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## Relating the number of human leukocytes antigen mismatches to pregnancy complications in oocyte donation pregnancies: study protocol for a prospective multicentre cohort study (DONOR study)

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## Title

**Relating the number of human leukocytes antigen mismatches to pregnancy complications in oocyte donation pregnancies: study protocol for a prospective multicentre cohort study (DONOR study)**

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### Keywords

Oocyte donation, pregnancy, HLA Antigens, preeclampsia, pregnancy induced hypertension, histocompatibility

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## Abstract

### Introduction

Oocyte donation (OD) enables women with reproductive failure to conceive. Compared to naturally conceived (NC) and in vitro fertilization (IVF) pregnancies, OD pregnancies are associated with a higher risk of pregnancy complications. The allogeneic nature of the fetus in OD pregnancies possibly plays a role in the development of these complications. The objective of the current study is therefore to study the number and nature of human leukocyte antigen (HLA) mismatches between fetus and mother and its association with the development of hypertensive pregnancy complications

### Methods and analysis

In this prospective multicentre cohort study, 200 patients visiting one of the eleven participating fertility centres in the Netherlands to perform oocyte or embryo donation or surrogacy will be invited to participate. These patients will be included as the exposed group. In addition, 146 patients with a naturally conceived pregnancy and 146 patients who applied for non-donor IVF are included as non-exposed subjects. These groups are frequency matched on age and ethnicity and only singleton pregnancies will be included. The primary clinical outcome of the study is the development of hypertensive disease during pregnancy. Secondary outcomes are the severity of the preeclampsia, time to development of preeclampsia and development of other pregnancy complications. The number and nature of HLA mismatches between mother and child will be determined and related to clinical outcome and pregnancy complication.

### Ethics and dissemination

This study has received ethical approval from the medical ethics committee in the Leiden University Medical Centre, the Netherlands (P16.048, ABR NL56308.058.16).

### Article summary

Strengths and limitations of this study

- This is the first prospective multicentre cohort study to assess the relationship between HLA mismatches and hypertensive complications in pregnancy.
- Control of bias by adjustment for important confounders that are identified by a directed acyclic graph.
- Minimizing selection bias by selecting women pregnant after oocyte donation, embryo donation or surrogacy as the exposed group and non-donor IVF and naturally conceived pregnancies as non-exposed group.
- By including both non-donor IVF/ICSI pregnancies and women with naturally conceived pregnancies as the unexposed group, we take into account the confounding effect of artificial conception of the embryo, as well as hormonal treatment of women
- True translation of fundamental research into a clinical setting.
- This study may be underpowered to show an association of HLA mismatches and other pregnancy complications than hypertensive complications.

## Introduction

Oocyte donation (OD) is a specific method of artificial reproductive technology that resembles the technique of *in vitro* fertilization (IVF), with the exception that an oocyte is obtained from a donor. Since the first successful OD pregnancy in 1984, thousands of OD procedures have been performed worldwide<sup>1-3</sup>. Whereas the original indication was premature ovarian failure<sup>4</sup>, nowadays the indication has been extended to other forms of infertility due to a diminished ovarian reserve<sup>5-7</sup>.

In OD pregnancies, the fetus can be completely allogeneic to the mother since the fetus carries paternal and donor derived genes, whereas in non-donor autologous pregnancies the fetus is semi-allogeneic and haplo-identical to the mother (Figure 1). It is presumed that during OD pregnancies the maternal immune system needs to adapt more, or differently, to tolerate this allogeneic fetus compared to naturally conceived (NC) and IVF pregnancies<sup>8</sup>.

Despite the increasing number of OD procedures, relatively little is known about the underlying biology and long-term complications. Most of the literature regarding outcome in OD pregnancies has been focussing on perinatal complications, such as preterm birth, growth retardation, and preeclampsia. Indeed, after correction for maternal age and plurality, OD pregnancies are accompanied with a higher risk for spontaneous miscarriages, pregnancy induced hypertension, caesarean section, and bleeding complications, when compared to NC and IVF pregnancies<sup>9-11</sup>. However, the pathophysiology of the higher incidence of pregnancy complication after OD remains unclear. A higher incidence of pregnancy induced hypertension has been shown when the oocyte donor is not genetically related to the recipient<sup>12</sup>. A possible explanation therefore suggests a relationship with the high level of immunogenetic dissimilarity, reflected by the number of human leukocyte antigens (HLA) mismatches<sup>13 14</sup>. Moreover, in uncomplicated OD pregnancies a significant higher level of HLA class I matching between mother and child was observed than expected by chance<sup>15</sup>. We therefore hypothesize in this study that the number of HLA mismatches between fetus and mother is related to the development of hypertensive pregnancy complications.

## Study Objectives

### Primary objective

The primary objective of the study is the association of high number of HLA mismatches between fetus and mother and the development of hypertensive disease during pregnancy, including pregnancy induced hypertension and preeclampsia. High number of HLA mismatches is defined as  $\geq 5$  fetal-maternal HLA mismatches on basis of discrepancy on the HLA-A, -B, -C, -DR and -DQ antigens.

### Secondary objectives

Secondary objective is the association of high number of HLA mismatches and the severity of the preeclampsia, time to the development of preeclampsia and development of other pregnancy complications, including spontaneous miscarriage, (severe) fetal growth restriction, HELLP, gestational diabetes mellitus and (severe) preterm birth. Furthermore, the association of these outcomes with total number of HLA class mismatches, HLA class I and II mismatches and mismatching at HLA locus specifically is studied.

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## Methods and analysis

### Study design

This project, the DONation of Oocytes in Reproduction (DONOR) study, will be performed as a prospective multicentre cohort study conducted at eleven fertility centres in the Netherlands, with the Leiden University Medical Centre (LUMC) as coordinating centre.

Data collection will continue until the required number of 492 patient inclusions has been reached and follow up has been completed. This is expected to take approximately 2 years.

### Eligibility

Inclusion criteria are:

- Patients who are pregnant after oocyte donation, embryo donation or surrogacy pregnancy;
- Patients who are pregnant after non-donor IVF or intra cytoplasmic sperm injection (ICSI);
- Patients with a naturally conceived pregnancy (spontaneously conceived and insemination)

Exclusion criteria are:

Patients with a multiple pregnancy, patients who are mentally or legally incapable of signing the informed consent, patients with known chromosomal abnormalities or fetal anomalies.

### Study population and recruitment

Study recruitment started at the coordinating centre on September 1<sup>st</sup> 2016. All other centres will start recruitment in 2019. Recruitment is expected to last until September 2021.

In this cohort study, the exposure of interest is the number of HLA mismatches. However, HLA typing of the fetus and therefore determination of number of HLA mismatches can only be determined after birth, and thus after the development of the outcome. Therefore, we will select women pregnant after oocyte donation, embryo donation or surrogacy as the exposed group. These women will be frequency matched for age (5 year categories) and ethnicity with two non- exposed women, represented by one non-donor IVF and one naturally conceived pregnancies.

The attending physician or nurse at the fertility department will ask eligible women who are pregnant after oocyte donation, embryo donation or surrogacy pregnancy. Age- and ethnicity matched non-donor IVF women (see section above) will be selected by a research nurse, involved in this study, and asked to participate. This research nurse will also select age- and ethnicity matched women with a naturally conceived pregnancy from the low-risk pregnancy population at the LUMC. These women will be recruited at their pregnancy intake visit in first trimester.

