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## **BMJ Open**

### Evaluating offspring genomic and epigenomic alterations after prenatal exposure to cancer treatment in pregnancy (GE-CIP): a multicentric observational study

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SCHOLARONE<sup>™</sup> Manuscripts Evaluating offspring genomic and <u>epigenomic</u> alterations after prenatal exposure to <u>cancer</u> treatment <u>in pregnancy</u> (GE-CIP): a multicentric observational study

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### Abstract

**Introduction:** Around 1 in 1000-2000 pregnancies are affected by a cancer diagnosis. Previous studies have shown that chemotherapy during pregnancy has reassuring cognitive and cardiac neonatal outcomes, and hence has been proposed as standard of care. However, although these children perform within normal ranges for their age, subtle differences have been identified. Given that chemotherapeutic compounds can cross the placenta, the possibility that prenatal chemotherapy exposure mutates the offspring's genome and/or epigenome, with potential deleterious effects later in life, urges to be investigated.

**Methods and analyses:** This multicentric observational study aims to collect cord blood, meconium and neonatal buccal cells at birth, as well as peripheral blood, buccal cells and urine from infants when 6, 18 and/or 36 months of age. Using bulk and single-cell approaches, we will compare samples from chemotherapy-treated pregnant cancer patients, pregnant cancer patients not treated with chemotherapy, and healthy pregnant women. Potential chemotherapy-related newborn genomic and/or epigenomic alterations, such as single nucleotide variants, copy number variants and DNAmethylation alterations, will be identified in mononuclear and epithelial cells, isolated from blood, buccal swabs and urine. DNA from maternal peripheral blood and paternal buccal cells will be used to determine *de novo* somatic mutations in the neonatal blood and epithelial cells. Additionally, the accumulated exposure of the fetus, and biological effective dose of alkylating agents, will be assessed in meconium and cord blood via mass spectrometry approaches.

**Ethics and dissemination:** The Ethics Committee Research of UZ/KU Leuven (EC Research) and the Medical Ethical Review Committee of University Medical Center Amsterdam have approved the study. Results of this study will be disseminated via presentations at (inter)national conferences, through peer-reviewed, open-access publications, via social media platforms aimed to inform patients and health care workers, and through the website of the International Network on Cancer, Infertility and Pregnancy (INCIP; <u>www.cancerinpregnancy.org</u>).

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### Strengths and limitations of this study

- This first study on identifying a genotoxic footprint in DNA of newborns that were prenatally exposed to chemotherapy relies on a variety of innovative bulk as well as single-cell techniques allowing the assessment of different types of genetic and epigenetic aberrations.
- In this study, different tissue types are investigated, i.e. cord blood and buccal epithelial cells, • which are both easy accessible and non-invasive sources of newborn DNA.
- This study will only provide information on the potential association, and not causal relation, between prenatal exposure to chemotherapy and genetic and/or epigenetic alterations in the newborn's DNA.
- , μ chemo. this study prot. of other drugs that . Since pregnant cancer patients, when treated with chemotherapy during pregnancy, usually • receive a cocktail of different chemotherapeutic agents, the effect of a single agent cannot be evaluated in this study.
- The methods presented in this study protocol can be applied for assessing the genotoxic effects to newborn DNA of other drugs that are applied during pregnancy.

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### Introduction

Cancer is the second leading cause of death during the reproductive years and complicates between 1 in 1000 to 2000 pregnancies (1). As women in developed societies tend to delay childbearing to the third or fourth decade of life, and since the incidence of several malignancies rises with increasing age, this rare coincidence is likely to become more common. In the past decades, the establishment of (inter)national registries, including the International Network on Cancer, Infertility and Pregnancy (INCIP), and an increasing number of smaller cohort studies, have provided insights for the management of cancer during pregnancy. Applying chemotherapy in the first trimester is to be avoided due to the increased risk of developing congenital malformations. Therefore, it is advised to start chemotherapy in the second or third trimester, i.e. after week 14 of pregnancy. Previous studies have shown that chemotherapy during the second and/or third trimester of pregnancy does not affect the short-term cardiac and neurocognitive development of children, which is reassuring (2-6). Yet, prenatal exposure to certain chemotherapeutic drugs has been associated with a higher risk for children being born small for their gestational age (1,7). Furthermore, although overall, children born from pregnancies complicated by maternal cancer perform within normal ranges for their age, chemotherapy-exposed children had a significantly lower verbal IQ at 6 year, and were 3 times more likely to wear glasses than non-exposed children. However, since there are many confounding factors in neurocognitive development of children, a direct link with prenatal chemotherapy exposure cannot be accurately established (1,2,4,5).

To date, data on long-term outcomes in these children are scarce, and thus, it remains unknown whether prenatal exposure to chemotherapy may provoke an increased risk of developing fertility issues, chronic health conditions or cancers later in life. Previous *in vivo, ex vivo* and clinical studies have shown that chemotherapeutic agents can cross the placental barrier (at passage rates that vary per drug) (8–14). Additionally, it is now well-known that chemotherapy treatment can leave a genotoxic footprint, consisting of point mutations and/or larger chromosomal abnormalities (15–18) and induce long-lasting epigenetic changes, such as alterations in the DNA methylation profile (19–21) in cancer tissue and even non-cancerous cells of cancer patients. Based on the above, it can be speculated that chemotherapeutic agents, administered during pregnancy, could target and damage the fetal DNA. This, in turn, could interfere with the offspring's long-term health. The aim of the methods presented in this protocol is to investigate the possibility of whether, and how, prenatal chemotherapy exposure is associated with genetic and epigenetic aberrations in the newborn DNA, and, if identified, how these aberrations evolve in early childhood years.

### Methods and analyses

### Objectives

The primary objectives of this study are (i) to determine whether chemotherapy administered during pregnancy affects the genome and/or epigenome of newborn cells, by identifying and contrasting acquired genetic and epigenetic alterations in cord blood and buccal cells derived from chemotherapy-treated and -untreated pregnant cancer patients, and healthy pregnant women, and (ii) to examine if the potential chemotherapy-related genomic and/or epigenomic alterations in newborn DNA are detectable at different time points, whether the mutation rate is altered, and if these changes lead to more complex downstream events in early childhood years. by analyzing peripheral blood cells and

epithelial cells from buccal and urinary tract tissue of infants aged 6, 18 and/or 36 months, and comparing the results with those obtained at birth. A secondary objective is to correlate the possibly identified genomic and/or epigenomic changes to the cumulative dose of chemotherapeutic compounds, and -adducts, measured in cord blood plasma and meconium samples taken after delivery.

### Study Cohort and eligibility criteria

This multicenter, prospective observational study will be carried out in Belgian and Dutch hospitals participating in the International Network on Cancer, Infertility and Pregnancy (INCIP, <u>www.cancerinpregnancy.org</u>). Eligible participants are women with (histologically proven) cancer of any type and stage during pregnancy and an ongoing pregnancy (≥ 24 weeks of gestation). The study group will consist of pregnant women who were diagnosed with cancer and treated with chemotherapy during pregnancy (termed "chemotherapy-treated pregnant cancer patients"). The control group will be pregnant women diagnosed with cancer during pregnancy who were not treated with chemotherapy during pregnancy (termed "chemotherapy-untreated pregnant cancer patients"). A second control group will consist of healthy pregnant women. For each included pregnant cancer patient, one healthy control case will be recruited from the general obstetric department of the hospital of inclusion or affiliated midwife practice, matched for the following criteria: maternal age, gestational age at birth and sex of the child.

Key eligibility criteria are listed in Table 1. For each patient, demographic (i.e. maternal and paternal age), obstetric (i.e. gestational age at delivery, sex newborn) and oncological (i.e. tumor type, subtype and staging, presence of a DNA repair gene mutation, chemotherapy schedule and cumulative dose, gestational age at diagnosis, gestational age at the start of the treatment, gestational age at the last chemotherapy administration, type of surgery, gestational age at surgery) data will be retrieved via the INCIP registry (pregnant cancer patients) or medical file (healthy pregnant women). Furthermore, biomaterials from eligible pregnant women will be collected in the respective participating hospitals and shipped to Catholic University Leuven for central processing. Lastly, a questionnaire is requested to be filled in at time of delivery (Supplementary 1).

	Inclusion criteria	
Study group: chemotherapy-treated pregnant cancer patients	Control group 1: chemotherapy-untreated pregnant cancer patients	Control group 2: healthy pregnant women
<ul> <li>Histologically proven cancer during pregnancy (any type and stage)</li> <li>Treatment during pregnancy with one or a combination of the following chemotherapeutic agents: Cyclophosphamide, Anthracyclines, Taxanes, Platinum derivates</li> <li>Gestational age (GA) at birth ≥24 weeks</li> </ul>	<ul> <li>Histologically proven cancer during pregnancy (any type and stage)</li> <li>No treatment during pregnancy or surgery only (subgroup 1)</li> <li>Radiotherapy and/or systemic treatment (other than chemotherapy) during pregnancy (subgroup 2)*</li> <li>GA at birth ≥24 weeks</li> </ul>	<ul> <li>Matched for maternal age gestational age at birth and infant sex with chemotherapy-treated group</li> <li>GA at birth ≥24 weeks</li> </ul>
	Exclusion criteria	

Any comorbidity that is associated with an increased micronucleus frequency, such as hypertensive disorders, preeclampsia, (gestational) diabetes, renal or cardiac pathology
 \*Subgroups 1 and 2 will be analyzed as separate control groups.

