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Impact of particulate matter on mothers and babies in Antwerp (IPANEMA), a prospective cohort study on the impact of pollutants and particulate matter in pregnancy: methodology and design

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Keywords:	air pollution, pregnancy, pre-eclampsia, particulate matter, fetal growth, hypertensive disorders in pregnancy

STUDY PROTOCOL: Impact of particulate matter on mothers and babies in Antwerp (IPANEMA), a prospective cohort study on the impact of pollutants and particulate matter in pregnancy: methodology and design

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ABSTRACT

Introduction

Air pollution is hot topic and is known to cause multiple health issues. Especially pregnant women seem to be vulnerable to environmental issues. There are data suggesting that exposure attributes to hypertensive disorders.

Methods and analysis

This study is a prospective cohort study. We aim to recruit 200 pregnant women. The outcome measurements will include maternal parameters, labour parameters and neonatal parameters.

Multiple samples will be analyzed such as maternal urine samples (8 oxo deoxyguanosine), maternal blood samples (routine blood sampling, biomarkers of pre-eclampsia and transcript and genetic markers), neonatal blood samples (transcript and genetic markers) combined with extensive questionnaires.

Ethics and dissemination

We obtain informed consent from each participant prior to enrollment in the study.

The study has received approval by the Ethical Committee of the Antwerp University Hospital (14/40/411) and is registered with ClinicalTrials.gov (14/40/411) on 22-10-2015.

IPANEMA is the first prospective study to assess the impact of particulate matter on mothers and babies in Antwerp, Belgium.

Findings from this study will contribute to improve knowledge on the impact of exposure to air pollution on mothers and babies and will also define biomarkers as predictors for pregnant women at risk.

Trial registration: ClinicalTrials.gov: 14/40/411. Registered 22-10-2015.

Key words: air pollution, pregnancy, fetal growth, pre-eclampsia, particulate matter, hypertensive disorders in pregnancy

STRENGHT AND LIMITATIONS OF THIS STUDY

- First prospective study to assess the impact of particulate matter on mother and babies in Antwerp, Belgium.
- Large questionnaires to minimalise bias from multiple factors.
- Only eligible for Dutch speaking women, which gives a bias.

INTRODUCTION

An emerging body of evidence indicates that there is an association between air pollution exposure in pregnancy and adverse pregnancy outcomes [1, 2]. Most studies have estimated personal exposure to air pollution by modeling data from outdoor monitoring stations and interpolating them to the home address of the study participants. The exposure data are averaged over different time periods and in general time spent in traffic or exposure at the workplace or indoors are not taken into account..

Particulate matter (PM) is an important component of outdoor air pollution. Particulate matter has different sizes, but especially fine particulate matter, with an aerodynamic diameter less than 2,5 µm ($PM_{2,5}$) is of great interest, because of its small size, the large specific surface area and long residence in air and thus more likely to adsorb harmful substances. $PM_{2,5}$ exists of a mixture of solid en liquid particles, emitted from a variety of sources. Because of its size it can penetrate in air ways until the alveoli from where the particles or the absorbed pollutants may be translocated into the blood stream[3].

A significant positive association between exposure to fine particulate matter during the third trimester and preeclampsia has been observed in a study of Davand et al among over 8,000 pregnant women in Barcelona, Spain.[2].

Exposure to local traffic-generated air pollution during pregnancy is also known to increase the risk of preeclampsia and preterm birth[4]. Proximity to major roads is associated with an

increased risk of preeclampsia, but not with a higher risk of gestational diabetes, placental abruption or placenta praevia [5].

Hypertensive disorders of pregnancy affect about 10% of all pregnant women. They are an important cause of severe morbidity and even mortality [6]. Pre-eclampsia complicates 2-8% of pregnancies and is a major contributor to maternal and neonatal mortality worldwide [7].

Pre-eclampsia is generally defined as de novo hypertension occurring after 20 weeks of pregnancy (with systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg), and proteinuria (≥ 300 mg in 24 h)[6-8].

The cause of pre-eclampsia still remains largely unknown, but A disturbed placental function, early in pregnancy has been suggested as an underlying mechanism. In normal pregnancy the uteroplacental arteries undergo a series of pregnancy specific changes in the first 20 weeks. Invasive trophoblasts replace endothelial cells and smooth muscle cells in the media, causing the arteries to loose elasticity and to dilate to wide tubes, without ability to contract effectively. They also lose their vasomotor control. This mechanism occurs in order to guarantee maternal blood supply to the placenta: the loss of maternal blood flow resistance and the increase of uteroplacental perfusion meet the requirements of the foetus [9]. In addition, circulating anti-angiogenic factors may play a role in the pathogenesis. The transmembrane protein vascular endothelial growth factor (VEGF) receptor fms-like tyrosine kinase 1 (Flt-1) binds with high affinity to VEGF and to placental growth factor (PlGF). Flt-1 is involved in normal angiogenesis. Soluble Flt-1 (sFlt-1) is a variant of Flt-1 that lacks a transmembrane protein, and is a naturally occurring antagonist of VEGF and PlGF[10]. Production of sFlt-1 appears to be expressed by endothelial cells and trophoblasts in response to reduced oxygen tensions [11, 12].In developing pre-eclamptic placenta the normal process of remodeling of the uterine spiral arteries is impaired, resulting in reduced perfusion, increased oxidative stress and inflammation[12].