All women will receive written information and the informed consent form, which includes a request to obtain permission for gathering data from medical records and storage of biomaterial for additional analyses related to the current study. Participants are informed that trial participation is voluntary and that they are free to withdraw at any time without any consequences for subsequent care. All members of the research team are aware of the guidelines for good clinical practice for obtaining consent. In case of participation, the informed consent form should be signed prior to inclusion in the study. Women pregnant after OD, embryo donation or surrogacy pregnancy in a foreign country will also be included in the first trimester of pregnancy.

## Study procedure

From all pregnancies, clinical characteristics will be documented (Table 1). Peripheral blood samples will be obtained from all subjects. Women in this study will have regular check-ups, according to Dutch guidelines<sup>16</sup>, and complications will be documented in their medical records. In case of pregnancy loss (gestational age > 8 weeks and < 24 weeks), the products of conception will be collected for pathologic investigation and fetal HLA typing. In case of a delivery (gestational age > 24 weeks), umbilical cord blood will be obtained, as previously described<sup>17 18</sup>. Material is pseudonomised by assigning a unique code after collection of materials and medical records.

## Control of bias

Since the design of this study is a prospective cohort study, there is need to control and adjust for confounding factors. Advanced maternal age, primiparity, obesity, IVF, and plurality are important risk factors for the development of hypertensive pregnancy complications<sup>19-21</sup>. All possible factors are visualized to provide insight into their effects by a directed acyclic graph (Figure 2) with the use of DAGitty program<sup>22</sup>. According to this DAG, adjustment for age, ethnicity, plurality and mode of conception is necessary to minimize the effect confounding. Therefore, we will select singleton pregnancies only. Next, with the inclusion of a women pregnant after oocyte donation, embryo donation or surrogacy, two women with autologous pregnancies will be included. These non-exposed women are frequency matched for ethnicity and age. One woman after natural conception and one after in vitro fertilization will be included for every exposed women. Finally, we will adjust for the confounder 'mode of conception' in the data analysis by multivariable analyses. By including two unexposed groups; non-donor IVF or intracytoplasmic sperm injection (ICSI) pregnancies as well as women with naturally conceived pregnancies, confounding is minimized. This is because artificial reproductive treatment is associated with more obstetric complications than naturally conceived pregnancies<sup>23</sup>. IVF pregnancies are therefore selected, with a comparable assisted reproductive technique as performed in OD. The hormonal treatment as part of this technique is however different between non-donor IVF and oocyte donation. In non-donor IVF, the women receive hormonal treatment for the retrieval of the oocytes and for induction of a proper endometrium before embryo transfer. In OD, the recipient only receives the treatment before embryo transfer, and the oocyte donor receives the hormonal treatment necessary for oocyte retrieval. The use of these two unexposed groups has been described in prior research<sup>14 24</sup>. The selection of the two unexposed groups is conducted early in pregnancy. Finally, information bias is limited by using standard measurement instruments for HLA typing and calculation of HLA mismatches only and by using information from medical records before birth outcome is known.

## Statistics

### *Sample size calculation*

No previous studies exist in which the number of fetal-maternal HLA mismatches is related to the development of hypertensive pregnancy complications, or other complications in pregnancy. As stated earlier, to prevent selection bias in this project the exposed group is represented by women pregnant after oocyte donation, embryo donation or surrogacy and the non-exposed group are non-donor IVF- and naturally conceived pregnancies. We based the sample size calculation on a study by Levron et al<sup>25</sup>, in which the rate of hypertensive disease of pregnancy was determined in women who conceived



through IVF using donor oocytes or autologous oocytes. In this study a stratification for maternal age and restriction to singleton pregnancies was performed. The rate of hypertensive diseases was significantly higher among donor oocyte recipients compared with autologous oocyte recipients in patients < 45 years (22% vs 10%,  $p=0.02$ ). Using  $\alpha=0.05$  and power = 80% and the assumed relative risk of 2.2 we would need to include 146 patients who conceived with donor oocytes and 146 patients who conceived with autologous oocytes to demonstrate a significant difference. In earlier studies, we showed that the median number of HLA mismatches in the OD group was 7 (3–10), in the naturally conceived pregnancy group 4 (0–5) and in the non-donor IVF group 3 (0–4)<sup>13</sup>. We will therefore select 200 patients conceived with donor oocytes to obtain around 146 exposed women with >5 HLA mismatches. As we will include two unexposed, autologous groups, both 146 patients with naturally conceived pregnancy and 146 patients with IVF pregnancy should be included. In the Netherlands, 285 OD procedures and 1 embryo donation were performed in 2012<sup>2</sup>. The centers performing these procedures are participating in this project and we therefore expect that the recruitment and inclusion will continue for approximately two years.

### *Statistical analysis plan*

Relative risks and 95% confidence intervals will be calculated for the dichotomous and categorical outcome measures. Differences between categorical variables will be compared between subgroups using the chi-square test. Normally and non normally distributed variables will be compared, respectively using the unpaired t-test and the Mann Whitney test.

To indicate an association between development of hypertensive complications (yes/no) and number of HLA mismatches multiple logistic regression analysis will be performed with adjustment for age, ethnicity and the aforementioned possible confounding variables

The relation between the number of HLA mismatches and time until development of hypertension will be visualized by Kaplan–Meier survival curves. The effect adjusted for age, ethnicity and confounders will be assessed by Cox proportional hazards regression.

Presence of Hardy-Weinberg equilibrium will be assessed using Pypop Software 0.7.0. All other statistical analyses are performed using SPSS Statistics 23 (IBM SPSS Software). For all tests a two-sided  $p<0.05$  or 95% confidence interval not including the null value is considered as significant.

### **Study outcomes**

#### *Clinical data*

We will document the obstetric and general medical history of all women participating in this study (Table 1). Furthermore, the following patient data is collected: date of birth, body weight and height, use of medication and ethnicity. In addition, paternal and donor age is collected.

During pregnancy subjects included in this study will have check-ups as with normal pregnancy controls<sup>26</sup> and the development of possible complications during pregnancy, birth or postpartum will be documented in the medical file. The complications registered during the pregnancies are spontaneous miscarriage, (severe) fetal growth restriction, pregnancy induced hypertension, preeclampsia, HELLP, gestational diabetes mellitus and (severe) preterm birth. Definition of the registered complications are listed hereafter. Finally, neonatal data on birth weight, gender and Apgar score amongst others will be documented (Table 1).

- Fetal growth restriction is defined as estimated fetal weight (EFW) less than the 10<sup>th</sup> percentile, an estimated abdominal circumference (AC) < p10 or deflection of the EFW and/or AC with > 20 percentiles over a period of >2 weeks<sup>27</sup>
- Pregnancy induced hypertension (PIH) is defined as systolic blood pressure  $\geq$  140 mmHg and/or diastolic blood pressure  $\geq$  90 mmHg detected after 20 weeks of gestation.
- Preeclampsia is defined as PIH in combination with proteinuria, as shown by  $\geq$  (300 mg/l) on dipstick testing, a protein to creatinine ratio of  $\geq$  30 mg/mmol on a random sample, or a urine protein excretion of  $\geq$  300 mg in 24 hours, or worsening of pre-existent hypertension and proteinuria<sup>28</sup>. Severe preeclampsia is defined when the blood pressure is >160 mmHg systolic or >110 mmHg diastolic or in presence of the HELLP syndrome (see further), independent of the amount of proteinuria<sup>29</sup>
- The Hemolysis Elevated Liver enzymes and Low Platelets (HELLP) syndrome is a gestational disease characterized by hemolysis, elevated liver enzymes and thrombocytopenia<sup>30</sup>.
- Gestational diabetes mellitus is defined as hyperglycemia during the pregnancy with an increased 75 grams OGTT > 7 mmol/l (sober) or > 7.8 mmol/l (after 2 hours) measured in venous blood plasma<sup>31</sup>.
- Spontaneous miscarriage is defined as the loss of pregnancy before the 24th week of gestation. In this study miscarriages before 8 weeks (before inclusion) of either the exposed or non-exposed group will not be documented
- Preterm birth is defined as birth  $\leq$  37 completed weeks of gestation<sup>32</sup>.