### Sample collection and processing

At, or shortly after birth, the following samples will be collected: cord blood, newborn buccal swabs, meconium (obtained during the first 48h of life), maternal peripheral blood and paternal buccal swabs. Further details on the use of these samples will be described in the respective assay sections below.

At the child's age of 6, 18 and/or 36 months, the following samples will be collected: child's peripheral blood, buccal swabs and urine samples. Potential parents' objection to sampling one of these biomaterials does not preclude further participation to the study.

All biomaterials are collected at the respective participating hospitals and shipped to Catholic University Leuven for central processing. Blood samples are to be shipped within 24 hours after collection for immediate processing. Buccal swabs, meconium and urine samples can be stored at the respective participating hospitals for bulk shipment at a chosen time. The conditions of transport, processing and storage for the different specimens are summarized in Table 2.

Table 2. Sample collection, transport, processing and storage details for the different specimens.			
Specimen	Sampling and storage conditions	Goal	
Cord blood and maternal blood	Heparin tube; direct shipment to central lab at room temperature	Direct isolation of blood mononuclear cells via density gradient separation and immediate use in the CBMN assay	
Cord blood and maternal blood	EDTA tubes; direct shipment to central lab at room temperature	Direct isolation of blood mononuclear cells via density gradient separation and storage in liquid nitrogen until further processing for sequencing to determine genetic aberrations	
		Serum and sediment separation and storage at - 80°C until LC/MS assessment of the biological effective chemotherapy dose (serum) and identification of epigenetic alterations (sediment) via bisulfite sequencing and methylation array	
Buccal Swabs	Isohelix™ Buccal Swabs; local storage at -20ºC	DNA extraction for the assessment of chromosomal abnormalities via low-pass sequencing and copy number profiling and identification of epigenetic alterations via bisulfite sequencing and methylation array	
Meconium	Cryotube; local storage at -20°C	Assessment of accumulated dose of chemotherapeutic compounds by MS	
	Cryotube; local storage at -20°C	DNA extraction for the assessment of the biological effective doses by LC/MS	
Urine	Urine bag; local storage at -20°C	Epithelial urinary tract cells for exploratory genetic analyses in early childhood years using single cell WGS and cell-free DNA analyses	
CBMN: cytokinesis-block micronucleus; EDTA: ethylenediaminetetraacetic acid; LC/MS: liquid			
chromatography–mass spectrometry; MS: mass spectrometry.			

### Sample size per group

Yearly, an average of 10 pregnant cancer patients are registered per participating center, of which 50% are treated with chemotherapy during pregnancy. Therefore, we envision to include 20 chemotherapy-treated pregnant cancer patients, 20 chemotherapy-untreated pregnant cancer patients and 40 healthy pregnant women per year. Since this is an exploratory study, with scarce data to rely on for

solid sample size calculations, post-hoc sample size calculations will be conducted after analyses of the first 10 subjects per group.

### Study Design

A schematic representation of the study design, patient groups, collected biomaterials and molecular analyses is given in Figure 1.



**Figure 1.** Scheme depicting the types of biomaterials that will be collected and the assays being performed. This scheme will be applied on every participant of each of the 3 study groups, i.e. chemotherapy-treated pregnant cancer patients, chemotherapy-untreated pregnant cancer patients, and healthy pregnant women. CBMN: cytokinesis-block micronucleus assay; LC/MS: liquid chromatography-mass spectrometry; MS: mass spectrometry; qPCR: quantitative polymerase chain reaction; WGS: whole genome sequencing.

#### **Study Methods**

### 1. Measuring genetic and epigenetic alterations in newborn cells

### 1.1 Studying the link between prenatal exposure to chemotherapy and a 'genotoxic footprint' in newborn cells

First, we will evaluate the presence of chemotherapy-associated gross chromosomal abnormalities in newborn cells, using two different techniques: (i) The cytokinesis-block micronucleus (CBMN) assay, and (ii) shallow whole-genome sequencing (WGS). The CBMN assay will be performed on T-lymphocytes, isolated from both cord blood and maternal peripheral blood (the latter as a positive control) from all participants. The CBMN assay will be performed as described by Fenech *et al.* (22). From each sample, a total of 1000 mononuclear cells will be microscopically scored to determine the micronucleus frequency (MNF) per 1000 binucleated T-lymphocytes. Shallow WGS will be performed using DNA isolated from mononuclear cells from cord blood.

In parallel to evaluating blood samples, we will also assess the presence of such *de novo* chromosomal abnormalities in DNA isolated from the newborn's buccal epithelial cells via low-pass sequencing (0.1x read depth) and copy number profiling (23). Maternal peripheral blood DNA and paternal buccal epithelial DNA will be used as reference to allow determining *de novo* (sub)chromosomal alterations in cord blood DNA. To maximize participation from the fathers, we prefer a non-invasive buccal swab sampling (for downstream DNA isolation) over blood sampling.

Second, we will investigate the possibility that chemotherapy-induced DNA alterations may be private to a single cell (24). We will do this using two **single-cell sequencing** techniques: (a) single-cell genome-plus-transcriptome-sequencing (G&T-seq) of single mononuclear cord blood cells to map structural

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variants, such as (sub)chromosomal deletions, amplifications and/or translocations (25,26), and, since current single-cell sequencing approaches are not yet optimal for accurate detection of point mutations, (b) WGS deep sequencing (paired-end, 15x) of clonal organoid cultures established from individual cord blood stem cells to detect single nucleotide variants (SNVs) and small insertions and deletions (indels) (27). Furthermore, this WGS information will be used to estimate telomere lengths in cord blood stem cells.

#### 1.2 Studying the link between prenatal exposure to chemotherapy and DNA-methylation changes in newborn cells

To study potential epigenetic changes associated with prenatal chemotherapy exposure, DNA will be isolated from the sediment fraction of cord blood and epithelial cells from buccal swabs of newborns. Then, the extracted DNA will be subjected to "oxidative" bisulfite conversion. After bisulfite sequencing, the DNA methylation status will be analyzed via array-based methylation assessment methods. Using bio-informatics, we will evaluate how identified changes in methylation signatures in newborn DNA are related to specific chromatin organizational states. Furthermore, to investigate the downstream effects of methylation changes, gene and pathway analyses will be performed, which allows assigning functional meanings to genes and genomes. Medication use of the mother up until 24 hours prior to delivery will be documented in the questionnaire (Supplementary 1), since this might influence the methylation profiles and needs to be taken into account during analyses.

#### 1.3 Studying the link between prenatal exposure to chemotherapy and gene-expression changes in newborn cells

To study potential gene-expression alterations associated with prenatal chemotherapy exposure, RNA will be isolated from single mononuclear cord blood cells (G&T-seq, cfr. 1.1), the sediment fraction of cord blood and epithelial cells from buccal swabs of newborns, and subjected to gene-expression analyses through single-cell RNA sequencing (scRNA-seq). DNA loci with significant differences in methylation status (cfr. 1.2), that correlate to transcribed genes and for which the transcript sequence is known, will be validated further via (single-cell) quantitative PCR (qPCR) approaches. Rationale for selection will include functional significance as well as statistical differences between chemotherapy-treated and chemotherapy-untreated samples.

### 2. Measuring chemotherapy concentrations and biological effective dose

To measure the concentration of applied chemotherapeutic agents and/or their metabolites, mass spectrometry (MS) will be used. The analytical protocol will be developed for blood, plasma, meconium and urine samples.

Additionally, for alkylating agents, the biological effective doses in the same biological matrices will be determined via measurement of DNA adducts, using liquid chromatography–mass spectrometry (LC-MS). As an indicator for DNA adducts in patients receiving alkylating agents (e.g. cisplatin, carboplatin and/or cyclophosphamide treatment), we will focus on the identification of bis-N7-guanine DNA-DNA cross-links. Maternal peripheral blood will be taken as a reference. Medication use of the mother up until 24 hours prior to delivery will be checked in the questionnaire (Supplementary 1), since this might interfere with the detection of chemotherapy compounds in both the cord and maternal blood samples.

### **3.** Examining the evolution of observed genomic and/or epigenomic alterations in infant cells If we identified genomic and/or epigenomic changes in DNA of newborns that were prenatally exposed to chemotherapy compared to newborns born to women in the control groups (see 1.1 and 1.2), we

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will monitor whether these genomic and/of epigenomic alterations are detectable at different time points, whether the mutation rate is altered, and if these changes lead to more complex downstream events in early childhood years. Therefore, we will sample peripheral blood cells and buccal epithelial cells from the infants of all 3 groups, at the ages of 6, 18 and/or 36 months and analyze the samples using the same techniques as mentioned above. To gain additional insight in the specific blood cell types that may be affected, we will perform single-cell WGS on different types of differentiated blood cells, separated via flow cytometry, from the chemotherapy-treated pregnant cancer patient group, complemented with phylogenetic tree construction in blood cells (27).