Although there are yet no validated biomarkers that allow to identify women at risk for pre-eclampsia, levels of angiogenic and anti-angiogenic factors are altered in women with pre-eclampsia. Levels of sFlt-1 are elevated and levels of PlGF are decreased, even before clinical symptoms of disease were overt [13, 14]. The ratio of sFlt-1/PlGF is a promising set of biochemical markers for prediction of pre-eclampsia. There is a pathogenic difference between early pre-eclampsia (onset of disease before 32-34 weeks of pregnancy) and late pre-eclampsia (onset of disease after 32-34 weeks of pregnancy). Early pre-eclampsia is characterized by a significant placental dysfunction, leading to a high risk of intrauterine growth restriction. In late pre-eclampsia there is more evidence for a pre-existing maternal inflammation[7, 15]. The distinction is not always that clear, and is under debate.

Cystatin C, a protease inhibitor, is also increased in pre-eclamptic women. Cystatin C can be used as an endogenous marker for renal function, synthesized by all nucleated cells, at a constant rate and exclusively eliminated by glomerular filtration[16]. Saleh et al found that levels of cystatin C were significantly higher in the second trimester of pregnancy in women who developed pre-eclampsia[17].

Higher exposure to $PM_{2,5}$ and O_3 during the first trimester are associated with preeclampsia, gestational hypertension and preterm delivery [18]

We hypothesize that exposure to $PM_{2,5}$ in the first trimester of pregnancy interferes with the maternal vascular remodeling process and that impaired remodeling between 9 and 18 weeks results in reduced placental perfusion and oxidative stress, [19].

In 2011 the World Health Organization stated in a report that supplementation of calcium during pregnancy for women at high risk of developing pre-eclampsia is recommended[6]. They also state that low-dose acetylsalicylic acid (75 mg/day) is recommend in the same population. A recent study of Souza et al (2014) confirmed that supplementation with 100 mg aspirin plus 2 g

calcium when started after 20 weeks of gestational age reduced the rate of superimposed pre-eclampsia and intra-uterine growth restriction in women with chronic hypertension and abnormal second-trimester uterine artery Doppler[20]. If this study can predict which women are at risk for hypertensive disorders during pregnancy or pre-eclampsia, supplementation of aspirin and calcium can be started in order to reduce perinatal risks.

Objectives and aims of the IPANEMA-study

This study aims to evaluate the effects of exposure to particulate matter and outdoor air pollutants on the clinical pregnancy outcome for mother and child and to determine which biochemical changes in maternal, placental and cord blood best explain this effect.

- Objective 1: Correlate estimated personal $PM_{2,5}$ exposures to angiogenesis related markers for hypertensive disorders in pregnant woman.
- Objective 2: Identifying a relationship between biomarkers for pre-eclampsia and biomarkers for exposure to $PM_{2,5}$ and such aid in elucidating the mechanism by which PM contributes to the pathology leading to pre-eclampsia.

METHODS AND ANALYSIS

Study design and setting

The IPANEMA is a regional prospective cohort study, recruiting pregnant women in the Antwerp region in Belgium.

Data are collected during pregnancy and at delivery, as well as immediately postpartum. Blood samples, hair samples and anthropometric measurements are obtained. Self-administered questionnaires are completed at different time frames in pregnancy, in relation to sample taking (see figure 1: Visits and interventions during the IPANEMA study.).

Study sample

Study cohort

Pregnant women are recruited within the network of the Antwerp University. Leading centre will be the University Hospital Antwerp (UZA), a tertiary centre with a maternal intensive care unit (MIC) and a neonatal intensive care unit (NIC) and 1000 deliveries a year.

The aim is to enroll 200 pregnant women over a 2 year recruitment period. An increase of 2% of pregnant women who develop pre-eclampsia or hypertensive disorders can be considered clinically relevant.

Diagnosis of pre-eclampsia (onset of a new episode of hypertension during pregnancy (with persistent diastolic blood pressure > 90 mmHg) with the occurrence of substantial proteinuria (> 0.3g/24h)) and hypertensive disorders are made using the WHO guidelines [6].