#### *Maternal peripheral blood and umbilical cord blood*

DNA will be extracted from peripheral blood and umbilical cord blood. In case of a miscarriage, pregnancy tissue will be collected for cell isolation and DNA extraction. HLA will be typed for loci HLA-A, -B, -C, -E, -G, -DQ and -DR using the Reverse Sequence Specific Oligonucleotides PCR technique<sup>33</sup>. For class I, a commercially available assay is applied (LIFECODES HLA-A,B and C SSO Typing kits from Immucor) , and HLA-DRB and HLA-DQB typing is performed with a locally developed SSO technique<sup>34</sup>. The maternal and fetal HLA allele frequencies will be tested for Hardy-Weinberg equilibrium<sup>35 36</sup>. The number of fetal-maternal HLA mismatches will be calculated at the Dutch national reference laboratory for histocompatibility testing (LUMC). On the basis of HLA-A, -B, -C, -DR, and -DQ antigens, the maximal number of (mis)matches between mother and child is 10. The nature of the HLA mismatches will be further analysed by determining the number of HLA epitope mismatches between mother and child, using the HLAMatchmaker program developed by Duquesnoy<sup>37</sup>. With HLAMatchmaker histocompatibility between mother and child is determined on basis of polymorphic amino acid configurations that represent defined areas of HLA epitopes on protein sequences of HLA-A, -B, and -C chains accessible to alloantibodies.

## **Discussion**

A successful pregnancy is an immunological paradox<sup>38</sup>. The fetus carries paternal and maternal genes, but is not rejected by the maternal immune system. In OD pregnancy, the fetus may be completely allogeneic compared to the mother. Therefore, it is reasonable to assume that the immune system needs to adapt more or differently to accept the allogeneic fetus. In this study we aim to determine if a

1 higher number of HLA mismatches contributes to a higher incidence of pregnancy hypertensive  
2 complications.

3 In solid organ transplantation, immunogenic dissimilarities are present between donor and recipient,  
4 and use of immunosuppressive drugs is necessary to prevent rejection of the graft<sup>8 39</sup>. As immunological  
5 acceptance of the fetus is often compared with the state of tolerance to an engrafted organ, the  
6 recognition of fetal antigens in pregnancy disorders, such as miscarriages and hypertensive  
7 complications, could be viewed as a kind of graft rejection<sup>40</sup>.

8 HLA typing and selection for an optimal number of HLA mismatches might therefore be useful as future  
9 strategies to induce immune tolerance and reduce complication rate in oocyte donation pregnancies<sup>39</sup>.

### 14 **Expected results**

- 15 • We expect to find a higher degree of pregnancy complications in OD pregnancies compared to IVF  
16 and NC pregnancies.
- 17 • We expect to find a higher number of HLA mismatches between mother and fetus in a pregnancy  
18 conceived through OD compared to IVF and NC pregnancies.
- 19 • We expect to find an association between the development of hypertensive pregnancy complications  
20 and a higher number of HLA mismatches.
- 21 • We expect to find a higher number of HLA mismatches between mother and fetus in women who  
22 conceived through OD with severe hypertensive complication, and that the development of the  
23 (severe) preeclampsia is at earlier gestational age.
- 24 • The results of this project may provide new strategies in increasing the chance of a successful OD  
25 pregnancy, for instance by defining the optimum number of HLA mismatches between donor and  
26 recipient before pregnancy. This would imply a possibility to HLA typing and matching of donors and  
27 recipients of oocytes, and extra medical care or use of specific medication during pregnancy to  
28 optimize the pregnancy outcome.
- 29 • The results of this project may lead to changes in guidelines and protocols considering OD  
30 pregnancies regarding an optimal number of HLA mismatches for OD pregnancies.

### 38 **Ethics and dissemination**

39 The study will be conducted according to the principles of the Declaration of Helsinki.

40 As mentioned before, all patients will obtain written information of the study. After a period of  
41 consideration, the patient has to decide about participation. In case of participation, the informed  
42 consent form should be signed prior to inclusion in the study. All women are treated according to local  
43 protocols. Research nurses will ensure that the samples are immediately pseudonymised by assigning a  
44 unique code. This unique code will also be used to associate clinical data with the samples, without the  
45 need for personal identifiers such as donor name, date of birth or patient hospital number on the  
46 sample container. Furthermore, the code will encompass the association of maternal, paternal, donor,  
47 and fetal samples from one family. Patient, donor, and partner will be asked to fill in webform  
48 questionnaires. Answers from this questionnaire will be linked to the Promise DONOR-database. These  
49 data, together with clinical data, will be saved semi-anonymised in the Promise DONOR-database. Only  
50 a minimum number of members of our project group will have access to this database. The principal  
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investigators will ensure that the database is maintained efficiently and that all information is up to date and accurate.

Approval was obtained from the LUMC Medical Research Ethics Committee protocol number P16.048

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## Author contribution

MLPH is the coordinating investigator. MLPH, EELOL drafted the protocol and then KB, MB and EELOL wrote the protocol in accordance with the co-authors' contributions. All authors contributed to the writing and reviewing of this article and gave final approval of the version to be published.

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## Competing interests statement

None declared.

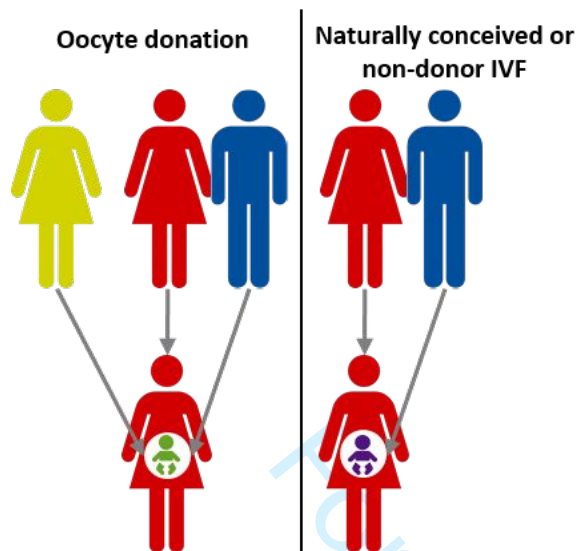
## Table and figure legends

**Table 1. Collection of data**

	Parameters
<i>Maternal characteristics</i>	Date of birth, maternal age, alcohol intake, smoking, caffeine intake, drugs intake, social economic class, weight, height, blood pressure, medical history (hypertension, diabetes mellitus, surgeries, previous blood transfusions), use of medication, education, ethnic origin, family history.
<i>Paternal characteristics</i>	Date of birth, age
<i>Donor characteristics</i>	Date of birth, age
<i>Obstetric history</i>	Parity, number of miscarriages, ectopic pregnancies or abortions, mode of delivery of previous births, gestational age at previous births, birth weight of children of previous births.
<i>During pregnancy</i>	Use of medication, miscarriage (spontaneously, medically induced or instrumental), hypertension, preeclampsia, pregnancy induced hypertension, HELLP syndrome, vaginal bleeding, fetal growth restriction.
<i>During delivery</i>	Gestational age, (if relevant) indication for induction of labour, indication for secondary caesarean section/instrumental delivery, mode of delivery, medication during labour other than oxytocin, gender of child, birth weight.
<i>Third stage</i>	Method of delivery of placenta, placenta weight, postpartum haemorrhage and blood transfusions, perineum lacerations.
<i>Neonatal data</i>	Live birth, fetal gender, birth weight, 5 and 10 minute Apgar scores, arterial umbilical cord pH, neonatal death, congenital anomalies, admission to intensive care unit.

