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Cohort Profile

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Cohort Profile: The ENVIRonmental influence *ON* early AGEing (ENVIR*ON*AGE): a birth cohort study

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Why was the cohort set up?

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

Age-related diseases often find their origin in risk factors operative in early life.[1,2] Neonates with adverse gestational outcomes, such as low birthweight and preterm birth, have a higher risk of developing cardiovascular,[3] metabolic,[4] respiratory[5] and even neurological pathologies[6,7] in adulthood. From conception onwards (and even preconceptionally), humans are exposed to a variety of environmental hazards that could lead to physiological and metabolic adaptations.[8] Relevant early life exposures include maternal factors such as nutrition, medication, alcohol consumption, tobacco smoke, physical activity, ambient noise, pesticides, radiation and air pollution, as well as hormonal and genetic determinants. In large areas of the world, fine-particle air pollution is an omnipresent environmental risk factor causing major health problems in adult populations.[9,10] Studies on the health effects of air pollution exposure during the most vulnerable stages in life, the *in utero* period, are still scarce.[11] The ENVIR*ON*AGE 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 ageing including mitochondrial function, telomere length, epigenetic mechanisms and DNA repair as the core axis of ageing. Specific objectives include:

- studying the association between environmental (e.g. air pollution) or lifestyle factors [e.g. maternal body mass

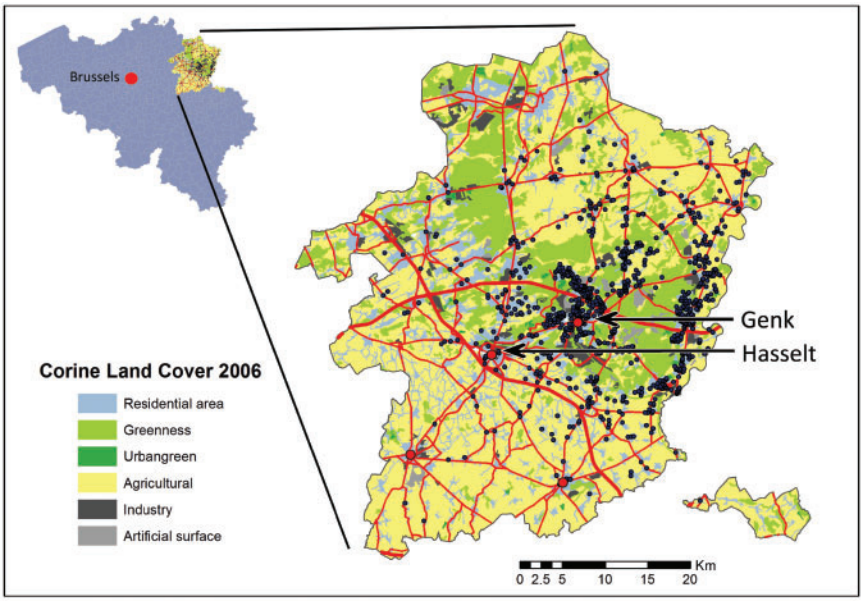

Figure 1. The catchment area of the ENVIR*ON*AGE birth cohort study is located in the north-east of Belgium (the province of Limburg, Flanders). Main roads are depicted in red and dots represent the residences of the current 1080 participating mothers. Although the catchment area is spread throughout the entire province, a great number of mothers are living close to the East-Limburg hospital in Genk.

index (BMI)] and molecular targets of ageing measured at birth and in childhood, including telomere length and mitochondrial function;

- studying the association between early life environmental or lifestyle factors and clinical outcomes in childhood including cardiovascular function and neurobehavioural performance;
- and exploring the associations between molecular targets of ageing (telomere length, mitochondria) or molecular signatures in early life (genetics, epigenetics, transcriptomics, proteomics, metabolomics) and clinical outcomes in childhood including cardiovascular function and neurobehavioural performance.

Who is in the cohort?