Eligibility criteria

The inclusion criteria are (see figure 2: Eligibility criteria)

- A singleton pregnancy
- > 12 weeks of gestational age
- Delivery planned in a hospital within the network of the Antwerp University
- Dutch speaking

The exclusion criteria are:

- Multiple pregnancy
- Planned home delivery or planned delivery in another hospital

Power calculation and statistical analyses

The incidence of preeclampsia in pregnancy is approximately 5%. An increase of 2% is of interest. A doubling in incidence is therefore clinically relevant. With a sample size of 200 subjects a doubling from 5 to 10 % can be detected with 80% power and 5% significance level.

Data analysis will be done using SPSS24.0.

Study visits

Women are recruited by the midwife or obstetrician at 12 weeks of gestational age, typically at the second routine antenatal visit.

Signed informed consent for participating in the study is obtained before the start.

Blood collections (maternal and umbilical cord)

Venous blood is drawn around 24 weeks of gestational age.

Table 1 Tests on maternal blood sample

Routine blood sampling (UZA)	Urea, Creatinin, CRP, LDH, AST, ALT, Uric acid, APTT, PT, Fibrinogen, D-dimers, Glucose, Erythrocytes/hematocrit, Hemoglobin, Thrombocytes, Leukocytes, Ferritin, Toxoplasmosis (IgG & IgM), CMV (IgG & IgM), Herpes simplex IgG, Varicella zoster IgG, Rubella IgG, Parvovirus (IgG & IgM), Syphilis (RPR & TPHAM), Indirect Coombs
Biomarkers of pre-eclampsia	sFlt-1, PlGF, Cystatin C
Transcript and genetic markers	m(i)RNA expression, telomere length, DNA methylation

Umbilical cord blood is taken at time of birth.

Table 2 Tests on umbilical blood sample

Transcript and genetic markers	m(i)RNA expression, telomere length, DNA methylation

Measurement of m(i)RNA expression, telomere length, DNA methylation

For analysis of the m(i)RNA profile, whole blood is collected in Tempus™ Blood RNA tubes (Applied Biosystems) and stored at -80°C until analysis. Telomere length and DNA methylation will be assessed on blood samples collected in EDTA tubes, which are stored at -80°C.

Urine collections

Urine samples are taken by the pregnant women themselves, after a minimum of 8 hours fasting (morning urine). Samples are handed to the midwife at the antenatal visit, and placed at -20°C freezer within 12 hours of collection. Analysis will be performed at the Flemish Institute for Technological Research (VITO).

Two urine samples are collected: at 20 weeks and at 30 weeks gestational age. 8 oxo deoxyguanosine will be determined in the samples.

Questionnaires

There are four different questionnaires:

- *Questionnaire on general habits, socio-economic factors, lifestyle and eating habits*

  We use a self-designed questionnaire to extensively collect information on ethnic origin and and education level, on employment, income and work environment, on family history of diseases and chronic disorders, on eating habits, on previous pregnancies and other habits (smoking, alcohol consumption).

- *Questionnaire on lifestyle during previous 3 days*

  We use a self-designed questionnaire to extensively collect information on contact with possible toxic factors and on eating pattern of the previous 3 days.

- *Questionnaire on residential facts*

We use a self-designed questionnaire to collect information about the exact location of the bedroom window and other possible factors influencing concentrations of particulate matter.

- *Questionnaire on stress factors and birth facts*

  We use a self-designed questionnaire to extensively collect information on stress levels during pregnancy, on birth facts and on medication during pregnancy.

Questionnaires will be taken at different visits:

Table 3 Questionnaires

	< 12 weeks	20 weeks	30 weeks	birth
Questionnaire on general habits, socio-economic factors, lifestyle and eating habits	Recruitment			
Questionnaire on lifestyle during previous 3 days		Urine sample 1	Urine sample 2	
Questionnaire on residential facts			4-8 weeks prior to visit	
Questionnaire on stress factors and birth facts				3 days after birth

Outcomes of the study

Diagnosis of cardiovascular disorders

Pre-eclampsia is defined as a the onset of a new episode of hypertension during pregnancy (with persistent diastolic blood pressure >90mmHg) with the occurrence of substantial proteinuria (>0.3 g/24h).

Pregnancy and delivery outcome data

Maternal data that are prospectively collected are: pregnancy duration, pre-eclampsia, hypertensive disorders,

Delivery data are: type of labor, type of delivery

Neonatal data are: birth weight, preterm delivery, Apgar score on 1, 5 and 10 minutes, congenital anomalies,…

Other study measurements

Measurement of sFlt-1, PlGF and Cystatin C

Blood samples taken at the study visit around 24 weeks of gestational age for the analyses of sFlt-1, PlGF and Cystatin C are collected, processed and aliquoted within 24 hours of collection. The serum samples are stored at -80 °C until analysis at Algemeen Medisch Laboratorium (AML). sFlt-1 and PlGF will be determined using an electrochemiluminescence based sandwich immunoassay on Cobas e 411 (Roche Diagnostics, Mannheim, Germany) [23, 24]. Cystatin C will be determined using a particle-enhanced immunonephelometric assay (N Latex Cystatin C, Siemens Healthcare Diagnostics, Marburg, Germany) by use of a BN II nephelometer (Siemens Healthcare Diagnostics). This assay has a calibration traceable to the first certified reference material for cystatin C in human serum (ERM-DA471/IFCC)[25].