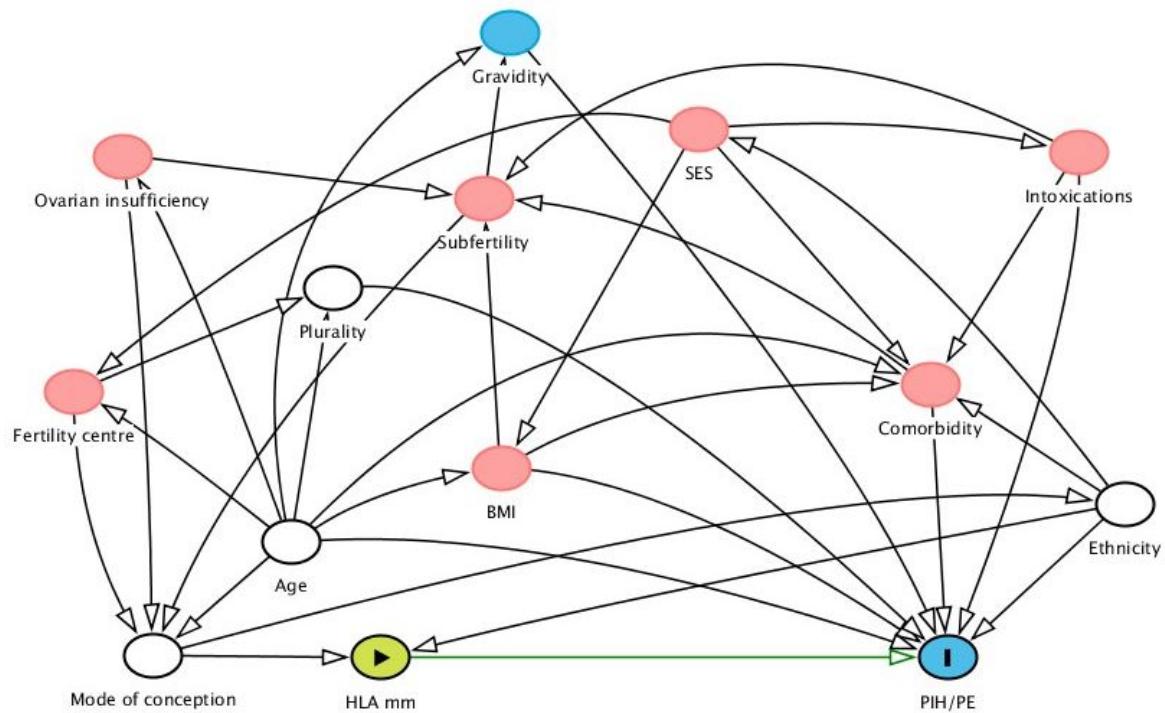
Abbreviations; BMI, body mass index; HELLP, hemolysis elevated liver enzymes and low platelets syndrome.





**Figure 1: Allogeneic situation in oocyte donation pregnancies**

In a NC or non-donor IVF pregnancy, the fetus inherits the genetic material from both the mother and the father (right in picture) leading to a semi-allogeneic situation. In an OD pregnancy involving an unrelated donor, the fetus may be completely allogeneic to the mother.



- ▶ exposure (HLA mismatches)
- ⓘ outcome (PIH/PE)
- ancestor of outcome (intoxications, BMI, comorbidity (endothelial dysfunction), subfertility and gravity)
- ancestor of exposure and outcome (SES (social economic status), fertility centre, ovarian insufficiency)
- adjusted variable (mode of conception, plurality, ethnicity, age)
- causal path

**Figure 2.** The directed acyclic graph of this study with all associating factors, available on [www.dagitty.net](http://www.dagitty.net). The minimal sufficient adjustment sets contains age, ethnicity, plurality and mode of conception for estimating the effect of HLA mm on PIH/PE.

## Abbreviations

1	
2	
3	ART artificial reproductive therapy
4	HELLP Hemolysis Elevated Liver enzymes and Low Platelets
5	
6	HLA human leukocyte antigen
7	ICSI intracytoplasmic sperm injection
8	IVF in vitro fertilization
9	
10	NC naturally conceived
11	OD oocyte donation
12	PE preeclampsia
13	PIH pregnancy induced hypertension
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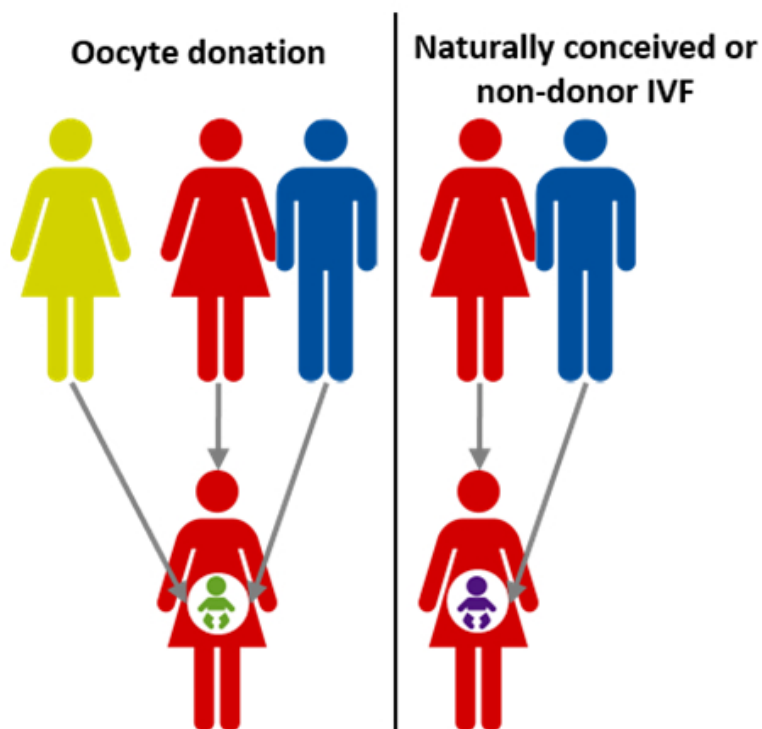
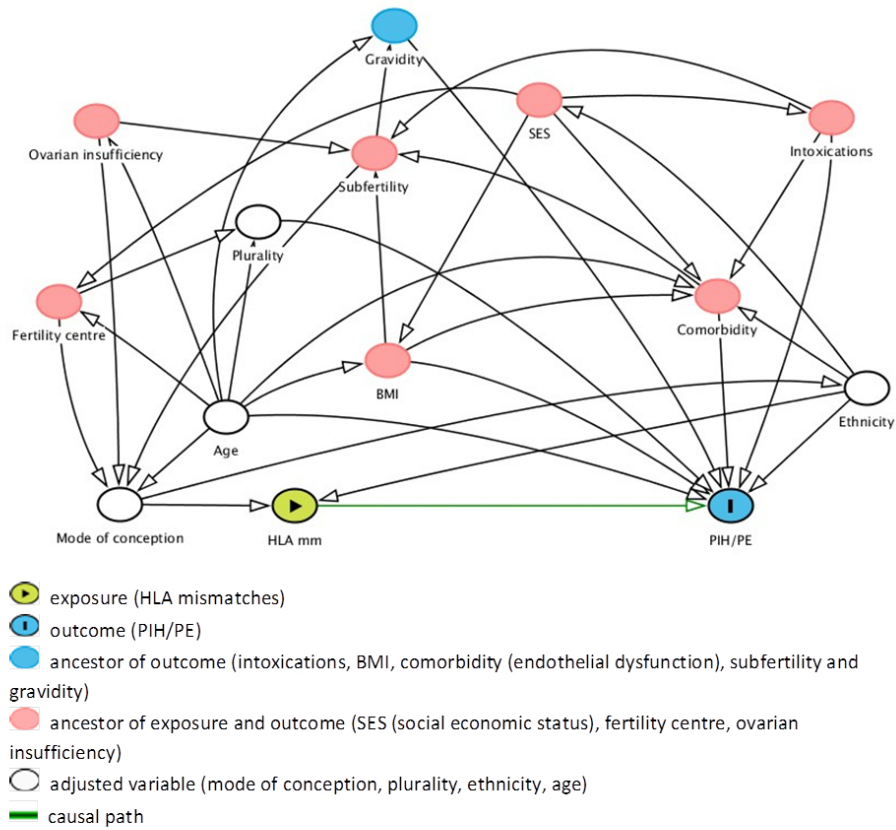