From February 2010 onward, mother-newborn pairs are recruited when they arrive for delivery at the East-Limburg Hospital in Genk (Belgium), following procedures approved by the Ethical Committee of Hasselt University and the East-Limburg Hospital. 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/km^2 (Figure 1). Each year, there are around 2000 live singleton births and 50 live twin births in the hospital. While recruitment is still ongoing, we enrolled during weekends between February 2010 and November 2015 a study population of 1080 singletons, making this the largest birth cohort with a prospective follow-up in Belgium. Mothers without planned caesarean section and able to fill out a Dutch language questionnaire are eligible for the cohort. Currently, the participation rate of eligible mothers is 61% and enrolment is spread equally over all seasons of the year. After birth, we follow these children throughout different stages in life (e.g. first follow-up at the age of 4 years). During follow-up, we collect lifestyle data (diet, exercise, education, etc.), medical data (BMI, history of diseases, etc.), and we perform clinical (blood pressure, microvasculature, arterial stiffness) and neurological measurements of both the child and the mother. The uniqueness of this cohort is that it combines molecular aspects and subclinical parameters of ageing with the interplay of environmental exposures in early life. Currently, 160 children have undergone follow-up measures. The preliminary response rate of the follow-up visits is 75%.

Demographic and lifestyle characteristics of the ENVIR*ON*AGE birth cohort participants (Table 1) are similar to those of the Flemish birth register of all births between 2002 and 2011 in the northern part of Belgium (Table 1).[12] Therefore, our mother-child cohort is representative for the gestational segment of the population in Flanders.

How often are cohort members being followed-up?

The ENVIR*ON*AGE birth cohort is a longitudinal study, starting with recruitment at birth and follow-up at the age of 4-6 years and at preadolescent age (Figure 2).

Table 1. Characteristics of the ENVIR*ON*AGE birth cohort participants compared with the characteristics of all births in Flanders (northern part of Belgium) from 2002 to 2011

	ENVIRONAGE ($n = 1080$)			Flanders ($n = 606877$)[a]	
Characteristic	Missing	Values		Values	
Mothers					
Age, years		29.4	[23-35]	29.5	[23.5-35.8]
< 25		160	(14.8)	98419	(16.2)
25-35		817	(75.6)	428781	(70.7)
> 35		103	(9.6)	79677	(13.1)
Pre-pregnancy BMI, kg/m^2	2	24.3	[19.5-30.7]	N/A	
Maternal education[b]	26				
Low		140	(13.3)	58743	(13.1)
Middle		385	(36.5)	183410	(40.8)
High		529	(50.2)	5968	(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)	284770	(46.9)
2		367	(34.0)	108134	(34.7)
≥ 3		130	(12.0)	114347	(18.4)
Caesarean section		44	(4.1)	N/A	
Epidural		861	(79.7)	N/A	
Newborns					
Sex					
Female		528	(48.9)	295257	(48.6)
Ethnicity[c]	5				
European-Caucasian		929	(86.4)	384522	(87.7)
Gestational age, weeks		39.1	[37-41]		
Season of delivery					
Winter (Dec-Mar)		249	(23.1)	147471	(24.3)
Spring (Mar-Jun)		283	(26.2)	152326	(25.1)
Summer (Jun-Sep)		251	(23.2)	157788	(26.0)
Autumn (Sep-Dec)		297	(27.5)	149292	(24.6)
Apgar score 5 min after birth					
7 or 8		595	(55.1)		
9		398	(36.8)		
10		87	(8.1)		
Birthweight, g		3384	[2795-4000]	3360	[2740-3965]
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]. N/A, not available.

[a]Cox *et al.* 2013.[12]

[b]Maternal education was categorized as 'low' (no diploma or primary school), 'middle' (high school) or 'high' (college or university degree).

[c]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.

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 filled out after delivery. Furthermore, daily concentrations of air pollutants are calculated at the mother's home address before and during the course of their pregnancy (see details below).