ETHICS AND DISSEMINATION

We obtain informed consent from each participant prior to enrollment in the study.

The study has received approval by the Ethical Committee of the Antwerp University Hospital (14/40/411) and is registered with ClinicalTrials.gov (14/40/411) on 22-10-2015.

IPANEMA is the first prospective study to assess the impact of particulate matter on mothers and babies in Antwerp, Belgium.

Exposure to particulate matter is debated on the political scene but robust data showing a relationship with hypertensive disorders of pregnancy are difficult to find. In Antwerp the design of a safe and functional tunnel for the highway is now focus of public discussion. Following the Barcelona experience a group of people want an urbal tunnel complex to lower the amount of particulate matter.

IPANEMA would like to create robust data on the correct impact of air pollution, in particular on pregnant women, a vulnerable population. IPANEMA uses prospective data from the beginning of the pregnancy till childbirth.

Available studies have been highly biased, probably in favor of detecting an effect even if this is minimal, by their retrospective nature (missing eg women who left the area before giving birth), not correcting for socioeconomic or ethic influences, using very raw and approximate models managing up to several square kilometers as identical areas of pollution, not compensating for time spent in traffic , at work or in other regions during weekends and holidays. Furthermore the discussion on the impact of air pollution had been partially taken over by politics, without robust data. Data from IPANEMA will enable to estimate the real individual dose effect relation for pregnant women.

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AVAILABILITY OF DATA AND MATERIALS

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

COMPETING INTERESTS

Lena Van den Eeden received a financial award from The Fondation Mustela.

Roche Belgium will provide laboratory reagents, free of charge.

FUNDING

No external funding has been awarded by a major funding body.

AUTHORS CONTRIBUTIONS

LVDE and YJ have made substantial contributions to the conception and the design of the study and have been involved in drafting the manuscript. NL, MB, VV & GS have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of

data. VDV & MB have been involved in revising it critically for important intellectual content. All authors have given final approval of the version to be published.

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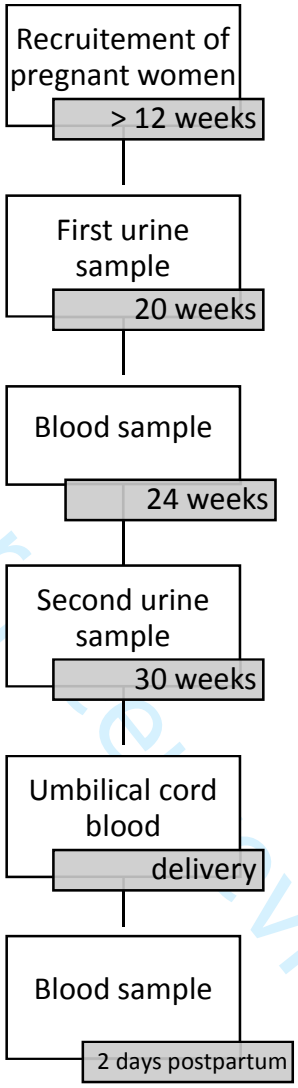

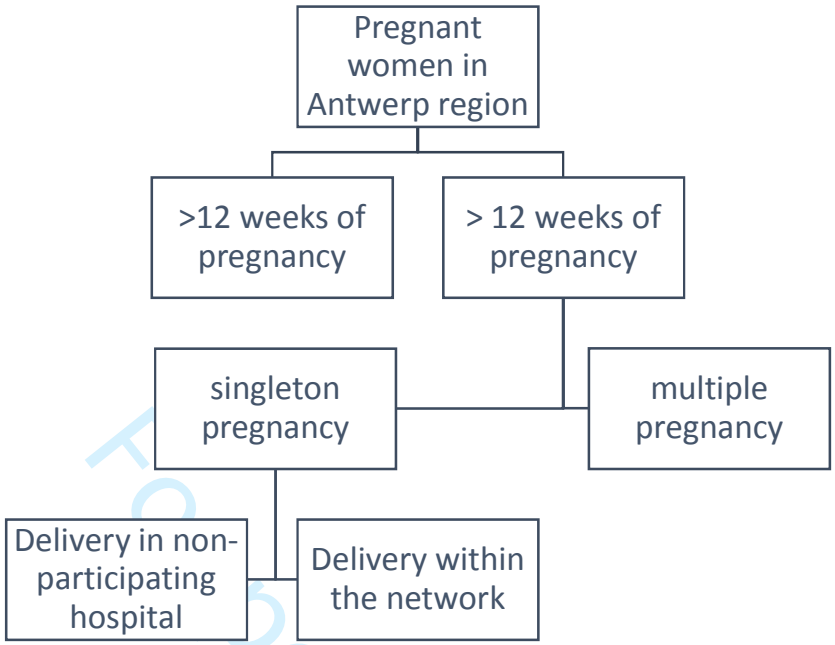

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Air pollution is hot topic and is known to cause multiple health issues. Especially pregnant women seem to be vulnerable to environmental issues. There are data suggesting that exposure contributes to hypertensive disorders.