Figure 1: Allogeneic situation in oocyte donation pregnancies. In a NC or non-donor IVF pregnancy, the fetus inherits the genetic material from both the mother and the father (right in picture) leading to a semi-allogeneic situation. In an OD pregnancy involving an unrelated donor, the fetus may be completely allogeneic to the mother.



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 37 The minimal sufficient adjustment sets contains age, ethnicity, plurality and mode of conception for  
 38 estimating the effect of HLA mm on PIH/PE.  
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# BMJ Open

## Relating the number of human leukocytes antigen mismatches to pregnancy complications in oocyte donation pregnancies: study protocol for a prospective multicentre cohort study (DONOR study)

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<b>Primary Subject Heading</b>:	Obstetrics and gynaecology
Secondary Subject Heading:	Immunology (including allergy)
Keywords:	Oocyte donation, Pregnancy, HLA Antigens, Preeclampsia, pregnancy induced hypertension, histocompatibility

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# Relating the number of human leukocytes antigen mismatches to pregnancy complications in oocyte donation pregnancies: study protocol for a prospective multicentre cohort study (DONOR study)

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## Abstract

### Introduction

Oocyte donation (OD) enables women with reproductive failure to conceive. Compared to naturally conceived (NC) and in vitro fertilization (IVF) pregnancies, OD pregnancies are associated with a higher risk of pregnancy complications. The allogeneic nature of the fetus in OD pregnancies possibly plays a role in the development of these complications. The objective of the current study is therefore to study the number and nature of human leukocyte antigen (HLA) mismatches between fetus and mother and its association with the development of hypertensive pregnancy complications

### Methods and analysis

In this prospective multicentre cohort study, 200 patients visiting one of the eleven participating fertility centres in the Netherlands to perform oocyte or embryo donation or surrogacy will be invited to participate. These patients will be included as the exposed group. In addition, 146 patients with a naturally conceived pregnancy and 146 patients who applied for non-donor IVF are included as non-exposed subjects. These groups are frequency matched on age and ethnicity and only singleton pregnancies will be included. The primary clinical outcome of the study is the development of hypertensive disease during pregnancy. Secondary outcomes are the severity of the preeclampsia, time to development of preeclampsia and development of other pregnancy complications. The association of high number of HLA mismatches (>5) between mother and fetus will be determined and related to clinical outcome and pregnancy complication.

### Ethics and dissemination

This study received ethical approval from the medical ethics committee in the Leiden University Medical Centre, the Netherlands (P16.048, ABR NL56308.058.16). Study findings will be presented at (inter) national conferences and published in peer-reviewed journals.

### Article summary

Strengths and limitations of this study

- Control of bias by adjustment for important confounders that are identified by a directed acyclic graph.
- Minimizing selection bias by selecting women pregnant after oocyte donation, embryo donation or surrogacy as the exposed group and non-donor IVF and naturally conceived pregnancies as non-exposed group.
- By including both non-donor IVF/ICSI pregnancies and women with naturally conceived pregnancies as the unexposed group, we take into account the confounding effect of artificial conception of the embryo, as well as hormonal treatment of women
- True translation of fundamental research into a clinical setting.
- Study may be underpowered to show an association of HLA mismatches and other pregnancy complications than hypertensive complications.



## Introduction

Oocyte donation (OD) is a specific method of artificial reproductive technology that resembles the technique of *in vitro* fertilization (IVF), with the exception that an oocyte is obtained from a donor. Since the first successful OD pregnancy in 1984, thousands of OD procedures have been performed worldwide<sup>1-3</sup>. Whereas the original indication was premature ovarian failure<sup>4</sup>, nowadays the indication has been extended to other forms of infertility due to a diminished ovarian reserve<sup>5-7</sup>. In OD pregnancies, the fetus can be completely allogeneic to the mother since the fetus carries paternal and donor derived genes, whereas in non-donor autologous pregnancies the fetus is semi-allogeneic and haplo-identical to the mother (Figure 1). It is presumed that during OD pregnancies the maternal immune system needs to adapt more, or differently, to tolerate this allogeneic fetus compared to naturally conceived (NC) and IVF pregnancies<sup>8</sup>.

Despite the increasing number of OD procedures, relatively little is known about the underlying biology and long-term complications. Most of the literature regarding outcome in OD pregnancies has been focussing on perinatal complications, such as preterm birth, growth retardation, and preeclampsia. Indeed, after correction for maternal age and plurality, OD pregnancies are accompanied with a higher risk for spontaneous miscarriages, pregnancy induced hypertension, caesarean section, and bleeding complications, when compared to NC and IVF pregnancies<sup>9-11</sup>. However, the pathophysiology of the higher incidence of pregnancy complication after OD remains unclear. A higher incidence of pregnancy induced hypertension has been shown when the oocyte donor is not genetically related to the recipient<sup>12</sup>. A possible explanation therefore suggests a relationship with the high level of immunogenetic dissimilarity, reflected by the number of human leukocyte antigens (HLA) mismatches<sup>13,14</sup>. Moreover, in uncomplicated OD pregnancies a significant higher level of HLA class I matching between mother and child was observed than expected by chance<sup>15</sup>. We therefore hypothesize in this study that the number of HLA mismatches between fetus and mother is related to the development of hypertensive pregnancy complications.

## Study Objectives

### Primary objective

The primary objective of the study is the association of high number of HLA mismatches between fetus and mother and the development of hypertensive disease during pregnancy, including pregnancy induced hypertension and preeclampsia. High number of HLA mismatches is defined as  $\geq 5$  fetal-maternal HLA mismatches on basis of discrepancy on the HLA-A, -B, -C, -DR and -DQ antigens.

### Secondary objectives

Secondary objective is the association of high number of HLA mismatches and the severity of the preeclampsia, time to the development of preeclampsia and development of other pregnancy complications, including spontaneous miscarriage, (severe) fetal growth restriction, HELLP, gestational diabetes mellitus and (severe) preterm birth. Furthermore, the association of these outcomes with total number of HLA class mismatches, HLA class I and II mismatches and mismatching at HLA locus specifically is studied.

## Methods and analysis

### Study design

This study is performed within the DONOR project; a project on the DONation of Oocytes in Reproduction. This study will be performed as a prospective multicentre cohort study conducted at eleven fertility centres in the Netherlands, with the Leiden University Medical Centre (LUMC) as coordinating centre.