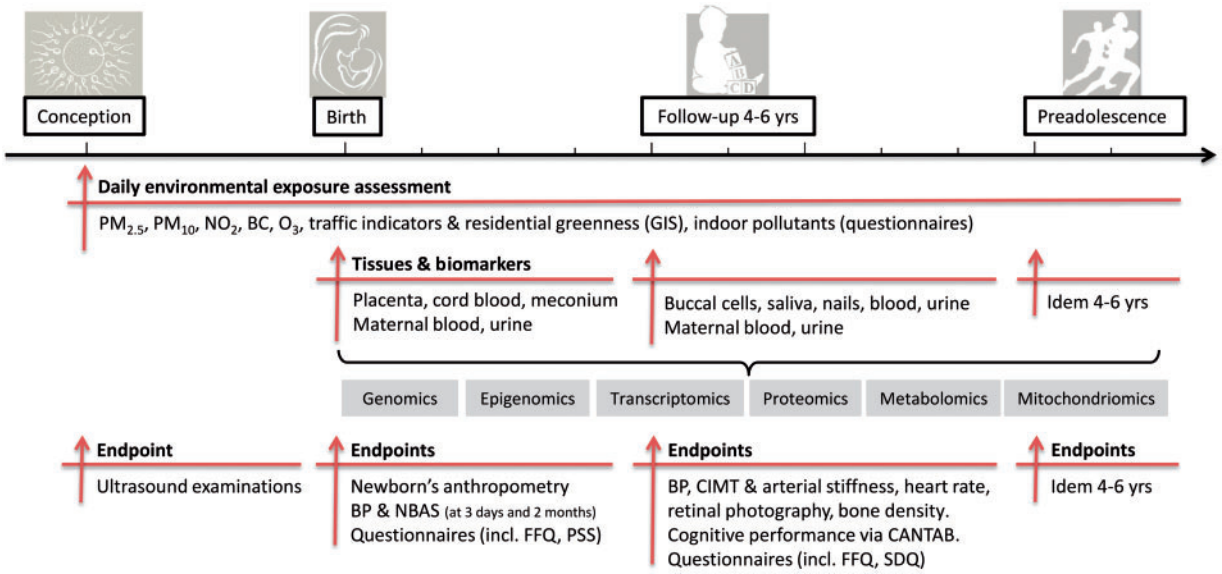

Figure 2. Study scheme of the ENVIR*ON*AGE birth cohort. Recruitment of the mother-newborn pairs started in February 2010 and is still ongoing. ENVIR*ON*AGE is a longitudinal study, starting with recruitment at birth and follow-up at the age of 4-6 years and at the pre-adolescent age. PM, particulate matter; NO_2, nitrogen dioxide; BC, black carbon; O_3, ozone; GIS, geographical information system; BP, blood pressure; NBAS, Neonatal Behaviour 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.

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 examination 3 days after delivery, maternal and neonatal blood pressures are measured and a cognitive development assessment is obtained using the Neonatal Behaviour Assessment Scale (NBAS).[13]

Follow-up 1 (at age four to six)

Mother-child pairs are re-invited at the age 4-6 years. During follow-up, we collect biological specimens, lifestyle data and medical data, and we perform clinical and cognitive measurements of both the child and mother (detailed in Table 2). The follow-up examination is conducted in a well-equipped child-friendly research room located at Hasselt University.

What has been measured?

The outline of the ENVIR*ON*AGE birth cohort study is summarized in Table 2 (and Table 3). We indicated in the table the different items as 'already performed: ☑'; 'planned to be performed: •'; and 'measured in a subgroup: S'.

Personal characteristics questionnaires

Extensive questionnaires are administered at birth and at each follow-up examination to collect information on anthropometry of the mother and father, maternal education, socio-demographic status, occupation, smoking status, alcohol consumption, BMI, diet, physical activity, medication, health status, use of assisted reproductive technologies and various lifestyle factors. Maternal stress during pregnancy is assessed using the Perceived Stress Scale (PSS), the most widely adopted psychological instrument for measuring the perception of stress.[14] At follow-up, the child's behaviour is assessed through a Strengths and Difficulties Questionnaire (SDQ) filled out by the mother. Environmental household exposures include indoor smoking, method of heating, ventilation, useage 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[15,16] and summed to obtain total dietary acrylamide intake.

Food questionnaire

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 months preceding the first follow-up examination. Antioxidant intake is estimated on

Table 2. Overview of questionnaires, analyses and measurements within the ENVIR*ON*AGE birth cohort