This study aims to evaluate the effects of exposure to particulate matter and outdoor air pollutants on the clinical pregnancy outcome for mother and child and to determine which biochemical changes in maternal, placental and cord blood best explain this effect.

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A significant positive association between exposure to fine particulate matter during the third trimester and preeclampsia has been observed in a study of Davand et al among over 8,000 pregnant women in Barcelona, Spain [2].

Exposure to local traffic-generated air pollution during pregnancy is also known to increase the risk of preeclampsia and preterm birth [4]. Proximity to major roads is associated with an increased risk of preeclampsia, but not with a higher risk of gestational diabetes, placental abruption or placenta praevia [5].

Hypertensive disorders of pregnancy affect about 10% of all pregnant women. They are an important cause of severe morbidity and even mortality [6]. Pre-eclampsia complicates 2-8% of pregnancies and is a major contributor to maternal and neonatal mortality worldwide [7].

Pre-eclampsia is generally defined as de novo hypertension occurring after 20 weeks of pregnancy (with systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg), and proteinuria (≥ 300 mg in 24 h) [6–8]

The cause of pre-eclampsia still remains largely unknown, but a disturbed placental function, early in pregnancy has been suggested as an underlying mechanism. In normal pregnancy the uteroplacental arteries undergo a series of pregnancy specific changes in the first 20 weeks. Invasive trophoblasts replace endothelial cells and smooth muscle cells in the media, causing the arteries to loose elasticity and to dilate to wide tubes, without ability to contract effectively. They also lose their vasomotor control. This mechanism occurs in order to guarantee maternal blood supply to the placenta: the loss of maternal blood flow resistance and the increase of uteroplacental perfusion meet the requirements of the foetus [9]. In addition, circulating anti-angiogenic factors may play a role in the pathogenesis. The transmembrane protein vascular endothelial growth factor (VEGF) receptor fms-like tyrosine kinase 1 (Flt-1) binds with high affinity to VEGF and to placental growth factor (PlGF) [10]. Flt-1 is involved in normal

angiogenesis. Soluble Flt-1 (sFlt-1) is a variant of Flt-1 that lacks a transmembrane protein, and is a naturally occurring antagonist of VEGF and PlGF. Production of sFlt-1 appears to be expressed by endothelial cells and trophoblasts in response to reduced oxygen tensions [11,12]. In developing pre-eclamptic placenta the normal process of remodeling of the uterine spiral arteries is impaired, resulting in reduced perfusion, increased oxidative stress and inflammation [12].

Although there are yet no validated biomarkers that allow to identify women at risk for pre-eclampsia, levels of angiogenic and anti-angiogenic factors are altered in women with pre-eclampsia. Levels of sFlt-1 are elevated and levels of PlGF are decreased, even before clinical symptoms of disease were overt [13,14].The ratio of sFlt-1/PlGF is a promising set of biochemical markers for prediction of pre-eclampsia. There is a pathogenic difference between early pre-eclampsia (onset of disease before 32-34 weeks of pregnancy) and late pre-eclampsia (onset of disease after 32-34 weeks of pregnancy). Early pre-eclampsia is characterized by a significant placental dysfunction, leading to a high risk of intrauterine growth restriction. In late pre-eclampsia there is more evidence for a pre-existing maternal inflammation and/or cardiovascular maladaptation [7,15]The distinction is not always that clear, and is under debate.

Cystatin C, a protease inhibitor, is also increased in pre-eclamptic women. Cystatin C can be used as an endogenous marker for renal function, synthesized by all nucleated cells, at a constant rate and exclusively eliminated by glomerular filtration [16]. Saleh et al found that levels of cystatin C were significantly higher in the second trimester of pregnancy in women who developed pre-eclampsia [17]. Angiogenesis- and stress-related pathways may underlie the vascular effects of PM2.5 exposure during pregnancy [10,18,19]. Endothelin-1 is an established biomarker of endothelium dysfunction [20]. NFAT contributes to diverse functions and was identified as a critical component of VEGF-induced angiogenesis [21].

Higher exposure to $PM_{2,5}$ and O_3 during the first trimester are associated with preeclampsia, gestational hypertension and preterm delivery [22].

We hypothesize that exposure to $PM_{2,5}$ in the first trimester of pregnancy interferes with the maternal vascular remodeling process and that impaired remodeling between 9 and 18 weeks results in reduced placental perfusion and oxidative stress [23].