Data collection will continue until the required number of 492 patient inclusions has been reached and follow up has been completed. This is expected to take approximately 2 years.

### Eligibility

Inclusion criteria are:

- Patients who are pregnant after oocyte donation, embryo donation or surrogacy pregnancy;
- Patients who are pregnant after non-donor IVF or intra cytoplasmic sperm injection (ICSI);
- Patients with a naturally conceived pregnancy (spontaneously conceived and insemination)

Exclusion criteria are:

Patients with a multiple pregnancy, patients who are mentally or legally incapable of signing the informed consent, patients with known chromosomal abnormalities or fetal anomalies.

### Study population and recruitment

Study recruitment started at the coordinating centre on September 1<sup>st</sup> 2016. All other centres will start recruitment in 2019. Recruitment is expected to last until September 2021.

In this cohort study, the exposure of interest is the number of HLA mismatches. However, HLA typing of the fetus and therefore determination of number of HLA mismatches can only be determined after birth, and thus after the development of the outcome. Therefore, we will select women pregnant after oocyte donation, embryo donation or surrogacy as the exposed group. These women will be frequency matched for age (5 year categories) and ethnicity with two non- exposed women, represented by one non-donor IVF and one naturally conceived pregnancies.

The attending physician or nurse at the fertility department will ask eligible women who are pregnant after oocyte donation, embryo donation or surrogacy pregnancy. Age- and ethnicity matched non-donor IVF women (see section above) will be selected by a research nurse, involved in this study, and asked to participate. This research nurse will also select age- and ethnicity matched women with a naturally conceived pregnancy from the low-risk pregnancy population at the LUMC. These women will be recruited at their pregnancy intake visit in first trimester.

All women will receive written information and the informed consent form, which includes a request to obtain permission for gathering data from medical records and storage of biomaterial for additional analyses related to the current study. Participants are informed that trial participation is voluntary and that they are free to withdraw at any time without any consequences for subsequent care. All members of the research team are aware of the guidelines for good clinical practice for obtaining consent. In case of participation, the informed consent form should be signed prior to inclusion in the study. Women pregnant after OD, embryo donation or surrogacy pregnancy in a foreign country will also be included in the first trimester of pregnancy.

## Study procedure

From all pregnancies, clinical characteristics will be documented (Table 1). Peripheral blood samples will be obtained from all subjects. Women in this study will have regular check-ups, according to Dutch guidelines<sup>16</sup>, and complications will be documented in their medical records. In case of pregnancy loss (gestational age > 8 weeks and < 24 weeks), the products of conception will be collected for pathologic investigation and fetal HLA typing. In case of a delivery (gestational age > 24 weeks), umbilical cord blood will be obtained, as previously described<sup>17,18</sup>. Material is pseudonomised by assigning a unique code after collection of materials and medical records.

## Control of bias

Since the design of this study is a prospective cohort study, there is need to control and adjust for confounding factors. Advanced maternal age, primiparity, obesity, IVF, and plurality are important risk factors for the development of hypertensive pregnancy complications<sup>19-21</sup>. All possible factors are visualized to provide insight into their effects by a directed acyclic graph (Figure 2) with the use of DAGitty program<sup>22</sup>. According to this DAG, adjustment for age, ethnicity, plurality and mode of conception is necessary to minimize the effect confounding. Therefore, we will select singleton pregnancies only. Next, with the inclusion of a women pregnant after oocyte donation, embryo donation or surrogacy, two women with autologous pregnancies will be included. These non-exposed women are frequency matched for ethnicity and age. One woman after natural conception and one after in vitro fertilization will be included for every exposed women. Finally, we will adjust for all pre-defined confounders including 'mode of conception' in the data analysis by multivariable analyses. In addition, since epidemiological studies show that limited seminal exposure or change of partner is associated with increased risk hypertensive complications<sup>23</sup>, the factor 'source of semen' will be included in the multivariable analysis

By including two unexposed groups; non-donor IVF or intracytoplasmic sperm injection (ICSI) pregnancies as well as women with naturally conceived pregnancies, confounding is minimized. This is because artificial reproductive treatment is associated with more obstetric complications than naturally conceived pregnancies<sup>24</sup>. IVF pregnancies are therefore selected, with a comparable assisted reproductive technique as performed in OD. The hormonal treatment as part of this technique is however different between non-donor IVF and oocyte donation. In non-donor IVF, the women receive hormonal treatment for the retrieval of the oocytes and for induction of a proper endometrium before embryo transfer. In OD, the recipient only receives the treatment before embryo transfer, and the oocyte donor receives the hormonal treatment necessary for oocyte retrieval. The use of these two unexposed groups has been described in prior research<sup>14,25</sup>. The selection of the two unexposed groups is conducted early in pregnancy. Finally, information bias is limited by using standard measurement instruments for HLA typing and calculation of HLA mismatches only and by using information from medical records before birth outcome is known.

## Statistics

### *Sample size calculation*

No previous studies exist in which the number of fetal-maternal HLA mismatches is related to the development of hypertensive pregnancy complications, or other complications in pregnancy. As stated earlier, to prevent selection bias in this project the exposed group is represented by women pregnant after oocyte donation, embryo donation or surrogacy and the non-exposed group are non-donor IVF- and naturally conceived pregnancies. We based the sample size calculation on a study by Levron et al<sup>26</sup>, in which the rate of hypertensive disease of pregnancy was determined in women who conceived through IVF using donor oocytes or autologous oocytes. In this study a stratification

1 for maternal age and restriction to singleton pregnancies was performed. The rate of hypertensive  
2 diseases was significantly higher among donor oocyte recipients compared with autologous oocyte  
3 recipients in patients < 45 years (22% vs 10%,  $p=0.02$ ). Using  $\alpha=0.05$  and power = 80% and the  
4 assumed relative risk of 2.2 we would need to include 146 patients who conceived with donor  
5 oocytes and 146 patients who conceived with autologous oocytes to demonstrate a significant  
6 difference. In earlier studies, we showed that the median number of HLA mismatches in the OD  
7 group was 7 (3–10), in the naturally conceived pregnancy group 4 (0–5) and in the non-donor IVF  
8 group 3 (0–4)<sup>13</sup>. We will therefore select 200 patients conceived with donor oocytes to obtain  
9 around 146 exposed women with >5 HLA mismatches. As we will include two unexposed,  
10 autologous groups, both 146 patients with naturally conceived pregnancy and 146 patients with IVF  
11 pregnancy should be included.

12 In the Netherlands, 285 OD procedures and 1 embryo donation were performed in 2012<sup>2</sup>. The  
13 centers performing these procedures are participating in this project and we therefore expect that  
14 the recruitment and inclusion will continue for approximately two years.

### 15 *Statistical analysis plan*

16 Relative risks and 95% confidence intervals will be calculated for the dichotomous and categorical  
17 outcome measures. Differences between categorical variables will be compared between subgroups  
18 using the chi-square test. Normally and non normally distributed variables will be compared,  
19 respectively using the unpaired t-test and the Mann-Whitney test.

20 To indicate an association between development of hypertensive complications (yes/no) and  
21 number of HLA mismatches multiple logistic regression analysis will be performed with adjustment  
22 for age, ethnicity and the aforementioned possible confounding variables

23 The relation between the number of HLA mismatches and time until development of hypertension  
24 will be visualized by Kaplan–Meier survival curves. The effect adjusted for age, ethnicity and  
25 confounders will be assessed by Cox proportional hazards regression.