Additionally, to investigate the range of tissue types that might be affected by prenatal exposure to chemotherapy, we will initiate exploratory genotoxicity analyses (using whole-genome single-cell sequencing and cell-free DNA analysis) in epithelial cells originating from the child's urinary tract.

#### Statistical analysis

Given that no published evidence exists to rely on, all statistical analyses are exploratory. Analysis will be performed with R Studio using 95% confidence intervals and a two-tailed *P*-value of 0.05. Continuous variables will be compared between the different patient groups to determine the potential association with prenatal chemotherapy exposure using Kruskal Wallis or ANOVA, depending on normality of the data. Linear regression models will be used to study the effect of chemotherapy-exposure on genotoxicity and methylation grade in the study groups, while adjusting for potential confounders such as maternal age, gestational age at delivery and sex of the newborn. Subgroup analyses related to the different chemotherapeutic compounds will be done when possible. Where necessary corrections for multiple testing will be applied.

#### Patient and public involvement statement

The ongoing research on cancer during pregnancy performed by the INCIP network was started based on the request of pregnant women who were confronted with a cancer diagnosis during their pregnancy. Clinical questions about how the treatment during pregnancy would affect their unborn child formed the basis of several studies investigating aspects of the children's development after birth. We aim to keep our patients informed of the research results by sharing them via our national social media platforms (www.kankerenzwangerschap.be; patient www.facebook.com/kankerenzwangerschap) and via the INCIP network (website www.cancerinpregnancy.org).

#### **Secondary findings**

As this study will not change treatment, nor randomize patients, participation in this study will not affect patients' outcomes. As this is an observational study, no additional risks from the study are expected. As described above, for the study of (sub)chromosomal abnormalities and mutations in the newborn's and infant's tissue (cord/peripheral blood, and buccal cells) potentially being associated with prenatal exposure to chemotherapy, only *de novo* alterations will be taken into account. The findings will be interpreted with the support of a clinical geneticist. Secondary findings might include genetic mutations, including point mutations and larger structural variants that could imply any pathogenicity, and will be managed conform the latest guidelines from the American College of Medical Genetics (28).

 Mutations causing highly penetrant disorders with validated evidence on the phenotype associated with the deletion or duplication. These are considered clinically relevant and will be reported.

- Mutations proven to be risk factors for developmental disorders with reduced penetrance and/or variable expression. The predictability of the future phenotype resulting from such CNVs remains very poor. Therefore, these susceptibility CNVs will not be reported.
- Mutations causing late-onset genetic disorders, typically cancer caused by the deletion of a tumor suppressor gene, will be communicated if undeniable health benefit can be expected for the participants (child or parent).

It is important to note that, by restricting the analyses to *de novo* findings in newborn's/infant's DNA, the number of 'to be reported secondary findings' is estimated to be limited.

Also, in the informed consent form, participants will be given the opportunity to opt out of being notified of secondary findings related to the newborn DNA.

### Availability of data and material

All clinical data are registered in a REDCap database. The participating physician ensures the confidentiality, accuracy, completeness, legibility and timeliness of the data recorded. Data handling and statistical analysis will be done in a coded fashion by the investigator, with the subject identification code list only available to the local investigator (and research nurse if applicable) working in the local center. The collection, processing and disclosure of personal data, such as patient health and medical information is subject to compliance with applicable personal data protection and the processing of personal data legislation (including, but not limited to the EU Directive 95/46/EC and Belgian Law of December 8, 1992 on the protection of the Privacy in relation to the Processing of Personal Data). Data and rest material (e.g. left-over DNA/RNA extracts) will be kept for 20 years. If archived samples may be useful for additional investigations, not mentioned in the current protocol, approval will be asked from the Ethical Committee. The genome-wide sequencing data are stored for 3 months at Genomics Core (KU Leuven) and subsequently transferred to the common lab drive. Similarly, data from the CBMN assay and the questionnaire are stored there. This lab drive is pass-word protected, with limited access to authorized persons.

### Ethics and dissemination

The study protocol was reviewed and approved by the Ethics Committee Research UZ / KU Leuven (EC Research) (S62388) and the Medical Ethical Review Committee of University Medical Center Amsterdam (NL76873.018.21\_S62388).

Eligible subjects will be asked, together with their partner, to sign a written informed consent form upon agreement to participate in the study. Participants are allowed to leave the study at any time for any reason, without any consequences, and are able to opt out for donating a particular sample type. The investigator can also decide to withdraw a subject from the study for urgent medical reasons. Participants in the study will not influence the clinical management of the patient. Furthermore, participants will be given the opportunity to opt out of being notified of secondary findings related to the newborn DNA, which they could indicate in the informed consent form. All data will be handled with strict confidentiality, assuring anonymity of the subjects.

Dissemination of the research results will be done via presentations at national and international conferences and seminars, and through the publication of peer-reviewed manuscripts in open-access journals. Additionally, we will communicate findings through our national patient social media platforms (www.kankerenzwangerschap.be; <u>www.facebook.com/kankerenzwangerschap</u>) and via the INCIP network (website <u>www.cancerinpregnancy.org</u>), created to increase knowledge among

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59 60 scientists, health care workers and the public (including patients). Further, the communication offices of both the Leuven Cancer Institute (LKI) and of KU Leuven guarantee optimal communication between KU Leuven and University Hospitals Leuven on the one hand, and outside university. Finally, a webbased software (Advisory Board Cancer In Pregnancy - ABCIP) was developed to offer remote multidisciplinary support to physicians that lack expertise to manage the treatment of cancer during pregnancy. Additionally, this platform will be used to distribute novel research insights to caregivers involved in the management of pregnant cancer patients.

### Discussion

This study will be the very first to explore a potential genotoxic effect of prenatal chemotherapy exposure to the newborn's DNA. We anticipate that the use of a variety of techniques, allowing the assessment of different types of genotoxicity, and the inclusion of pregnant cancer patients treated with different treatment regimens, will give an unprecedented insight in treatment-dependent genotoxic footprints, and link this with the level of accumulated dose of chemotherapeutic compounds in the fetus. Genomic insults in the perinatal period have been linked to the development of diseases, such as cancer and neurodevelopmental disorders, and may be more important than in other life stages given the higher probability that mutated and genomically unstable cells could populate rapidly growing tissues of an infant (29,30). This project will address the question of whether prenatal chemotherapy-treatment is associated with a 'genotoxic footprint' in newborn tissue, at the earliest time point in life, and whether and how this evolves in early-childhood years. The combined analysis of signatures of point mutations and larger structural variants might also reveal information about the potential mechanisms underlying the identified aberrations. If positive, our findings will give an indication of which functional genomic regions and pathways might be affected. Data on the safety of administering chemotherapy during pregnancy are highly warranted, as both clinicians and patients remain reluctant about this issue. If we would not find any 'genotoxic footprint', this would indicate DNA abnormalities following prenatal chemotherapy exposure occur at a very low frequency, suggesting a limited clinical impact. Alternatively, a positive outcome may trigger further fundamental and clinical research to further asses the clonality of observed aberrations, the most affected cells and the potential clinical impact. Though preliminary, the results of this study may aid in the identification of potentially harming agents to the unborn child, and hence provide an opportunity to optimize therapeutic regimens for pregnant women confronted with cancer.

### Abbreviations

CBMN: Cytokinesis-block micronucleus; CIP: Cancer In Pregnancy; CNV: Copy Number Variation; GE-CIP: Genomic and Epigenomic alterations after Cancer treatment In Pregnancy; indels: insertions and deletions; INCIP: International Network on Cancer, Infertility and Pregnancy; LC-MS: Liquid chromatography-mass spectrometry; MNF: micronucleus frequency; MS: Mass Spectrometry; scRNAseq: single-cell RNA sequencing; SNV: single nucleotide variant; WGS: Whole Genome Sequencing; qPCR: quantitative Polymerase Chain Reaction

### Author contributions

IS, LL, VW, CV and FA made substantial contributions to the design and writing of the study protocol. IS, LL and CV will be conducting the sample collection and different study parts. FA is the founder of INCIP and will be, together with KvC, informing and including the eligible patients. LG, BT, RvB and TV gave their expert opinion in designing genetic and epigenetic analyses of the biomaterials. KD will be consulted when secondary findings occur. HS was consulted to work out the follow-up study parts. All authors were involved in revising the manuscript draft and approved the final manuscript.

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### **Competing interests statement**

TV is co-inventor on licensed patents WO/2011/157846 (Methods for haplotyping single cells), WO/2014/053664 (High-throughput genotyping by sequencing low amounts of genetic material) and WO/2015/028576 (Haplotyping and copy number typing using polymorphic variant allelic frequencies).