In 2011 the World Health Organization stated in a report that supplementation of calcium during pregnancy for women at high risk of developing pre-eclampsia is recommended [6]. They also state that low-dose acetylsalicylic acid (75 mg/day) is recommend in the same population. A recent study of Souza et al (2014) confirmed that supplementation with 100 mg aspirin plus 2 g calcium when started after 20 weeks of gestational age reduced the rate of superimposed pre-eclampsia and intra-uterine growth restriction in women with chronic hypertension and abnormal second-trimester uterine artery Doppler [24]. If this study can predict which women are at risk for hypertensive disorders during pregnancy or pre-eclampsia, supplementation of aspirin and calcium can be started in order to reduce perinatal risks.

Objectives and aims of the IPANEMA-study

This study aims to evaluate the effects of exposure to particulate matter and outdoor air pollutants on the clinical pregnancy outcome for mother and child and to determine which biochemical changes in maternal, placental and cord blood best explain this effect.

- Objective 1: Correlate estimated personal $PM_{2,5}$ exposures to angiogenesis related markers for hypertensive disorders in pregnant woman.
- Objective 2: Gain more insight in the pathophysiology behind cardiovascular effects of air pollution during pregnancy, plausible underlying molecular pathways will be assessed e.g. stress- and angiogenesis-related pathways.

METHODS AND ANALYSIS

Study design and setting

The IPANEMA is a regional prospective cohort study, recruiting pregnant women in the Antwerp region in Belgium.

Data are collected during pregnancy and at delivery, as well as immediately postpartum. Blood samples, hair samples and anthropometric measurements are obtained. Self-administered questionnaires are completed at different time frames in pregnancy, in relation to sample taking (see figure 1: Visits and interventions during the IPANEMA study.).

Study sample

Study cohort

Pregnant women are recruited within the network of the Antwerp University. Leading center will be the University Hospital Antwerp (UZA), a tertiary center with a maternal intensive care unit (MIC) and a neonatal intensive care unit (NIC) and 1000 deliveries a year.

The aim is to enroll 200 pregnant women over a 3 year recruitment period. An increase of 2% of pregnant women who develop pre-eclampsia or hypertensive disorders can be considered clinically relevant.

Diagnosis of pre-eclampsia (onset of a new episode of hypertension during pregnancy (with persistent diastolic blood pressure > 90 mmHg) with the occurrence of substantial proteinuria (> 0.3g/24h)) and hypertensive disorders are made using the WHO guidelines [6].

Eligibility criteria

The inclusion criteria are (see figure 2: Eligibility criteria)

- A singleton pregnancy
- > 12 weeks of gestational age
- Delivery planned in a hospital within the network of the Antwerp University

- Dutch speaking

The exclusion criteria are:

- Multiple pregnancy
- Planned home delivery or planned delivery in another hospital

Power calculation and statistical analyses

The incidence of preeclampsia in pregnancy is approximately 5%. An increase of 2% is of interest. A doubling in incidence is therefore clinically relevant. With a sample size of 200 subjects a doubling from 5 to 10 % can be detected with 80% power and 5% significance level.

Data analysis will be done using SPSS24.0.

Recruitment started in summer of 2015 and we aim to collect all participants by the end of 2018.

Study visits

Women are recruited by the midwife or obstetrician at 12 weeks of gestational age, typically at the second routine antenatal visit.

Signed informed consent for participating in the study is obtained before the start.

Blood collections (maternal and umbilical cord)

Venous blood is drawn around 24 weeks of gestational age (table 1).

Table 1 Tests on maternal blood sample

Routine blood sampling (UZA)	Urea, Creatinin, CRP, LDH, AST, ALT, Uric acid, APTT, PT, Fibrinogen, D-dimers, Glucose, Erythrocytes/hematocrit, Hemoglobin, Thrombocytes, Leukocytes, Ferritin, Toxoplasmosa (IgG & IgM), Cytomegalovirus (IgG & IgM), Herpes simplex IgG, Varicella zoster IgG, Rubella IgG, Parvovirus (IgG &

	IgM), Syphilis (RPR & TPHA), Indirect Coombs
Biomarkers of pre-eclampsia	sFlt-1, PlGF, Cystatin C, Endothelin
Molecular pathway and transcript markers	m(i)RNA expression, telomere length, DNA methylation

Umbilical cord blood is taken at time of birth (table 2).

Table 2 Tests on umbilical blood sample

Transcript markers	m(i)RNA expression, telomere length, DNA methylation

*Measurement of molecular pathway and transcript markers*For analysis of the m(i)RNA profile, whole blood is collected in Tempus™ Blood RNA tubes (Applied Biosystems) and stored at -80°C until analysis. Telomere length and DNA methylation will be assessed on blood samples collected in EDTA tubes, which are stored at -80°C.