26 Presence of Hardy-Weinberg equilibrium will be assessed using Pypop Software 0.7.0. All other  
27 statistical analyses are performed using SPSS Statistics 23 (IBM SPSS Software). For all tests a two-  
28 sided  $p<0.05$  or 95% confidence interval not including the null value is considered as significant.

### 29 **Study outcomes**

#### 30 *Clinical data*

31 We will document the obstetric and general medical history of all women participating in this study  
32 (Table 1). Furthermore, the following patient data is collected: date of birth, body weight and height,  
33 use of medication and ethnicity. In addition, paternal and donor age is collected.

34 During pregnancy subjects included in this study will have check-ups as with normal pregnancy  
35 controls<sup>27</sup> and the development of possible complications during pregnancy, birth or postpartum will  
36 be documented in the medical file. The complications registered during the pregnancies are  
37 spontaneous miscarriage, (severe) fetal growth restriction, pregnancy induced hypertension,  
38 preeclampsia, HELLP, gestational diabetes mellitus and (severe) preterm birth. Definition of the  
39 registered complications are listed hereafter. Finally, neonatal data on birth weight, gender and  
40 Apgar score amongst others will be documented (Table 1).

- 41 - Fetal growth restriction is defined as estimated fetal weight (EFW) less than the 10<sup>th</sup>  
42 percentile, an estimated abdominal circumference (AC) < p10 or deflection of the EFW  
43 and/or AC with > 20 percentiles over a period of >2 weeks<sup>28</sup>

- Pregnancy induced hypertension (PIH) is defined as systolic blood pressure  $\geq 140$  mmHg and/or diastolic blood pressure  $\geq 90$  mmHg detected after 20 weeks of gestation.
- Preeclampsia is defined as PIH in combination with proteinuria, as shown by  $\geq (300$  mg/l) on dipstick testing, a protein to creatinine ratio of  $\geq 30$  mg/mmol on a random sample, or a urine protein excretion of  $\geq 300$  mg in 24 hours, or worsening of pre-existent hypertension and proteinuria<sup>29</sup>. Severe preeclampsia is defined when the blood pressure is  $>160$  mmHg systolic or  $>110$  mmHg diastolic or in presence of the HELLP syndrome (see further), independent of the amount of proteinuria<sup>30</sup>
- The Hemolysis Elevated Liver enzymes and Low Platelets (HELLP) syndrome is a gestational disease characterized by hemolysis, elevated liver enzymes and thrombocytopenia<sup>31</sup>.
- Gestational diabetes mellitus is defined as hyperglycemia during the pregnancy with an increased 75 grams OGTT  $> 7$  mmol/l (sober) or  $> 7.8$  mmol/l (after 2 hours) measured in venous blood plasma<sup>32</sup>.
- Spontaneous miscarriage is defined as the loss of pregnancy before the 24th week of gestation. In this study miscarriages before 8 weeks (before inclusion) of either the exposed or non-exposed group will not be documented
- Preterm birth is defined as birth  $\leq 37$  completed weeks of gestation<sup>33</sup>.

#### *Maternal peripheral blood and umbilical cord blood*

DNA will be extracted from peripheral blood and umbilical cord blood. In case of a miscarriage, pregnancy tissue will be collected for cell isolation and DNA extraction. HLA will be typed for loci HLA-A, -B, -C, -E, -G, -DQ and -DR using the Reverse Sequence Specific Oligonucleotides PCR technique<sup>34</sup>. For class I, a commercially available assay is applied (LIFECODES HLA-A,B and C SSO Typing kits from Immucor) , and HLA-DRB and HLA-DQB typing is performed with a locally developed SSO technique<sup>35</sup>.

The maternal and fetal HLA allele frequencies will be tested for Hardy-Weinberg equilibrium<sup>36,37</sup>.

The number of fetal-maternal HLA mismatches will be calculated at the Dutch national reference laboratory for histocompatibility testing (LUMC). On the basis of HLA-A, -B, -C, -DR, and -DQ antigens, the maximal number of (mis)mismatches between mother and child is 10.

The nature of the HLA mismatches will be further analysed by determining the number of HLA epitope mismatches between mother and child, using the HLAMatchmaker program developed by Duquesnoy<sup>38</sup>. With HLAMatchmaker histocompatibility between mother and child is determined on basis of polymorphic amino acid configurations that represent defined areas of HLA epitopes on protein sequences of HLA-A, -B, and -C chains accessible to alloantibodies.

#### **Patient and Public Involvement statement**

The Dutch society for patients with fertility problems (Freya) was consulted during the protocol development. To further facilitate the recruitment of patients, in addition to informing patients and their partners at the fertility centers, advertisement of this study will be done by the website of Freya. We will use their communication forms with their fellow sufferers to increase the recognition of the study and present results during their thematic meetings to inform on (progress of) the study. In addition, social media will be used to signpost publications and conference presentations and highlight important findings.

## Discussion

A successful pregnancy is an immunological paradox<sup>39</sup>. The fetus carries paternal and maternal genes, but is not rejected by the maternal immune system. In OD pregnancy, the fetus may be completely allogeneic compared to the mother. Therefore, it is reasonable to assume that the immune system needs to adapt more or differently to accept the allogeneic fetus. In this study we aim to determine if a higher number of HLA mismatches contributes to a higher incidence of pregnancy hypertensive complications.

In solid organ transplantation, immunogenic dissimilarities are present between donor and recipient, and use of immunosuppressive drugs is necessary to prevent rejection of the graft<sup>8,40</sup>. As immunological acceptance of the fetus is often compared with the state of tolerance to an engrafted organ, the recognition of fetal antigens in pregnancy disorders, such as miscarriages and hypertensive complications, could be viewed as a kind of graft rejection<sup>41</sup>.

HLA typing and selection for an optimal number of HLA mismatches might therefore be useful as future strategies to induce immune tolerance and reduce complication rate in oocyte donation pregnancies<sup>40</sup>.

## Expected results

- We expect to find a higher degree of pregnancy complications in OD pregnancies compared to IVF and NC pregnancies.
- We expect to find a higher number of HLA mismatches between mother and fetus in a pregnancy conceived through OD compared to IVF and NC pregnancies.
- We expect to find an association between the development of hypertensive pregnancy complications and a higher number of HLA mismatches.
- We expect to find a higher number of HLA mismatches between mother and fetus in women who conceived through OD with severe hypertensive complication, and that the development of the (severe) preeclampsia is at earlier gestational age.
- The results of this project may provide new strategies in increasing the chance of a successful OD pregnancy, for instance by defining the optimum number of HLA mismatches between donor and recipient before pregnancy. This would imply a possibility to HLA typing and matching of donors and recipients of oocytes, and extra medical care or use of specific medication during pregnancy to optimize the pregnancy outcome.
- The results of this project may lead to changes in guidelines and protocols considering OD pregnancies regarding an optimal number of HLA mismatches for OD pregnancies.

## Ethics and dissemination

The study will be conducted according to the principles of the Declaration of Helsinki.