Section	Pregnancy and birth	Infancy 2 months	Follow-up 1 (age 4-6 years)	Comment
Questionnaires				
Lifestyle factors (nutrition, smoking, etc.)	☑		☑	Medication use, physical activity, indoor heating
Anthropometric data	☑		☑	Height, weight, BMI
Allergy symptoms	☑		☑	
Perceived Stress Scale (PSS)	☑		☑	
Strengths & Difficulties Questionnaire (SDQ)			☑	
Dietary acrylamide intake	☑		☑	
Food questionnaire			☑	Food Frequency Questionnaire (FFQ), antioxidant intake, Mediterranean diet
Environmental exposures[a]				
PM_{10}, $PM_{2.5}$, BC, NO_2, O_3	☑	☑	☑	Spatiotemporal interpolation model
Traffic indicators & residential greenness	☑	☑	☑	Geographic Information System
Indoor air pollution		S		BTEX (benzene, toluene, ethylbenzene, xylene) & formaldehyde
Temperature, humidity, UV, sunlight	☑	☑	☑	By the Royal Meteorological Institute of Belgium
Biological specimens				
Placenta & cord blood	☑			Different placental biopsies (Supplementary Figure 1)
Newborn meconium	☑			
Maternal specimens	☑		☑	Blood, urine
Child specimens			☑	Blood, urine, buccal cells, saliva, nails
Blood analysis				
Haematology	☑		☑	Complete blood cell count
Blood biochemistry	☑		☑	Ferritin, cholesterol, LDL and HDL cholesterol, cystatin C, FT_3, FT_4, TSH, folic acid, homocysteine, insulin, vitamin D, estradiol, IL-6, IgE, LH, FSH, SHBG
Acrylamide and glycidamide haemoglobin adducts				
Toxic and essential metals	☑		☑	Arsenic, cadmium, lead, mercury, manganese and selenium measured in the placenta and blood
Phenotypic measurements				
Pregnancy ultrasound data	☑			Estimated weight, nuchal translucency (first echo), CRL, BPD, OFD, HC, TD, AC and FL
Retinal photography			☑	Microvasculature
Carotid ultrasound			☑	Arterial stiffness, intima-media thickness
Blood pressure	☑	S	☑	
Heart rate			☑	
Bone density			☑	
Cambridge Neuropsychological Test Automated Battery (CANTAB)			☑	
Neonatal Behaviour Assessment Scale (NBAS)	☑	☑		

☑ have been performed; S, measured in a subgroup.

[a]Biomarkers measured in blood reflecting environmental exposures are given in the blood section of this table.

PM, particulate matter; BC, black carbon; NO_2, nitrogen dioxide; O_3, ozone; BTEX, benzene, toluene, ethylbenzene and xylene.

Table 3. Details of molecular measurements at birth in the ENVIR*ON*AGE birth cohort

Molecular measurements	Placental tissue	Cord blood	Maternal blood	Comment or targets
Genomics				
Single nucleotide polymorphisms		☑		Candidate SNPs in inflammation, ageing, cognition and metabolism
Epigenomics				
Epigenome-wide DNA methylation (450K)	☑	☑		By the Illumina 450K platform
Global DNA methylation	S	•		By UPLC/MS-MS; [5-mdC / (5-mdC + dC)] %
Targeted DNA methylation				
Mitochondrial targets	S	S	•	*PPARG, PPARGC1A, TFAM*
Ageing-related targets	S	S	•	*SIRT1, TP53, D4Z4* (subtelomeric region)
Cognitive targets	S		•	*BDNF, HTR2A, SLC6A4, NR3C1, FKBP5*
Circadian clock targets	S		•	*ARNTL, CLOCK, CRY1, CRY2, NPAS2, PER1, PER2, PER3*
Other targets	S		•	*CYP1A1, HERVW, OX1R, LEP*
Targeted microRNA expression	S	•	•	miR-16, miR-20a, miR-21, miR-34a, miR-146a, miR-210, miR-222
Transcriptomics				
Microarrays gene expression	S	S	•	By Agilent platform
Targeted gene expression				
Ageing-related targets		S	•	*TP53, BAX, DNMT1, MYC, Rb1, CHEK1, CHEK2,*
Cognitive targets	S		•	*BDNF, PLCγ, SOS, AKT, SYN1, SLC6A4*
miRNA targets	S		•	*PTEN, ISCU, COX10, HIF1a, RAD52, PTPN1*
Other targets		S	•	*PCNA, p21, PER1, DOK3, KRAS, RAB5C, VEGFA*
Proteomics				
Targeted protein levels	S	S		TP53
Metabolomics				
Targeted metabolites		☑		PGD2, PGE2, PGF2α, TXB2, 5,6-DHET, 8,9-DHET, 11,12-DHET, 14,15-DHET, 5(6)-EET, 8(9)-EET, 11(12)-EET, 14(15)-EET, 9,10-DiHOME, 12,13-DiHOME, 9(10)-EpOME, 12(13)-EpOME, 20-HETE, 5-HETE, 5-oxoETE, 9(S)-HODE, LTB4, 9,10,13-TriHOME, 9,12,13-TriHOME, 8-HETE, 9-HETE, 11-HETE, 12-HETE, 12(S)-HEPE, 12-oxoETE, 15-HETE, 15(S)-HETrE, 15-oxoETE, 13-HODE, 13-oxoODE
Ageing-related targets				
Telomere length	☑	☑	☑	Telomere repeat copy number relative to a single gene copy number (T/S ratio)
Mitochondrial DNA content	☑	☑	☑	*MTF3212/R3319, MT-ND1*
Mitochondrial DNA methylation	S	S	•	*D-loop, MT-RNR1*
Mitochondrial 8-OHdG		S	S	