In order to gain more insight in pathophysiological pathways, relevant biomarkers and transcript markers will be analysed. Specific transcript markers will be selected that were previously associated with exposure to particulate matter, intrauterine growth retardation or stress- and angiogenesis pathways e.g. Glucocorticoid Receptor Signaling pathway, Nuclear Factor of Activated T cells (NFAT) signaling pathway, … .[25].

Urine collections

Urine samples are taken by the pregnant women themselves, after a minimum of 8 hours fasting (morning urine). Samples are handed to the midwife at the antenatal visit, and placed at -20°C

freezer within 12 hours of collection. Analysis will be performed at the Flemish Institute for Technological Research (VITO).

Two urine samples are collected: at 20 weeks and at 30 weeks gestational age. 8 oxo deoxyguanosine will be determined in the samples.

Hair collections

Being incorporated into the growing hair, Hair Cortisol Concentrations (HCC) provide a retrospective reflection of integrated cortisol secretion over periods of several months [26].

Hair samples are drawn by the midwife around 24 weeks of gestational age and three days after delivery. Long periods of stress are associated with increased hair cortisol concentrations [26] . Confounding variables are low maternal education, season of delivery, smoking during pregnancy and obesity [27]. Titanium scissors are cleaned with denaturated ethanol and the midwife wears disposable gloves in order to limit contamination. A lock of hair with a thickness of a match (2 mm) and a length of 4 cm will be taken and put in an envelope. Analysis will be performed at the University of Southern Denmark.

Statistical analysis

Regression models will be calculated. In particular, birth weight will be correlated with PM2.5 values using linear regression, pre-eclampsia with blood pressure evolution and PM2.5 using logistic regression, birth weight, birth weight (dichotomised) and preeclampsia with sFlt-1, plGF, Cystatin C and PM2.5 using linear and logistic regression respectively and birth weight (dichotomised) versus inflammatory parameters using logistic regression. The effect of the following a priori covariates will be analysed: history of pre-eclampsia, diabetes, BMI at start,

age, smoking, intake of low dose aspirin and preexisting hypertension). These factors will however not be a reason for exclusion.

Questionnaires

There are four different questionnaires:

- *Questionnaire on general habits, socio-economic factors, lifestyle and eating habits*

  We use a self-designed questionnaire to extensively collect information on ethnic origin and education level, on employment, income and work environment, on family history of diseases and chronic disorders, on eating habits, on previous pregnancies and other habits (smoking, alcohol consumption).

- *Questionnaire on lifestyle during previous 3 days*

  We use a self-designed questionnaire to extensively collect information on contact with possible toxic factors and on eating pattern of the previous 3 days.

- *Questionnaire on residential facts*

  We use a self-designed questionnaire to collect information about the exact location of the bedroom window and other possible factors influencing concentrations of particulate matter.

- *Questionnaire on stress factors and birth facts*

  We use a self-designed questionnaire to extensively collect information on stress levels during pregnancy, on birth facts and on medication during pregnancy.

Questionnaires will be taken at different visits (table 3)

Table 3 Questionnaires

	< 12 weeks	20 weeks	30 weeks	birth
Questionnaire on general habits, socio-economic factors, lifestyle and eating habits	Recruitment			
Questionnaire on lifestyle during previous 3 days		Urine sample 1	Urine sample 2	
Questionnaire on residential facts			4-8 weeks prior to visit	
Questionnaire on stress factors and birth facts				3 days after birth

Additional clinical information and data about medication will be obtained by detailed data extraction from the hospital records.

Outcomes of the study

Diagnosis of cardiovascular disorders

Pre-eclampsia is defined as a the onset of a new episode of hypertension during pregnancy (with persistent diastolic blood pressure >90mmHg) with the occurrence of substantial proteinuria (>0.3 g/24h).

Pregnancy and delivery outcome data

Maternal data that are prospectively collected are: pregnancy duration, pre-eclampsia, hypertensive disorders,

Delivery data are: type of labor, type of delivery

Neonatal data are: birth weight, preterm delivery, Apgar score on 1, 5 and 10 minutes, congenital anomalies,…

Other study measurements

Measurement of sFlt-1, PlGF and Cystatin C

Blood samples taken at the study visit around 24 weeks of gestational age for the analyses of sFlt-1, PlGF and Cystatin C are collected, processed and aliquoted within 24 hours of collection. The serum samples are stored at -80 °C until analysis at Algemeen Medisch Laboratorium (AML). sFlt-1 and PlGF will be determined using an electrochemiluminescence based sandwich immunoassay on Cobas e 411 (Roche Diagnostics, Mannheim, Germany) [28,29] Cystatin C will be determined using a particle-enhanced immunonephelometric assay (N Latex Cystatin C, Siemens Healthcare Diagnostics, Marburg, Germany) by use of a BN II nephelometer (Siemens Healthcare Diagnostics). This assay has a calibration traceable to the first certified reference material for cystatin C in human serum (ERM-DA471/IFCC) [30].

ETHICS AND DISSEMINATION

We obtain informed consent from each participant prior to enrollment in the study.