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Ziezony

For peer review only - http://bmjopen.bmj.com/site/about/guidelines.xhtml

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### Supplementary 1

### Questionnaire protocol GE-CIP study (S62388)

Patient ID:	•	
Date:		

### 1. Do you smoke or have you ever smoked?

- Never
- I quit smoking before I got pregnant
- □ I smoked during my pregnancy; amount: ..... cigarettes/week

### 2. Did you or someone else smoke indoors during your pregnancy?

- □ No
- 🗆 Yes

### 3. Did you take any medication/supplements/vitamins during your pregnancy? (E.g. folic acid, vitamin D, zinc supplements)

- □ No
- $\square$  Yes

If yes, please enter the details in the table below:

Medication/vitamin/supplement	Pregnancy timeframe
	week up to week

### 4. Do you drink or have you drunk alcohol during your pregnancy?

- Never
- □ Very occasionally
- □ More frequently, in particular ..... units/week

### 5. Have you been ill (other than cancer) during your pregnancy?

- □ No
- Yes, in particular: .....
- 6. Have you gone through a major change during your pregnancy (other than the cancer diagnosis), such as divorce, moving house, passing of someone close to you...
- $\square$  No
- Yes, in particular: .....
- 7. What is the highest level of education you and your partner attained? (a completed course with degree or certificate)

Mother	Father

Primary school		Primary school	
□ Secondary school		Secondary school	
□ Bachelor degree		Bachelor degree	
□ Master degree		□ Master degree	
□ Other:			
8. In which country w	8. In which country were you and your partner born?		
N 4 - th - w			
Mother:			
Father			
<u>Mother</u>			
Age ye			
Height: cm			
Weight before getting	pregnant: kg		
Fother.			
Father			
Age: ye	ars		
<u>Child</u>			
This is my 🗆 first 🗆 seco	This is my		
Your baby's sex: 🗆 girl			
Delivery - coentanee			
caesarean	Delivery:  spontaneous and vaginal  induced (vaginal)  planned caesarean section  emergency caesarean		
cacsarcan			
Season in which the ba	Season in which the baby was delivered: $\square$ winter $\square$ spring $\square$ summer $\square$ autumn		
Gestation at the time of	of delivery: weel	ks and days	



Figure 1. Scheme depicting the types of biomaterials that will be collected and the assays being performed. This scheme will be applied on every participant of each of the 3 study groups, i.e. chemotherapy-trated pregnant cancer patients, and healthy pregnant women. CBMV: cytokinesis-block micronucleus assay; LC/MS: liquid chromatography-mass spectrometry; MS: mass spectrometry; qPCR: quantitative polymerase chain reaction; WGS: whole genome sequencing.

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2		
3 4		
5	Supplementary 1	
6		
/ 8	Questionnaire protocol GE-CIP stud	lv (S62388)
9		,, (002000)
10		
11		
12		<b>,</b>
13	1. Do you smoke of have you ever smoked:	ſ
15	□ Never	
16	I quit smoking before I got pregnant	
17	I smoked during my pregnancy; amount:	cigarettes/week
10		
20	2. Did you or someone else smoke indoors	during your pregnancy?
21		
22		
23 24		
25	3. Did you take any medication/supplements/vitamins during your	
26	(E.g. folic acid, vitamin D, zinc supplemer	nts)
27		
28 20		
30	Yes	
31	If yes, please enter the details in the table below:	
21		
32	Medication/vitamin/supplement	Pregnancy time
32 33 34	Medication/vitamin/supplement	Pregnancy times
32 33 34 35	Medication/vitamin/supplement	Pregnancy time week up
32 33 34 35 36	Medication/vitamin/supplement	Pregnancy time           week         up           week         up
32 33 34 35 36 37	Medication/vitamin/supplement	Pregnancy time       week       week       week       week
32 33 34 35 36 37 38 30	Medication/vitamin/supplement	Pregnancy time         week       up         week       up         week       up
32 33 34 35 36 37 38 39 40	Medication/vitamin/supplement	Pregnancy time         week       up         week       up         week       up         week       up
32 33 34 35 36 37 38 39 40 41	Medication/vitamin/supplement	Pregnancy time:         week       up         week       up         week       up         week       up         week       up
32 33 34 35 36 37 38 39 40 41 42	Medication/vitamin/supplement	Pregnancy time:         week       up         week       up         week       up         week       up         during your pregnancy?
32 33 34 35 36 37 38 39 40 41 42 43	Medication/vitamin/supplement	Pregnancy time:         week       up         week       up         week       up         week       up         during your pregnancy?
32 33 34 35 36 37 38 39 40 41 42 43 44	Medication/vitamin/supplement	Pregnancy time:         week       up         week       up         week       up         week       up         during your pregnancy?
32 33 34 35 36 37 38 39 40 41 42 43 44 45 46	Medication/vitamin/supplement	Pregnancy times week up week up week up week up during your pregnancy?
32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47	Medication/vitamin/supplement	Pregnancy time:         weekup         weekup         weekup         weekup         weekup         during your pregnancy?
32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48	Medication/vitamin/supplement	Pregnancy time:         weekup         weekup         weekup         weekup         during your pregnancy?         eek         ring your pregnancy?
32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50	Medication/vitamin/supplement	Pregnancy time:         weekup         weekup         weekup         weekup         weekup         during your pregnancy?         eek         ring your pregnancy?
32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51	Medication/vitamin/supplement	Pregnancy time:         weekup         weekup         weekup         weekup         during your pregnancy?         eek         ring your pregnancy?
32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52	Medication/vitamin/supplement	Pregnancy time:         weekup         weekup         weekup         weekup         weekup         during your pregnancy?         eek         ring your pregnancy?
32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53	Medication/vitamin/supplement	Pregnancy time:         weekup         weekup         weekup         weekup         weekup         during your pregnancy?         eek         ring your pregnancy?         during vour pregnancy?
32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55	Medication/vitamin/supplement	Pregnancy time:         weekup         weekup         weekup         weekup         weekup         during your pregnancy?         eek         ring your pregnancy?         during your pregnancy?         during your pregnancy?
32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56	Medication/vitamin/supplement	Pregnancy time:         weekup         weekup         weekup         weekup         weekup         weekup         during your pregnancy?         eek         ring your pregnancy?         during your pregnancy?         during your pregnancy?         during your pregnancy (of someone close to you.
32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57	Medication/vitamin/supplement	Pregnancy time:         weekup         weekup         weekup         weekup         weekup         during your pregnancy?         eek         ring your pregnancy?         during your pregnancy?         during your pregnancy?
32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58	Medication/vitamin/supplement	Pregnancy time:         weekup         weekup         weekup         weekup         weekup         weekup         during your pregnancy?         eek         ring your pregnancy?         during your pregnancy?         during your pregnancy?         during your pregnancy (or         of someone close to you.
31         32         33         34         35         36         37         38         39         40         41         42         43         44         45         46         47         48         49         50         51         52         53         54         55         56         57         58         59	Medication/vitamin/supplement	Pregnancy time:         weekup         weekup         weekup         weekup         weekup         during your pregnancy?         eek         ring your pregnancy?         during your pregnancy?         during your pregnancy?         during your pregnancy?         during your pregnancy (or of someone close to you.

Patient ID: ..... Date:....

# supplements/vitamins during your pregnancy?

### supplements)

Medication/vitamin/supplement	Pregnancy timeframe
	week up to week

### k alcohol during your pregnancy?

### cancer) during your pregnancy?

- or change during your pregnancy (other than the cancer diagnosis), , passing of someone close to you...