☑ have been performed; • are planned; S, measured in a subgroup.
SNPs, single nucleotide polymorphisms; 5-mdC, 5-methyl-2'-deoxycytidine; dC, 2'-deoxycytidine; 8-OHdG, 8-hydroxy-2'-deoxyguanosine.

the basis of a modified validated FFQ[17] comprising 54 items that contribute most to the antioxidant intake (fruits, vegetables, cereals, etc). Furthermore, since adherence to a Mediterranean diet has been described to protect against obesity[18,19] and has been shown to reduce overall mortality in a European cohort,[20] we also included a validated 14-item Mediterranean diet assessment tool[21] including questions on the consumption of olive oil and of fish, preference for red or white meat, and fruit and vegetable intake.

Exposure characterization

Ambient air pollution

Residential particulate matter ($PM_{2.5}$ and PM_{10}: particles with an aerodynamic diameter $\leq$ 2.5 μm and $\leq$ 10 μm, respectively), black carbon (BC) and nitrogen dioxide (NO_2) exposure levels ($\mu g/m^3$) are interpolated for each mother's residential address using a spatiotemporal interpolation method[22] that takes into account land-cover data obtained from satellite images (CORINE land-cover dataset) and pollution data from fixed monitoring stations in combination with a dispersion model.[23,24] This model provides daily interpolated exposure values in a high-resolution receptor grid using data from the Belgian telemetric air quality networks, point sources and line sources. Overall model performance was evaluated by leave-one-out cross-validation including 34 monitoring points for $PM_{2.5}$, 14 for BC and 44 for NO_2. For Flanders, the validation statistics of the interpolation tool gives a spatiotemporal explained variance of more than 0.80 for $PM_{2.5}$, 0.74 for BC[24] and 0.78 for NO_2.[25] With this method we calculate the daily concentrations of air pollutants at the mother's residential address during the entire pregnancy, specific exposure windows of interest during gestation (e.g. separate trimesters, past month, etc.) and the preconception period. In addition, we calculate long-term exposure to air pollutants to which child and parents have been subject before the first follow-up study. In our exposure modelling, we always take into account address changes.

Indoor air pollution

In a subgroup of the ENVIRONAGE birth cohort, we measure indoor air pollution. We apply a hanging air-sampling system in each participant's living room for a period of 2 weeks to evaluate concentrations of NO_2, BTEX (benzene, toluene, ethylbenzene and xylene) and formaldehyde.

Traffic indicators and residential greenness

We collect information on two traffic indicators at the mother's residence, i.e. distance to major roads and traffic density using Geographic Information System (GIS) functions with ArcGIS 10 software. Semi-natural, forested and agricultural areas (greenness) as well as residential and industrial areas are estimated in a 5000-m radius or less from the residential address, based on CORINE land-cover 2006 (European Environment Agency) (Figure 1). In addition, residential surrounding greenness was also assessed using the Normalized Difference Vegetation Index (NDVI) based on Moderate Resolution Imaging Spectroradiometer (MODIS) images with a 250-m resolution.[26]

Biomarkers of internal acrylamide exposure

Acrylamide and glycidamide haemoglobin adducts are assessed in cord blood as biomarkers of integrated internal acrylamide exposure during approximately the previous 4 months (lifespan of red blood cells) using the modified Edman degradation and a high-performance liquid chromatography-tandem mass spectrometry (HPLC/MS/MS).[27]

Toxic and essential metals

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

Biological specimens

At birth, we collect approximately 42 ml of 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 (Supplementary Figure 1, available as Supplementary data at *IJE* online).[28] Briefly, we take biopsy samples of approximately 1-2 cm^3 at each quadrant of the fetal side across the middle region of the placenta approximately 4 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. One day after delivery, we collect maternal blood (20 ml) and urine, as well as newborn's meconium samples.

At the follow-up examination at age 4 years, we collect buccal cells from the children using SK-2 Isohelix buccal swabs (Cell Projects, 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.