The study has received approval by the Ethical Committee of the Antwerp University Hospital (14/40/411) and is registered with ClinicalTrials.gov (14/40/411) on 22-10-2015.

Results from this study will be disseminated at regional and international conferences and in peer-reviewed journals.

DISCUSSION

IPANEMA is the first prospective study to assess the impact of particulate matter on mothers and babies in Antwerp, Belgium.

Exposure to particulate matter is debated on the political scene but robust data showing a relationship with hypertensive disorders of pregnancy are difficult to find. In Antwerp the design of a safe and functional tunnel for the highway is now focus of public discussion. Following the Barcelona experience a group of people want an urbal tunnel complex to lower the amount of particulate matter.

IPANEMA would like to create robust data on the correct impact of air pollution, in particular on pregnant women, a vulnerable population. IPANEMA uses prospective data from the beginning of the pregnancy till childbirth.

Available studies have been highly biased, probably in favor of detecting an effect even if this is minimal, by their retrospective nature (missing eg women who left the area before giving birth), not correcting for socioeconomic or ethic influences, using very raw and approximate models managing up to several square kilometers as identical areas of pollution, not compensating for time spent in traffic, at work or in other regions during weekends and holidays. Furthermore the discussion on the impact of air pollution had been partially taken over by politics, without robust data. Data from IPANEMA will enable to estimate the real individual dose effect relation for pregnant women.

AVAILABILITY OF DATA AND MATERIALS

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

COMPETING INTERESTS

Lena Van den Eeden received a financial award from The Fondation Mustela.

Roche Belgium will provide laboratory reagents, free of charge.

FUNDING

No external funding has been awarded by a major funding body.

AUTHORS CONTRIBUTIONS

LVDE and YJ have made substantial contributions to the conception and the design of the study and have been involved in drafting the manuscript. NL, MB, VV & GS have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data. VDV & MB have been involved in revising it critically for important intellectual content. All authors have given final approval of the version to be published.

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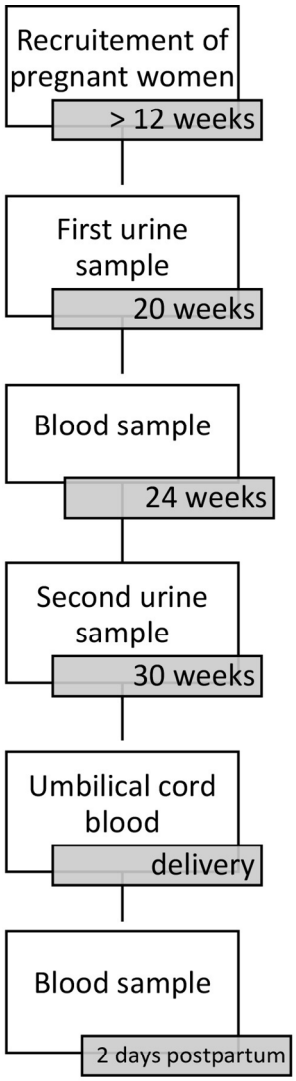

Visits and interventions during the IPANEMA study

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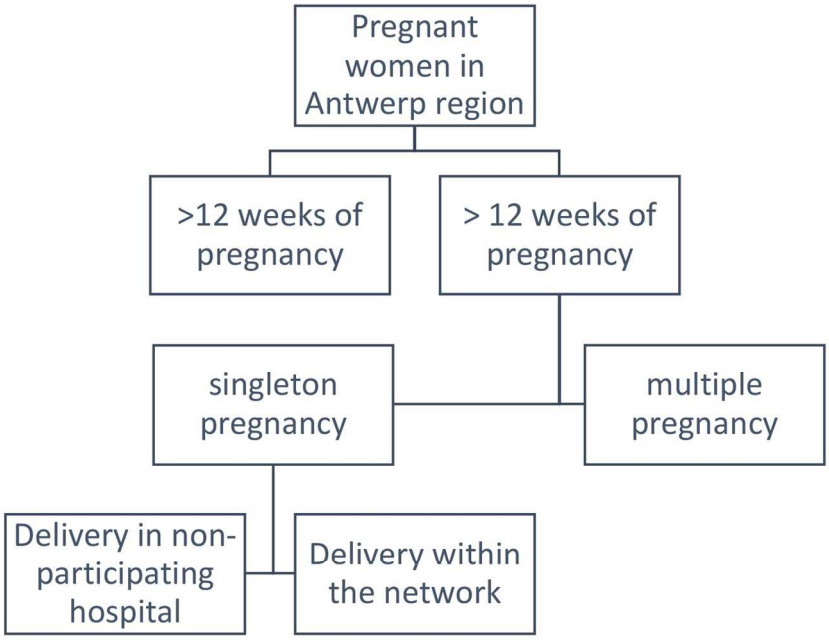

Eligibility criteria

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