Yes, in particular:	
---------------------	--

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7.	What is the highest level of education you and your partner attained? (a completed course with
	degree or certificate)

Mother	Father
Primary school	Primary school
□ Secondary school	□ Secondary school
Bachelor degree	Bachelor degree
Master degree	Master degree
□ Other:	□ Other:

8.	In which	country	were	vou	and	vour	partner	born?
				,		,	P	

Mother:					
Father:					
<u>Mother</u>					
Age:	years				
Height:	cm				
Weight befor	e getting pregnant: kg				
Father					
Age:	years				
<u>Child</u>					
This is my $\square$ fi	irst $\Box$ second $\Box$ third $\Box$ child				
Your baby's s	ex: 🗆 girl 🗆 boy				
Delivery: 🗆 sı caesarean	pontaneous and vaginal $\square$ induced (vaginal) $\square$ planned caesarean section $\square$ emergency				
Season in which the baby was delivered: $\square$ winter $\square$ spring $\square$ summer $\square$ autumn					
Gestation at t	he time of delivery: weeks and days				

### STROBE Statement—Checklist of items that should be included in reports of cohort studies

	Item No	Recommendation	Page No
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the	1-2
		abstract	
		(b) Provide in the abstract an informative and balanced summary of what was	
		done and what was found	
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being	4
		reported	
Objectives	3	State specific objectives, including any prespecified hypotheses	4-5
Methods			
Study design	4	Present key elements of study design early in the paper	5-7
Setting	5	Describe the setting, locations, and relevant dates, including periods of	5-7
		recruitment, exposure, follow-up, and data collection	
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of	5
		participants. Describe methods of follow-up	
		(b) For matched studies, give matching criteria and number of exposed and	
		unexposed	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and	7-9
		effect modifiers. Give diagnostic criteria, if applicable	
Data sources/	8*	For each variable of interest, give sources of data and details of methods of	7-9
measurement		assessment (measurement). Describe comparability of assessment methods if	
		there is more than one group	
Bias	9	Describe any efforts to address potential sources of bias	11
Study size	10	Explain how the study size was arrived at	6
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable,	9
		describe which groupings were chosen and why	
Statistical methods	12	(a) Describe all statistical methods, including those used to control for	9
		confounding	
		(b) Describe any methods used to examine subgroups and interactions	
		(c) Explain how missing data were addressed	
		(d) If applicable, explain how loss to follow-up was addressed	
		(e) Describe any sensitivity analyses	
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially	NA
		eligible, examined for eligibility, confirmed eligible, included in the study,	
		completing follow-up, and analysed	
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social)	NA
		and information on exposures and potential confounders	
		(b) Indicate number of participants with missing data for each variable of interest	
		(c) Summarise follow-up time (eg. average and total amount)	

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### **BMJ** Open

Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their	NA
		precision (eg, 95% confidence interval). Make clear which confounders were adjusted for	
		and why they were included	
		(b) Report category boundaries when continuous variables were categorized	
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a	
		meaningful time period	
Other analyses	17	Report other analyses done-eg analyses of subgroups and interactions, and sensitivity	NA
		analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	NA
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision.	3
		Discuss both direction and magnitude of any potential bias	
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations,	NA
		multiplicity of analyses, results from similar studies, and other relevant evidence	
Generalisability	21	Discuss the generalisability (external validity) of the study results	11
Other informati	on		
Funding	22	Give the source of funding and the role of the funders for the present study and, if	12
		applicable, for the original study on which the present article is based	

\*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.

## **BMJ Open**

### Evaluating offspring genomic and epigenomic alterations after prenatal exposure to cancer treatment in pregnancy (GE-CIP): a multicentric observational study

Journal:	BMJ Open
Manuscript ID	bmjopen-2023-081833.R1
Article Type:	Protocol
Date Submitted by the Author:	29-Feb-2024
Complete List of Authors:	Struys, Ilana; KU Leuven, Department of Oncology Velázquez, Carolina; KU Leuven, Department of Oncology Devriendt , Koenraad; University hospital leuven, Department of Human Genetics Godderis, Lode; KU Leuven, Department of Public Health and Primary Care; External Service for Prevention and Protection at Work Segers, Heidi; University hospital Leuven, Department of Paediatric Oncology Thienpont, Bernard; KU Leuven, Department of Human Genetics van Boxtel, Ruben; Princess Maxima Center for Pediatric Oncology Van Calsteren, Kristel; University hospital Leuven, Department of Obstetrics and Gynecology Voet, Thierry; KU Leuven, Department of Human Genetics; KU Leuven, Institute for Single Cell Omics (LISCO) Wolters, Vera; Netherlands Cancer Institute, Gynecologic Oncology Lenaerts, Liesbeth; KU Leuven, Department of Obstetrics and Gynaecology
<b>Primary Subject Heading</b> :	Oncology
Secondary Subject Heading:	Genetics and genomics, Obstetrics and gynaecology, Research methods
Keywords:	GENETICS, CHEMOTHERAPY, NEONATOLOGY



Evaluating offspring genomic and epigenomic alterations after prenatal exposure to cancer treatment in pregnancy (GE-CIP): a multicentric observational study

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#### 

### Abstract

**Introduction:** Around 1 in 1000-2000 pregnancies are affected by a cancer diagnosis. Previous studies have shown that chemotherapy during pregnancy has reassuring cognitive and cardiac neonatal outcomes, and hence has been proposed as standard of care. However, although these children perform within normal ranges for their age, subtle differences have been identified. Given that chemotherapeutic compounds can cross the placenta, the possibility that prenatal chemotherapy exposure mutates the offspring's genome and/or epigenome, with potential deleterious effects later in life, urges to be investigated.

**Methods and analyses:** This multicentric observational study aims to collect cord blood, meconium and neonatal buccal cells at birth, as well as peripheral blood, buccal cells and urine from infants when 6, 18 and/or 36 months of age. Using bulk and single-cell approaches, we will compare samples from chemotherapy-treated pregnant cancer patients, pregnant cancer patients not treated with chemotherapy, and healthy pregnant women. Potential chemotherapy-related newborn genomic and/or epigenomic alterations, such as single nucleotide variants, copy number variants and DNAmethylation alterations, will be identified in mononuclear and epithelial cells, isolated from blood, buccal swabs and urine. DNA from maternal peripheral blood and paternal buccal cells will be used to determine *de novo* somatic mutations in the neonatal blood and epithelial cells. Additionally, the accumulated exposure of the fetus, and biological effective dose of alkylating agents, will be assessed in meconium and cord blood via mass spectrometry approaches.

**Ethics and dissemination:** The Ethics Committee Research of UZ/KU Leuven (EC Research) and the Medical Ethical Review Committee of University Medical Center Amsterdam have approved the study. Results of this study will be disseminated via presentations at (inter)national conferences, through peer-reviewed, open-access publications, via social media platforms aimed to inform patients and health care workers, and through the website of the International Network on Cancer, Infertility and Pregnancy (INCIP; <u>www.cancerinpregnancy.org</u>).

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### Strengths and limitations of this study

- In this study, the mutational consequences of prenatal chemotherapy exposure in newborn DNA will be investigated using a variety of innovative bulk and single-cell techniques, allowing a comprehensive assessment of different types of genetic and epigenetic aberrations.
- Different tissue types will be investigated, i.e. cord blood and buccal epithelial cells, which are both easy accessible and non-invasive sources of newborn DNA.
- By applying the same methodology to blood samples from the same children at 6, 18 and/or 36 months of age, the evolution of possible identified mutational consequences can be tracked.
- This study will only provide information on the potential association, and not causal relation, between prenatal exposure to chemotherapy and genetic and/or epigenetic alterations in the newborn's DNA.

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### Introduction

Cancer is the second leading cause of death during the reproductive years and complicates between 1 in 1000 to 2000 pregnancies (1). As women in developed societies tend to delay childbearing to the third or fourth decade of life, and since the incidence of several malignancies rises with increasing age, this rare coincidence is likely to become more common. In the past decades, the establishment of (inter)national registries, including the International Network on Cancer, Infertility and Pregnancy (INCIP), and an increasing number of smaller cohort studies, have provided insights for the management of cancer during pregnancy. Applying chemotherapy in the first trimester is to be avoided due to the increased risk of developing congenital malformations (2). Therefore, it is advised to start chemotherapy in the second or third trimester, i.e. after week 14 of pregnancy (3–5). Previous studies have shown that chemotherapy during the second and/or third trimester of pregnancy does not affect the short-term cardiac and neurocognitive development of children, which is reassuring (2,6-9). Additionally, administering chemotherapy treatment to pregnant women has led to an increase in livebirths and decrease in iatrogenic preterm deliveries (1). Yet, prenatal exposure to certain chemotherapeutic drugs has been associated with a higher risk for children being born small for their gestational age (1,10). Furthermore, although overall, children born from pregnancies complicated by maternal cancer perform within normal ranges for their age, chemotherapy-exposed children had a significantly lower verbal IQ at 6 year, and were 3 times more likely to wear glasses than non-exposed children. However, since there are many confounding factors in neurocognitive development of children, a direct link with prenatal chemotherapy exposure cannot be accurately established (1,6-8).

To date, data on long-term outcomes in these children are scarce, and thus, it remains unknown whether prenatal exposure to chemotherapy may provoke an increased risk of developing fertility issues, chronic health conditions or cancers later in life. Previous *in vivo, ex vivo* and clinical studies have shown that chemotherapeutic agents can cross the placental barrier (at passage rates that vary per drug) (11–17). Additionally, increasing evidence is showing that chemotherapy treatment can leave a genotoxic footprint, consisting of point mutations and/or larger chromosomal abnormalities (18–21) and induce long-lasting epigenetic changes, such as alterations in the DNA methylation profile (22–24) in cancer, and even non-cancerous cells, of directly exposed cancer patients. Based on the above, it can be speculated that chemotherapeutic agents, administered during pregnancy, could target and damage the fetal DNA. This, in turn, could interfere with the offspring's long-term health. The aim of the methods presented in this protocol is to investigate the possibility of whether, and how, prenatal chemotherapy exposure is associated with genetic and epigenetic aberrations in the newborn DNA, and, if identified, how these aberrations evolve in early childhood years.