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, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol, cystatin C, thyroid hormones [free triiodothyronine (T_3), free thyroxine (T_4) and thyroid-stimulating hormone (TSH)], folic acid, homocysteine, insulin, vitamin D, estradiol, interleukin-6, immunoglobulin E (IgE), luteinizing hormone (LH), follicle-stimulating hormone (FSH) and sex hormone-binding globulin (SHBG) are measured with an electro-chemiluminescence

immunoassay using the Modular E170 automatic analyser (Roche, Basel, Switzerland).

Phenotypic measures

Pregnancy and birth

On three antenatal visits at fixed time points during gestation, the gynaecologist performs fetal ultrasound examinations to establish gestational age and fetal growth patterns such as estimated weight, nuchal translucency (first echo), crown-rump length (CRL), biparietal diameter (BPD), occipital frontal diameter (OFD), head circumference (HC), transcerebellar diameter (TC), abdominal contour (AC) and femur length (FL). Maternal height and weight are 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, sex, Apgar score and pregnancy complications.

At the postpartum examination (hospital) as well as at the age of 2 months (home visit), a well-trained academic midwife/researcher performs the NBAS test. The NBAS assesses the neonate's behavioural repertoire on 28 items, covering four domains of neurobehavioural functioning, i.e. autonomic regulation, motor organization, self-control and social interaction. The scale also includes an assessment of the infant's neurological status on 18 reflex items. Scoring the neonatal behaviour provides a comprehensive profile of a full range of neurobehavioural functioning as well as identifying areas of difficulty or deviation.[13] In addition, neonatal and maternal blood pressures are recorded up to five times with 1-min intervals using the Welch Allyn Vital Signs Monitor 6000 Series and Omron 705IT (Omron Corporation) respectively.

Follow-up 1

Children and their mothers receive an extensive 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 are measured. Furthermore, cognitive performance of children is assessed using the Cambridge Neuropsychological Test Automated Battery (CANTAB). These tests are used as measures of the neuropsychological domains of attention (sustained), reaction time, visual memory and executive function. The mother's stress levels are assessed using the PSS.

Molecular 'omics' measurements

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. Untargeted 'omics' technologies provide unique opportunities for the discovery of new biomarkers of exposure or risk assessment and have the potential to augment our observations with mechanistic evidence. The ENVIRONAGE birth cohort combines both upstream (metabolomics, adductomics) as well as downstream 'omic' signatures including epigenomics and transcriptomics. Using high-resolution mass spectrometry (MS) coupled to UPLC, we determined metabolites in cord plasma samples and measured global DNA methylation in placental tissue. Transcription patterns (Agilent Microarray; number of signals per sample: 44K) are determined in cord blood and placental tissue ($n = 200$). The epigenome-wide DNA methylation profile (Illumina Platform; number of signals per sample: 450k) is determined in cord blood ($n = 200$). The discovery phase is partly embedded in the European EXPOsOMICS consortium.

In addition to the untargeted platforms, we perform a targeted-based 'omic' approach based on *a priori* hypotheses or to validate the targets derived from the untargeted approach in larger numbers. Measured targets in specific tissues are listed in Table 3.

Ageing-related molecular targets

Telomeres

Telomere length declines with each cell division and is considered as a marker of biological ageing.[29] We have special interest in exploring the role of early environmental exposures on telomere length, the distal hexameric repeats at the end of the chromosomes, which provide stability and protection to the coding DNA. 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 polymerase chain reaction (PCR)-based telomere assay by Cawthon.[30]

Mitochondria

Maintenance of mitochondrial function has been suggested to be an important mechanism of extending lifespan, whereas decreased mitochondrial function, impaired ATP

generation and increased reactive oxygen species (ROS) production are associated with ageing.[31] A study in knockout mice[32] and cross-sectional studies in humans[33–35] provide evidence of the relationship between mitochondrial DNA (mtDNA) content and telomere length and form a mechanistic platform for age-related disease.[36] There is a new area of research that stretches beyond the nuclear genome, called mitochondriomics.[37] Mitochondriomics is dedicated to clarifying whether mitochondria are novel biosensors or mediators of environmental effects, by exploring mtDNA abundance, mutations and deletions, epigenetics and mtDNA-encoded proteins.

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). We have shown that air pollution exposure during pregnancy induces molecular,[38–42] cellular[43–45] and hormonal[46] 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 is given in Table 4.

