel geleerd tijdens de "Wednesday afternoon masterclasses". Ook nog een woordje voor mijn lieftallige bureaugenoten Hanne en Nele. Jullie zorgden altijd voor leven in de brouwerij en zorgden er ook voor dat ik op tijd mijn benen kon strekken voor (wat anders dan) koffie natuurlijk!

Tot slot nog enkele woorden voor familie en vrienden. Om zeker niemand te vergeten ga ik algemeen alle vrienden van de handbal, studentenclub, en anderen bedanken om me de deugddoende ontspanning te geven die van cruciaal belang was tijdens deze 4 jaren. Ik ben dankbaar voor al mijn familie die me de voorbije jaren heeft gesteund. Ma en pa, het is vanzelfsprekend dat dit niet zonder jullie gelukt was. Ik wil terugrijpen naar het citaat in het begin van dit boek dat zegt: "Het verleden vergezelt ons altijd en bepaalt hoe wij zijn". Jullie hebben me sinds mijn geboorte altijd alle kansen gegeven, gesteund in alles wat ik deed en me vertrouwen gegeven, wat resulteert in de persoon die ik nu geworden ben, bedankt hiervoor. Gert en Hans, het "studentje" gaat nu eindelijk "afstuderen". Dikke merci om (toch wel wat) interesse te tonen in mijn onderzoek ☺.

Last but not least, Lene, bedankt voor jou onvoorwaardelijke steun, geduld (lees: vél geduld) en liefde. Ik zou niet weten hoe ik dit boekje moest klaarspelen zonder jou. Het was soms wel wat druk maar je speelde het altijd klaar om me te kalmeren en gerust te stellen. Hoewel de scherpe kantjes van onze vierkante tafel mooi zijn afgerond tijdens deze 4 hectische jaren ☺, ben ik er van overtuigd dat ze eeuwig zal blijven rechtstaan...

Bram Janssen

Juni 2016

"Op de deur naar succes

staat altijd DUW"