### Methods and analyses

### Objectives

The primary objectives of this study are (i) to determine whether chemotherapy administered during pregnancy affects the genome and/or epigenome of newborn cells, by identifying and contrasting acquired genetic and epigenetic alterations in cord blood and buccal cells derived from chemotherapy-treated and -untreated pregnant cancer patients, and healthy pregnant women, and (ii) to examine if the potential chemotherapy-related genomic and/or epigenomic alterations in newborn DNA are detectable at different time points, whether the mutation rate is altered, and if these changes lead to

more complex downstream events in early childhood years. by analyzing peripheral blood cells and epithelial cells from buccal and urinary tract tissue of infants aged 6, 18 and/or 36 months, and comparing the results with those obtained at birth. A secondary objective is to correlate the possibly identified genomic and/or epigenomic changes to the cumulative dose of chemotherapeutic compounds, and -adducts, measured in cord blood plasma and meconium samples taken after delivery.

### Study Cohort and eligibility criteria

This multicenter, prospective observational study will be carried out in Belgian (University Hospital Leuven, University Hospital Gent and University Hospital Antwerpen) and Dutch (Amsterdam University Medical Center and University Medical Center Groningen) hospitals participating in the International Network on Cancer, Infertility and Pregnancy (INCIP, <u>www.cancerinpregnancy.org</u>). Eligible participants are women with (histologically proven) cancer of any type and stage during pregnancy and an ongoing pregnancy (≥ 24 weeks of gestation). The study group will consist of pregnant women who were diagnosed with cancer and treated with chemotherapy during the second and/or third trimester of pregnancy (termed "chemotherapy-treated pregnant cancer patients"). The control group will be pregnancy (termed "chemotherapy-untreated pregnant cancer patients"). A second control group will consist of healthy pregnant women. For each included pregnant cancer patient, one healthy control case will be recruited from the general obstetric department of the hospital of inclusion or affiliated midwife practice, matched for the following criteria: maternal age, gestational age at birth and sex of the child.

Key eligibility criteria are listed in Table 1. For each patient, demographic (i.e. maternal and paternal age), obstetric (i.e. gestational age at delivery, sex newborn) and oncological (i.e. tumor type, subtype and staging, presence of a DNA repair gene mutation, chemotherapy schedule and cumulative dose, gestational age at diagnosis, gestational age at the start of the treatment, gestational age at the last chemotherapy administration, type of surgery, gestational age at surgery) data will be retrieved via the INCIP registry (pregnant cancer patients) or medical file (healthy pregnant women). Furthermore, biomaterials from eligible pregnant women will be collected in the respective participating hospitals and shipped to Catholic University Leuven for central processing. Lastly, a questionnaire is requested to be filled in at time of delivery (Supplementary 1).

Table 1. Study inclusion and exclusion criteria							
Inclusion criteria							
Study group:	Control group 1:	Control group 2:					
chemotherapy-treated pregnant cancer patients	chemotherapy-untreated pregnant cancer patients	healthy pregnant women					
<ul> <li>Histologically proven cancer during pregnancy (any type and stage)</li> <li>Treatment during the second and/or third trimester of pregnancy with one or a combination of the following chemotherapeutic agents: Cyclophosphamide, Anthracyclines, Taxanes, Platinum derivates</li> <li>Gestational age (GA) at birth &gt;24 weeks</li> </ul>	<ul> <li>Histologically proven cancer during pregnancy (any type and stage)</li> <li>No treatment during pregnancy or surgery only (subgroup 1)</li> <li>Radiotherapy and/or systemic treatment (other than chemotherapy) during pregnancy (subgroup 2)*</li> </ul>	<ul> <li>Matched for maternal age, gestational age at birth and infant sex with chemotherapy-treated group</li> <li>GA at birth ≥24 weeks</li> </ul>					

	• GA at birth ≥24 weeks				
	Exclusion criteria				
•	• GA at birth <24 weeks (miscarriage or termination of pregnancy)				
•	• Inability to give informed consent e.g. due to mental disabilities				
•	• Any comorbidity that is associated with an increased micronucleus frequency, such as hypertensive disorders,				
	preeclampsia, (gestational) diabetes, renal or cardiac pathology				
*	Subgroups 1 and 2 will be analyzed as separate control groups.				

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### Sample collection and processing

At, or shortly after birth, the following samples will be collected: cord blood, newborn buccal swabs, meconium (obtained during the first 48h of life), maternal peripheral blood and paternal buccal swabs. Further details on the use of these samples will be described in the respective assay sections below.

At the child's age of 6, 18 and/or 36 months, the following samples will be collected: child's peripheral blood, buccal swabs and urine samples. Potential parents' objection to sampling one of these biomaterials does not preclude further participation to the study.

All biomaterials are collected at the respective participating hospitals and shipped to Catholic University Leuven for central processing. Blood samples are to be shipped within 24 hours after collection for immediate processing. Buccal swabs, meconium and urine samples can be stored at the respective participating hospitals for bulk shipment at a chosen time. The conditions of transport, processing and storage for the different specimens are summarized in Table 2.

Table 2. Sample collection, transport, processing and storage details for the different specimens.						
Specimen	Sampling and storage conditions	Goal				
Cord blood and maternal blood	Heparin tube; direct shipment to central lab at room temperature	Direct isolation of blood mononuclear cells via density gradient separation and immediate use in the CBMN assay				
		Direct isolation of blood mononuclear cells via density gradient separation and storage in liquid nitrogen until further processing for sequencing to determine genetic aberrations				
Cord blood and maternal blood	central lab at room temperature	Serum and sediment separation and storage at - 80°C until LC/MS assessment of the biological effective chemotherapy dose (serum) and identification of epigenetic alterations (sediment) via bisulfite sequencing and methylation array				
Buccal Swabs	Isohelix™ Buccal Swabs; local storage at -20ºC	DNA extraction for the assessment of chromosomal abnormalities via low-pass sequencing and copy number profiling and identification of epigenetic alterations via bisulfite sequencing and methylation array				
Meconium	Cryotube; local storage at -20°C	Assessment of accumulated dose of chemotherapeutic compounds by MS				
Meconium	Cryotube; local storage at -20°C	DNA extraction for the assessment of the biological effective doses by LC/MS				
Urine	Urine bag; local storage at -20°C	Epithelial urinary tract cells for exploratory genetic analyses in early childhood years using single cell WGS and cell-free DNA analyses				
CBMN: cytokinesis-block micronucleus; EDTA: ethylenediaminetetraacetic acid; LC/MS: liquid chromatography–mass spectrometry; MS: mass spectrometry.						

#### Sample size per group

 Yearly, an average of 10 pregnant cancer patients are registered per participating center, of which 50% are treated with chemotherapy during pregnancy. Therefore, we envision to include 20 chemotherapy-treated pregnant cancer patients, 20 chemotherapy-untreated pregnant cancer patients and 40 healthy pregnant women per year. Since this is an exploratory study, with scarce data to rely on for solid sample size calculations, post-hoc sample size calculations will be conducted after analyses of the first 10 subjects per group.

### Study Design

A schematic representation of the study design, patient groups, collected biomaterials and molecular analyses is given in Figure 1. Start and end date of the international study: September 2022 – December 2030.

### Study Methods

### 1. Measuring genetic and epigenetic alterations in newborn cells

#### 1.1 Studying the link between prenatal exposure to chemotherapy and a 'genotoxic footprint' in newborn cells

First, we will evaluate the presence of chemotherapy-associated gross chromosomal abnormalities in newborn cells, using two different techniques: (i) The cytokinesis-block micronucleus (CBMN) assay, and (ii) shallow whole-genome sequencing (WGS). The CBMN assay will be performed on T-lymphocytes, isolated from cord blood from all participants. The CBMN assay will be performed as described by Fenech *et al.* (25). From each sample, a total of 1000 mononuclear cells will be microscopically scored to determine the micronucleus frequency (MNF) per 1000 binucleated T-lymphocytes. Shallow WGS will be performed using DNA isolated from mononuclear cells from cord blood.

In parallel to evaluating blood samples, we will also assess the presence of such *de novo* chromosomal abnormalities in DNA isolated from the newborn's buccal epithelial cells via low-pass sequencing (0.1x read depth) and copy number profiling (26). Maternal peripheral blood DNA and paternal buccal epithelial DNA will be used as reference to allow determining *de novo* (sub)chromosomal alterations in cord blood DNA. To maximize participation from the fathers, we prefer a non-invasive buccal swab sampling (for downstream DNA isolation) over blood sampling.

Second, we will investigate the possibility that chemotherapy-induced DNA alterations may be private to a single cell (27). We will do this using two **single-cell sequencing** techniques: (a) single-cell genomeplus-transcriptome-sequencing (G&T-seq) of single mononuclear cord blood cells to map structural variants, such as (sub)chromosomal deletions, amplifications and/or translocations (28,29), and, since current single-cell sequencing approaches are not yet optimal for accurate detection of point mutations, (b) WGS deep sequencing (paired-end, 15x) of clonal organoid cultures established from individual cord blood stem cells to detect single nucleotide variants (SNVs) and small insertions and deletions (indels) (30). Furthermore, this WGS information will be used to estimate telomere lengths in cord blood stem cells.