As mentioned before, all patients will obtain written information of the study. After a period of consideration, the patient has to decide about participation. In case of participation, the informed consent form should be signed prior to inclusion in the study. All women are treated according to local protocols. Research nurses will ensure that the samples are immediately pseudonimised by assigning a unique code. This unique code will also be used to associate clinical data with the samples, without the need for personal identifiers such as donor name, date of birth or patient hospital number on the sample container. Furthermore, the code will encompass the association of

1 maternal and fetal samples from one family. Patients will be asked to fill in webform questionnaires.  
2 Answers from this questionnaire will be linked to the Promise DONOR-database. These data,  
3 together with clinical data, will be saved semi-anonymised in the Promise DONOR-database. Only a  
4 minimum number of members of our project group will have access to this database. The principal  
5 investigators will ensure that the database is maintained efficiently and that all information is up to  
6 date and accurate.  
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8 Approval was obtained from the LUMC Medical Research Ethics Committee protocol number  
9 P16.048. Study findings will be presented at (inter) national conferences and published in peer-  
10 reviewed journals.  
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### **Contributorship statement**

MLPH is the coordinating investigator. MLPH and ELOL drafted the protocol and then KB, MB and ELOL wrote the protocol in accordance with the co-authors' contributions. All authors contributed to the writing and reviewing of this article; ME, SH and FC complemented on the immunological questions in this protocol and SIC improved the methodological aspects. All authors gave final approval of the version to be published.

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### **Competing interests statement**

None declared.

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## Table and figure legends

**Table 1. Collection of data**

	Parameters
<i>Maternal characteristics</i>	Date of birth, maternal age, alcohol intake, smoking, caffeine intake, drugs intake, social economic class, weight, height, blood pressure, medical history (hypertension, diabetes mellitus, surgeries, previous blood transfusions), use of medication, education, ethnic origin, family history.
<i>Paternal characteristics</i>	Date of birth, age
<i>Donor characteristics</i>	Date of birth, age
<i>Obstetric history</i>	Parity, number of miscarriages, ectopic pregnancies or abortions, mode of delivery of previous births, gestational age at previous births, birth weight of children of previous births.
<i>During pregnancy</i>	Use of medication, miscarriage (spontaneously, medically induced or instrumental), hypertension, preeclampsia, pregnancy induced hypertension, HELLP syndrome, vaginal bleeding, fetal growth restriction.
<i>During delivery</i>	Gestational age, (if relevant) indication for induction of labour, indication for secondary caesarean section/instrumental delivery, mode of delivery, medication during labour other than oxytocin, gender of child, birth weight.
<i>Third stage</i>	Method of delivery of placenta, placenta weight, postpartum haemorrhage and blood transfusions, perineum lacerations.
<i>Neonatal data</i>	Live birth, fetal gender, birth weight, 5 and 10 minute Apgar scores, arterial umbilical cord pH, neonatal death, congenital anomalies, admission to intensive care unit.

Abbreviations; BMI, body mass index; HELLP, hemolysis elevated liver enzymes and low platelets syndrome.

### Figure 1: Allogeneic situation in oocyte donation pregnancies

In a NC or non-donor IVF pregnancy, the fetus inherits the genetic material from both the mother and the father (right in picture) leading to a semi-allogeneic situation. In an OD pregnancy involving an unrelated donor, the fetus may be completely allogeneic to the mother.

**Figure 2.** The directed acyclic graph of this study with all associating factors, available on [www.dagitty.net](http://www.dagitty.net). The minimal sufficient adjustment sets contains age, ethnicity, plurality and mode of conception for estimating the effect of HLA mm on PIH/PE.

## Abbreviations

ART	artificial reproductive therapy
HELLP	Hemolysis Elevated Liver enzymes and Low Platelets
HLA	human leukocyte antigen
ICSI	intracytoplasmic sperm injection
IVF	in vitro fertilization
NC	naturally conceived
OD	oocyte donation
PE	preeclampsia
PIH	pregnancy induced hypertension

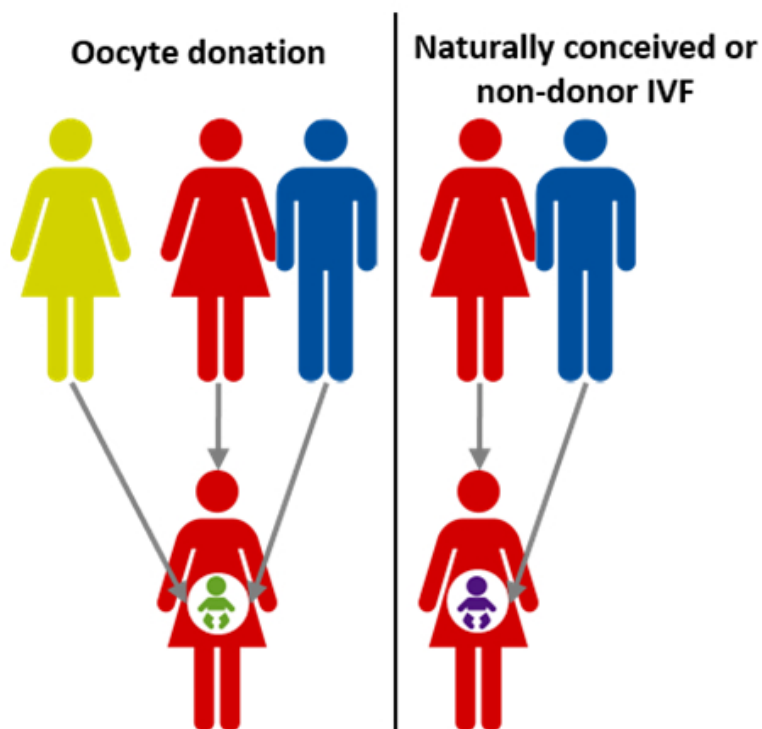
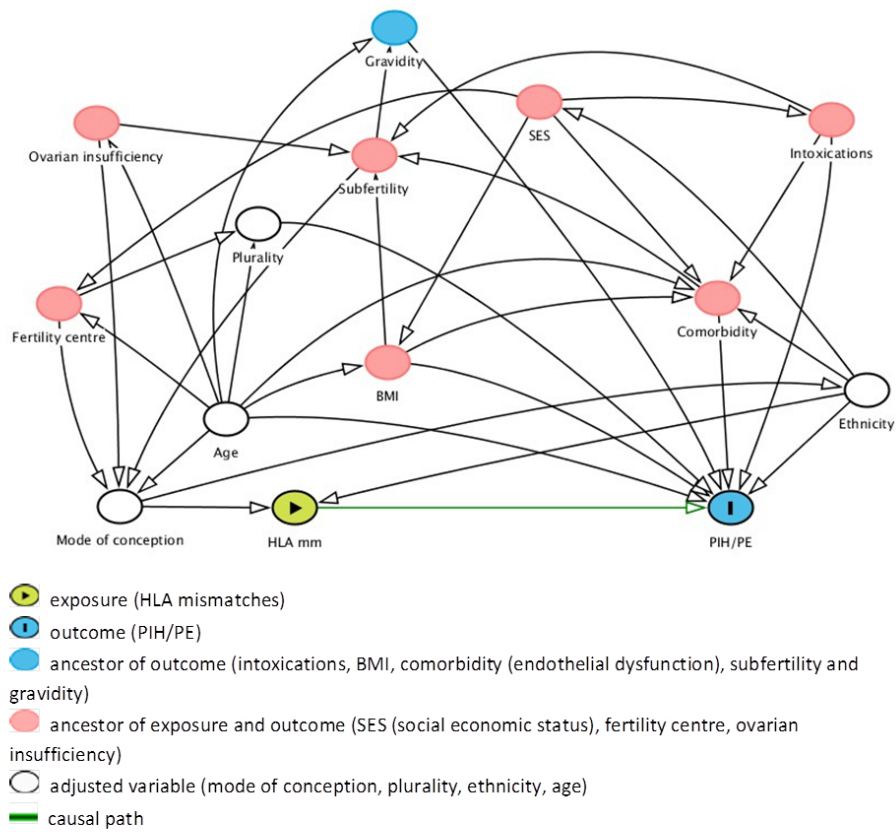


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