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 the entire pregnancy exposure to $PM_{2.5}$ and BC. These findings highlight the relevance of placental 3-NTp as marker of cumulative airborne particulate matter-induced prenatal oxidative stress.[43]

Epigenetics

With the exception of imprinted genes, all DNA methylation patterns are re-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 during adulthood.[47] 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 time for methylation reprogramming, was a highly sensitive window for $PM_{2.5}$ exposure on placental DNA methylation at birth.[38]

We analysed miRNA expression by qRT-PCR in a subset ($n = 210$) of placenta samples. We observed negative associations between placental expression of miRNAs (miR-21, miR-146a, miR-222) and second-trimester $PM_{2.5}$ exposure. In addition, placental expression of tumour suppressor phosphatase and tensin homologue (*PTEN*), a common target of the miRNAs, is positively associated with third-trimester $PM_{2.5}$ exposure in our study population.[39]

Transcriptomics

Early life exposure to $PM_{2.5}$ negatively influenced placental transcription of brain-derived neurotrophic factor (*BDNF*) and *Synapsin 1*, 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 (SOS) signalling cascades of the *BDNF* pathway.[40]

Mitochondriomics

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 shown (each mitochondrion carries 2-10 copies of mtDNA).[48] We found a lower mtDNA copy number in placental tissue in association with *in utero* exposure to PM_{10}, which reflects signs of mitophagy and mitochondrial death.[44] In a subsequent study which combines two independent European cohorts, INMA (INfancia y Medio Ambiente) located in Spain, and ENVIRONAGE, we demonstrated that mtDNA copy number is one of the potential mediators in the association between antenatal air pollution exposure and birthweight.[45] 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 are regulated.[41] Last, we showed that antenatal PM air pollution exposure was positively associated with mitochondrial 8-OHdG in maternal and cord blood, a marker of oxidative DNA damage.[42] 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, in both mother and fetus.

Table 4. Summary table of published articles of the ENVIR*ON*AGE birth cohort study (2012-16)

Reference	N	Target tissue	Effect measure	Exposure	Effect [95% CI]	Conclusion
Janssen *et al.* (2012)[44]	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 and was associated with maternal residential air pollution exposure
Janssen *et al.* (2013)[38]	240	Placenta	Global DNA methylation	A 5-μg/m^3 increment in $PM_{2.5}$ during pregnancy	Associated with a lower global DNA methylation (relative -2.19% [-3.65, -0.73]), with higher estimates in the first trimester (implantation) (-2.13% [-3.71, -0.54])	Our findings give mechanistic plausibility to the hypothesis that air pollution is linked to fetal programming
Janssen *et al.* (2015)[41]	381	Placenta	mtDNA methylation	An IQR (7.8 μg/m^3) increment in $PM_{2.5}$ during pregnancy	Associated with a higher mtDNA methylation (absolute +0.47% [0.20, 2.23]) with higher estimates in the first trimester (0.75% [0.16, 1.34])	mtDNA methylation substantially mediates the association between gestational $PM_{2.5}$ exposure and placental mtDNA content
Saenen *et al.* (2015)[40]	90	Placenta	Gene expression in *BDNF* pathway	A 5-μg/m^3 increment in $PM_{2.5}$ during the first 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 of neurodevelopment, decreased with exposure to $PM_{2.5}$
Clemente *et al.* (2016)[45]	726[a]	Placenta	mtDNA content & birthweight	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 birthweight of 48 g [-87, -9]	mtDNA content can be a potential mediator of the association between prenatal air pollution exposure and birthweight
Grevendonk *et al.* (2016)[42]	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, in both mother and fetus
	293	Cord blood	mtDNA 8-OHdG	An IQR increment in PM_{10} during trimesters 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	

(Continued)

Table 4. Continued

Reference	N	Target tissue	Effect measure	Exposure	Effect [95% CI]	Conclusion
Saenen *et al.* (2016)[43]	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	Our findings suggest a pivotal relation between PM exposure and placental 3-nitrotyrosine
Tsamou *et al.* (2016)[39]	210	Placenta	miR-21, miR-146a, miR-222	A 5-μg/m^3 increment in $PM_{2.5}$ during the second trimester of pregnancy	Associated with a relative decrease in miR-21 (-33.7% [-53.2, -6.2]), miR-146a (-30.9% [-48.0, -8.1]), and 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 $PM_{2.5}$ during the third trimester of pregnancy	Associated with a relative increase in *PTEN* (+59.6% [26.9, 100.7]	
Janssen *et al.* (2016)[46]	499	Cord blood Maternal blood	TSH, FT_3, FT_4, birthweight	An IQR (8.2 μg/m^3) increment in $PM_{2.5}$ during the 3rd trimester of pregnancy	Associated with a decrease in FT_4/FT_3 ratio of -62.7% [-91.6, -33.8] which was mainly attributed to a reduction in cord FT_4 concentrations No association with maternal thyroid hormones	Fetal FT_4 levels in cord blood substantially mediate the association between third trimester $PM_{2.5}$ exposure and birthweight