#### 1.2 Studying the link between prenatal exposure to chemotherapy and DNA-methylation changes in newborn cells

To study potential epigenetic changes associated with prenatal chemotherapy exposure, DNA will be isolated from the sediment fraction of cord blood and epithelial cells from buccal swabs of newborns.

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Then, the extracted DNA will be subjected to "oxidative" bisulfite conversion. After bisulfite sequencing, the DNA methylation status will be analyzed via array-based methylation assessment methods. Using bio-informatics, we will evaluate how identified changes in methylation signatures in newborn DNA are related to specific chromatin organizational states. Furthermore, to investigate the downstream effects of methylation changes, gene and pathway analyses will be performed, which allows assigning functional meanings to genes and genomes. Medication use of the mother up until 24 hours prior to delivery will be documented in the questionnaire (Supplementary 1), since this might influence the methylation profiles and needs to be taken into account during analyses.

### 1.3 Studying the link between prenatal exposure to chemotherapy and gene-expression changes in newborn cells

To study potential gene-expression alterations associated with prenatal chemotherapy exposure, RNA will be isolated from single mononuclear cord blood cells (G&T-seq, cfr. 1.1), the sediment fraction of cord blood and epithelial cells from buccal swabs of newborns, and subjected to gene-expression analyses through single-cell RNA sequencing (scRNA-seq). DNA loci with significant differences in methylation status (cfr. 1.2), that correlate to transcribed genes and for which the transcript sequence is known, will be validated further via (single-cell) quantitative PCR (qPCR) approaches. Rationale for selection will include functional significance as well as statistical differences between chemotherapy-treated and chemotherapy-untreated samples.

### 2. Measuring chemotherapy concentrations and biological effective dose

To measure the concentration of applied chemotherapeutic agents and/or their metabolites, mass spectrometry (MS) will be used. The analytical protocol will be developed for blood, plasma, meconium and urine samples.

Additionally, for alkylating agents, the biological effective doses in the same biological matrices will be determined via measurement of DNA adducts, using liquid chromatography–mass spectrometry (LC-MS). As an indicator for DNA adducts in patients receiving alkylating agents (e.g. cisplatin, carboplatin and/or cyclophosphamide treatment), we will focus on the identification of bis-N7-guanine DNA-DNA cross-links. Maternal peripheral blood will be taken as a reference. Medication use of the mother up until 24 hours prior to delivery will be checked in the questionnaire (Supplementary 1), since this might interfere with the detection of chemotherapy compounds in both the cord and maternal blood samples.

### 3. Examining the evolution of observed genomic and/or epigenomic alterations in infant cells

If we identified genomic and/or epigenomic changes in DNA of newborns that were prenatally exposed to chemotherapy compared to newborns born to women in the control groups (see 1.1 and 1.2), we will monitor whether these genomic and/of epigenomic alterations are detectable at different time points, whether the mutation rate is altered, and if these changes lead to more complex downstream events in early childhood years. Therefore, we will sample peripheral blood cells and buccal epithelial cells from the infants of all 3 groups, at the ages of 6, 18 and/or 36 months and analyze the samples using the same techniques as mentioned above. To gain additional insight in the specific blood cell types that may be affected, we will perform single-cell WGS on different types of differentiated blood cells, separated via flow cytometry, from the chemotherapy-treated pregnant cancer patient group, complemented with phylogenetic tree construction in blood cells (30).

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Additionally, to investigate the range of tissue types that might be affected by prenatal exposure to chemotherapy, we will initiate exploratory genotoxicity analyses (using whole-genome single-cell sequencing and cell-free DNA analysis) in epithelial cells originating from the child's urinary tract.

### Statistical analysis

Given that no published evidence exists to rely on, all statistical analyses are exploratory. Analysis will be performed with R Studio using 95% confidence intervals and a two-tailed *P*-value of 0.05. Continuous variables will be compared between the different patient groups to determine the potential association with prenatal chemotherapy exposure using Kruskal Wallis or ANOVA, depending on normality of the data. Linear regression models will be used to study the effect of chemotherapyexposure on genotoxicity and methylation grade in the study groups, while adjusting for potential confounders such as maternal age, gestational age at delivery and sex of the newborn. Subgroup analyses related to the different chemotherapeutic compounds will be done when possible. Where necessary corrections for multiple testing will be applied.

### Patient and public involvement statement

The ongoing research on cancer during pregnancy performed by the INCIP network was started based on the request of pregnant women who were confronted with a cancer diagnosis during their pregnancy. Clinical questions about how the treatment during pregnancy would affect their unborn child formed the basis of several studies investigating aspects of the children's development after birth. We aim to keep our patients informed of the research results by sharing them via our national social media platforms (www.kankerenzwangerschap.be; patient www.facebook.com/kankerenzwangerschap) INCIP and via the network (website www.cancerinpregnancy.org).

#### Secondary findings

As this study will not change treatment, nor randomize patients, participation in this study will not affect patients' outcomes. As this is an observational study, no additional risks from the study are expected. As described above, for the study of (sub)chromosomal abnormalities and mutations in the newborn's and infant's tissue (cord/peripheral blood, and buccal cells) potentially being associated with prenatal exposure to chemotherapy, only *de novo* alterations will be taken into account. The findings will be interpreted with the support of a clinical geneticist. Secondary findings might include genetic mutations, including point mutations and larger structural variants that could imply any pathogenicity, and will be managed conform the latest guidelines from the American College of Medical Genetics (31).

- Mutations causing highly penetrant disorders with validated evidence on the phenotype associated with the deletion or duplication. These are considered clinically relevant and will be reported.
- Mutations proven to be risk factors for developmental disorders with reduced penetrance and/or variable expression. The predictability of the future phenotype resulting from such CNVs remains very poor. Therefore, these susceptibility CNVs will not be reported.
- Mutations causing late-onset genetic disorders, typically cancer caused by the deletion of a tumor suppressor gene, will be communicated if undeniable health benefit can be expected for the participants (child or parent).

It is important to note that, by restricting the analyses to *de novo* findings in newborn's/infant's DNA, the number of 'to be reported secondary findings' is estimated to be limited.

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Also, in the informed consent form, participants will be given the opportunity to opt out of being notified of secondary findings related to the newborn DNA.

### Availability of data and material

All clinical data are registered in a REDCap database. The participating physician ensures the confidentiality, accuracy, completeness, legibility and timeliness of the data recorded. Data handling and statistical analysis will be done in a coded fashion by the investigator, with the subject identification code list only available to the local investigator (and research nurse if applicable) working in the local center. The collection, processing and disclosure of personal data, such as patient health and medical information is subject to compliance with applicable personal data protection and the processing of personal data legislation (including, but not limited to the EU Directive 95/46/EC and Belgian Law of December 8, 1992 on the protection of the Privacy in relation to the Processing of Personal Data). Data and rest material (e.g. left-over DNA/RNA extracts) will be kept for 20 years. If archived samples may be useful for additional investigations, not mentioned in the current protocol, approval will be asked from the Ethical Committee. The genome-wide sequencing data are stored for 3 months at Genomics Core (KU Leuven) and subsequently transferred to the common lab drive. Similarly, data from the CBMN assay and the questionnaire are stored there. This lab drive is pass-word protected, with limited access to authorized persons.

### Ethics and dissemination

The study protocol was reviewed and approved by the Ethics Committee Research UZ / KU Leuven (EC Research) (S62388) and the Medical Ethical Review Committee of University Medical Center Amsterdam (NL76873.018.21\_S62388).

Eligible subjects will be asked, together with their partner, to sign a written informed consent form upon agreement to participate in the study. Participants are allowed to leave the study at any time for any reason, without any consequences, and are able to opt out for donating a particular sample type. The investigator can also decide to withdraw a subject from the study for urgent medical reasons. Participation in the study will not influence the clinical management of the patient. Furthermore, participants will be given the opportunity to opt out of being notified of secondary findings related to the newborn DNA, which they could indicate in the informed consent form. All data will be handled with strict confidentiality, assuring anonymity of the subjects.

Dissemination of the research results will be done via presentations at national and international conferences and seminars, and through the publication of peer-reviewed manuscripts in open-access journals. Additionally, we will communicate findings through our national patient social media platforms (www.kankerenzwangerschap.be; <u>www.facebook.com/kankerenzwangerschap</u>) and via the INCIP network (website <u>www.cancerinpregnancy.org</u>), created to increase knowledge among scientists, health care workers and the public (including patients). Further, the communication offices of both the Leuven Cancer Institute (LKI) and of KU Leuven guarantee optimal communication between

KU Leuven and University Hospitals Leuven on the one hand, and outside university. Finally, a webbased software (Advisory Board Cancer In Pregnancy - ABCIP) was developed to offer remote multidisciplinary support to physicians that lack expertise to manage the treatment of cancer during pregnancy. Additionally, this platform will be used to distribute novel research insights to caregivers involved in the management of pregnant cancer patients.

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### Discussion

This study will be the very first to explore a potential genotoxic effect of prenatal chemotherapy exposure to the newborn's DNA. We anticipate that the use of a variety of techniques, allowing the assessment of different types of genotoxicity, and the inclusion of pregnant cancer patients treated with different treatment regimens, will give an unprecedented insight in treatment-dependent genotoxic footprints, and link this with the level of accumulated dose of chemotherapeutic compounds in the fetus. Genomic insults in the perinatal period have been linked to the development of diseases, such as cancer and neurodevelopmental disorders, and may be more important than in other life stages given the higher probability that mutated and genomically unstable cells could populate rapidly growing tissues of an infant (32,33). This project will address the question of whether prenatal chemotherapy-treatment is associated with a 'genotoxic footprint' in newborn tissue, at the earliest time point in life, and whether and how this evolves in early-childhood years. The combined analysis of signatures of point mutations and larger structural variants might also reveal information about the potential mechanisms underlying the identified aberrations. If positive, our findings will give an indication of which functional genomic regions and pathways might be affected. Data on the safety of administering chemotherapy during pregnancy are highly warranted, as both clinicians and patients remain reluctant about this issue. If we would not find any 'genotoxic footprint', this would indicate DNA abnormalities following prenatal chemotherapy exposure occur at a very low frequency, suggesting a limited clinical impact. Alternatively, a positive outcome may trigger further fundamental and clinical research to further asses the clonality of observed aberrations, the most affected cells and the potential clinical impact. Though preliminary, the results of this study may aid in the identification of potentially harming agents to the unborn child, and hence provide an opportunity to optimize therapeutic regimens for pregnant women confronted with cancer.

### Abbreviations

CBMN: Cytokinesis-block micronucleus; CIP: Cancer In Pregnancy; CNV: Copy Number Variation; GE-CIP: Genomic and Epigenomic alterations after Cancer treatment In Pregnancy; indels: insertions and deletions; INCIP: International Network on Cancer, Infertility and Pregnancy; LC-MS: Liquid chromatography-mass spectrometry; MNF: micronucleus frequency; MS: Mass Spectrometry; scRNAseq: single-cell RNA sequencing; SNV: single nucleotide variant; WGS: Whole Genome Sequencing; qPCR: quantitative Polymerase Chain Reaction

### Author contributions

IS, LL, VW, CV and FA made substantial contributions to the design and writing of the study protocol. IS, LL and CV will be conducting the sample collection and different study parts. FA is the founder of INCIP and will be, together with KvC, informing and including the eligible patients. LG, BT, RvB and TV gave their expert opinion in designing genetic and epigenetic analyses of the biomaterials. KD will be consulted when secondary findings occur. HS was consulted to work out the follow-up study parts. All authors were involved in revising the manuscript draft and approved the final manuscript.

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#### **Competing interests statement**

TV is co-inventor on licensed patents WO/2011/157846 (Methods for haplotyping single cells), WO/2014/053664 (High-throughput genotyping by sequencing low amounts of genetic material) and WO/2015/028576 (Haplotyping and copy number typing using polymorphic variant allelic frequencies).

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, like ermore, w participate in th. The manuscript's authors would like to thank all the experts who contributed to conceptualize and design the GE-CIP Study. Furthermore, we thank the patients, their families, institutions, physicians and nurses who are willing to participate in this study.

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### Figure legend:

**Figure 1.** Scheme depicting the types of biomaterials that will be collected and the assays being performed. This scheme will be applied on every participant of each of the 3 study groups, i.e. chemotherapy-treated pregnant cancer patients, chemotherapy-untreated pregnant cancer patients, and healthy pregnant women. CBMN: cytokinesis-block micronucleus assay; LC/MS: liquid chromatography-mass spectrometry; MS: mass spectrometry; qPCR: quantitative polymerase chain reaction; WGS: whole genome sequencing.

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Supplementary 1	
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### Questionnaire protocol GE-CIP study (S62388)

Patient ID:	 		 	•••	•••	•••	•••		•
Date:	 	•••	 	•••	•••		•••	•••	•

### 1. Do you smoke or have you ever smoked?

- $\square$  Never
- I quit smoking before I got pregnant
- □ I smoked during my pregnancy; amount: ..... cigarettes/week

### 2. Did you or someone else smoke indoors during your pregnancy?

- $\square$  No
- Yes

## 3. Did you take any medication/supplements/vitamins during your pregnancy? (E.g. folic acid, vitamin D, zinc supplements)

- $\square$  No
- 🗆 Yes

If yes, please enter the details in the table below:

Medication/vitamin/supplement	Pregnancy timeframe
	week up to week

### 4. Do you drink or have you drunk alcohol during your pregnancy?

- $\square \ Never$
- □ Very occasionally
- □ More frequently, in particular ..... units/week

### 5. Have you been ill (other than cancer) during your pregnancy?

- $\square$  No
- Yes, in particular: .....
- 6. Have you gone through a major change during your pregnancy (other than the cancer diagnosis), such as divorce, moving house, passing of someone close to you...
- 🗆 No

60

Yes, in particular: .....

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7.	What is the highest level of education you and your partner attained? (a completed course with
	degree or certificate)

Mother	Father
Primary school	Primary school
Secondary school	□ Secondary school
Bachelor degree	Bachelor degree
□ Master degree	Master degree
□ Other:	□ Other:

8.	In which coun	trv were	vou and v	vour pa	artner born?	)
			,			

Mother:					
rather.					
<u>Mother</u>					
Age:	years				
Height:	cm				
Weight before	e getting pregnant: kg				
<b>Father</b>					
Age:	years				
<u>Child</u>					
This is my 🗆 fi	irst 🗆 second 🗆 third 🗆 child				
Your baby's se	ex: □ girl □ boy				
Delivery:  Spontaneous and vaginal  induced (vaginal)  planned caesarean section  emergency caesarean					
Season in whi	ich the baby was delivered: $\square$ winter $\square$ spring $\square$ summer $\square$ autumn				
Gestation at t	he time of delivery: weeks and days				

### STROBE Statement—Checklist of items that should be included in reports of cohort studies

	Item No	Recommendation	Page No
Title and abstract	1	( <i>a</i> ) Indicate the study's design with a commonly used term in the title or the abstract	1-2
		(b) Provide in the abstract an informative and balanced summary of what was	
		done and what was found	
Introduction			1
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	4-5
Methods			
Study design	4	Present key elements of study design early in the paper	5-7
Setting	5	Describe the setting, locations, and relevant dates, including periods of	5-7
0		recruitment, exposure, follow-up, and data collection	
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of	5
1		participants. Describe methods of follow-up	
		(b) For matched studies, give matching criteria and number of exposed and	
		unexposed	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and	7-9
		effect modifiers. Give diagnostic criteria, if applicable	
Data sources/	8*	For each variable of interest, give sources of data and details of methods of	7-9
measurement		assessment (measurement). Describe comparability of assessment methods if	
		there is more than one group	
Bias	9	Describe any efforts to address potential sources of bias	11
Study size	10	Explain how the study size was arrived at	6
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable,	9
		describe which groupings were chosen and why	
Statistical methods	12	(a) Describe all statistical methods, including those used to control for	9
		confounding	
		(b) Describe any methods used to examine subgroups and interactions	
		(c) Explain how missing data were addressed	
		(d) If applicable, explain how loss to follow-up was addressed	
		(e) Describe any sensitivity analyses	
Doculto			
Particinants	13*	(a) Report numbers of individuals at each stage of study—eq numbers potentially	NA
i articipanto	15	eligible examined for eligibility confirmed eligible included in the study	
		completing follow-up, and analysed	
		(b) Give reasons for non-narticipation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data	1/1*	(a) Give characteristics of study participants (eq demographic clinical social)	NA
Descriptive data	14	and information on exposures and potential confounders	
		(b) Indicate number of participants with missing data for each variable of interact	
		(a) Summarise follow up time (ag. sverage and total amount)	
Outcome data	15*	C) Summarise follow-up time (eg, average and total amount)	NA
Ouicome data	13*	Report numbers of outcome events or summary measures over time	1171

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Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their	NA
		precision (eg, 95% confidence interval). Make clear which confounders were adjusted for	
		and why they were included	
		(b) Report category boundaries when continuous variables were categorized	
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a	
		meaningful time period	
Other analyses	17	Report other analyses done-eg analyses of subgroups and interactions, and sensitivity	NA
		analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	NA
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision.	3
		Discuss both direction and magnitude of any potential bias	
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations,	NA
		multiplicity of analyses, results from similar studies, and other relevant evidence	
Generalisability	21	Discuss the generalisability (external validity) of the study results	11
Other informati	ion		
Funding	22	Give the source of funding and the role of the funders for the present study and, if	12
		applicable, for the original study on which the present article is based	

\*Give information separately for exposed and unexposed groups.

**Note:** An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.

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