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

IQR, interquartile range; CI, confidence interval; *BDNF*, brain-derived neurotrophic factor; *SYN1*, synapsin 1; 8-OHdG, 8-hydroxy-2'–deoxyguanosine; *PTEN*, tumour suppressor phosphatase and tensin homologue; TSH, thyroid-stimulating hormone; FT_3, free triiodothyronine; FT_4, free thyroxine.

Fetal thyroid function and *in utero* exposure to particulate air pollution

During prenatal life, the surges of maternal and fetal thyroid hormones are critical for placental and fetal growth and development.[49] In normal healthy pregnancies, we showed that exposure to $PM_{2.5}$ air pollution during the third trimester of pregnancy negatively influences newborn TSH levels and the FT_4/FT_3 ratio in cord blood but not in maternal blood.[46] Moreover, our study demonstrated that the effect of $PM_{2.5}$ exposure during the last trimester of pregnancy on birthweight was mediated by cord blood FT_4 levels.

What are the main strengths and weaknesses?

The major strength of the ongoing ENVIR*ON*AGE birth cohort study is that detailed repeated physiological, clinical and 'omics' measurements 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 molecular epidemiology depends on innovative strategies and new technologies, both in the measurement of environmental agents and in their pathophysiological modes of action and outcomes.[50] Deep phenotyping of participants is of crucial importance to understand the dynamic interrelationships between biological systems and environmental exposures and the developmental programming of the disease outcome. So far, there is no generally accepted metric to differentiate normal from dysfunctional development or healthy from unhealthy ageing. ENVIR*ON*AGE is a resource consisting of deeply phenotyped newborns and 4-year-old children, 'omics' data and bio-banked samples. Deep phenotyping is combined with a variety of environmental, occupational, societal and lifestyle determinants that potentially influence the onset and progression of dysfunctional development or less successful ageing. Children undergo phenotyping for macro- and microcirculation, blood pressure, body composition, bone density, neurobehavioural performance and renal function. Another strength is biobanking of an extensive collection of target biospecimens which are representative of fetal exposures and which include not only cord blood and multiple samples of placental tissue but also maternal samples. Our cohort is embedded in international structures including the Epigenetic Placenta Consortium (led by Karin Michels, Department of Epidemiology, UCLA School of Public Health, Los Angeles, CA) and the EXPOsOMICS consortium [http://www.exposomicsproject.eu/]. Last, we have daily residential air pollution concentrations from pre-conception onwards on a high-resolution scale which enables us to explore the impact of air pollution exposures during different exposure windows.

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 behaviour 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 childhood, including mechanistic molecular targets, we can obtain a better understanding of disease onset. Findings from the ENVIR*ON*AGE 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 ENVIR*ON*AGE birth cohort can be obtained via the website: [www.environage.be].

Profile in a nutshell

- The ongoing population-based prospective ENVIR*ON*AGE birth cohort study is designed with a strong focus on molecular mechanisms to understand the determinants of molecular ageing in early life and its role in the developmental origins of health and disease.
- From February 2010, we recruited 1080 mother-child pairs from the East-Limburg Hospital in Genk, Belgium (recruitment ongoing).
- The first follow-up examination (age 4-6 years) comprises deep phenotyping including micro-and macrovascular phenotypes, body composition, bone density, neurobehavioural performance and renal function. The preliminary response rate is 75%.
- The dataset comprises detailed air pollution exposure assessment from pre-conception onwards, questionnaire and anthropometric data, extensive

biological specimens collected at birth (placental tissue, cord blood, meconium, maternal blood and urine) and during childhood (blood, urine, buccal cells, saliva and nails) with in-depth molecular measures and clinical phenotyping.
- Any researcher interested in collaborating with the ENVIR*ON*AGE birth cohort should contact Prof. Dr Tim Nawrot [tim.nawrot@uhasselt.be].

Supplementary Data

Supplementary data are available at *IJE* online.

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