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Genetic and epidemiological aspects of implantation defects

Studies on recurrent miscarriage, preeclampsia and oocyte donation

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Abstract

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Implantation requires complex molecular and cellular events involving coagulation, angiogenesis and immunological processes that need to be well regulated for a pregnancy to establish and progress normally. The overall aim of this thesis was to study different models associated with atypical angiogenesis, impaired implantation and/or placentation, such as recurrent miscarriage (RM), oocyte donation (OD) and preeclampsia.

Histidine-rich glycoprotein (HRG), a serum protein with angiogenic potential has been previously shown to have an impact on implantation and fertility. In two retrospective case-control studies, women suffering from RM (Study I) and gestational hypertensive disorders (GHD) (Study IV) have been compared to healthy control women, regarding carriership of HRG genotypes (HRG A1042G and C633T SNP, respectively). According to the findings of this thesis, heterozygous carriers of the HRG A1042G SNP suffer from RM more seldom than homozygous carriers (Study I). Additionally, the presence of the HRG 633T allele was associated with increased odds of GHD (GHD IV). Studies II and III comprised a national cohort of relatively young women with optimal health status conceiving singletons with donated oocytes versus autologous oocytes (spontaneously or via IVF). We explored differences in various obstetric (Study II) and neonatal (Study III) outcomes from the Swedish Medical Birth Register. Women conceiving with donated oocytes had a higher risk of GHD, induction of labor and cesarean section, as well as postpartum hemorrhage and retained placenta, when compared to autologously conceiving women. OD infants had higher odds of prematurity and lower birthweight and length when born preterm, compared to neonates from autologous oocytes. With regard to the indication of OD treatment, higher intervention but neverthelss favourable neonatal outcomes were observed in women with diminished ovarian reserve; the risk of GHD did not differ among OD recipients after adjustment.

In conclusion, HRG genetic variation appears to contribute to placental dysfunction disorders. HRG is potential biomarker that may contribute in the prediction of the individual susceptibility for RM and GHD. Regarding OD in Sweden, the recipients-despite being of optimal age and health status- need careful preconceptional counselling and closer prenatal monitoring, mainly due to increased prevalence of hypertensive disorders and prematurity.

Keywords: Angiogenesis, genetic polymorphism, gestational hypertensive disorders, Histidine-rich glycoprotein, HRG, implantation defect, oocyte donation, placentation, preeclampsia, recurrent miscarriage, SNP.

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To my family

for giving me
roots and wings

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Elenis E, Lindgren KE, Karypidis H, Skalkidou A, Hosseini F, Bremme K, Landgren BM, Skjöldebrand-Sparre L, Stavreus-Evers A, Sundström-Poromaa I, Åkerud H. (2014) The histidine-rich glycoprotein A1042G polymorphism and recurrent miscarriage: a pilot study. *Reprod Biol Endocrinol*. 12:70

II Elenis E, Svanberg AS, Lampic C, Skalkidou A, Åkerud H, Sydsjö G. (2015) Adverse obstetric outcomes in pregnancies resulting from oocyte donation; a retrospective cohort case study in Sweden. *BMC Pregnancy and Childbirth.* 15:247

III Elenis E, Sydsjö G, Skalkidou A, Lampic C, Svanberg AS. (2016) Neonatal outcomes in pregnancies resulting from oocyte donation: a cohort study in Sweden. *BMC Pediatrics.* 16:170.

IV Elenis E, Skalkidou A, Svanberg AS, Sydsjö G, Stavreus-Evers A, Åkerud H. (2016) HRG C633T polymorphism and risk of gestational hypertensive disorders: a pilot study. *(Submitted).*

Contents

Abbreviations

ACOG	American College of Obstetricians and Gynecologists
ART	Assisted Reproductive Technology
ASRM	American Society of Reproductive Medicine
BMI	Body Mass Index
CI	Confidence Interval
ESHRE	European Society of Human Reproduction & Embryology
GHD	Gestational Hypertensive Disorders
HRG	Histidine-rich glycoprotein
IVF	*In Vitro* Fertilization
ICSI	Intracytoplasmic Sperm Injection
LBW	Low Birth Weight (<2500 gr)
LMWH	Low Molecular Weight Heparin
MBR	Medical Birth Register
NICE	National Institute for Health and Clinical Excellence
OD	Oocyte Donation
OR	Odds Ratio
PlgF	Placental Growth Factor
POI	Premature Ovarian Insufficiency
RCOG	Royal College of Obstetricians and Gynaecologists
RM	Recurrent Miscarriage
SGA	Small for Gestational Age
SNP	Single Nucleotide Polymorphism
TS	Turner Syndrome
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

Preface

Through the years, as a clinician I have come in contact with several couples facing the heart-wrenching diagnosis of recurrent miscarriage. Despite the evaluation work-up, no real answer about the reason could be given to more than half of them, leaving them feeling helpless and we physicians feeling frustrated. During the past years, it has come to light that even different gynecological diseases share common steps in the process, for example, preeclampsia and oocyte donation. Though challenging, my lack of answers was the reason motivating me to look deeper into the world of implantation, where it all begins.

Introduction

Infertility

Infertility is defined as failure to conceive during a 12 month-period or longer of regular unprotected vaginal sexual intercourse [1, 2] and it affects as many as 8–15% of reproductive-aged couples worldwide [3, 4]. According to the WHO calculations, the global infertility prevalence is estimated to be around 186 million in women aged 15 to 49 years [5].

Primary infertility is defined as the inability to conceive without a prior pregnancy, whereas secondary infertility is preceded by a previous pregnancy with or without livebirth [4, 6]. Some prefer the term "subfertility" in order to describe couples who are not sterile but exhibit decreased reproductive efficiency [7].

Diagnostic evaluation and clinical assessment is recommended after a twelve-month interval. Earlier evaluation is warranted after 6 months of unsuccessful efforts to conceive in women aged 35 years [2] or 36 years or over [1] due to the observed age-related decline in fertility as a woman approaches age 40. Furthermore, if a history of predisposing factors for subfertility exists, earlier referral for specialist consultation is justified [1]. In Sweden, no specific age threshold exists, but usually the evaluation process is accelerated for women above the age of 38 years [8].

The most common causes of infertility are: factors in the male (35%), ovulatory disorders (15%), tubal damage and pelvic pathology (ex endometriosis) (35%), uterine factors (5%), or unexplained infertility (10%) [7]. The investigations include history and physical examination, semen analysis of the male, assessment of ovulation and hormone status of the female, screening for tubal occlusion with hysterosalpingo-contrast-ultrasonography (HyCo-Sy) or hysterosalpingography (HSG), uterine cavity abnormalities and susceptibility to rubella and other sexually transmitted infections (STI) (ex HIV, hepatitis B and C, HTLV1-2 and syphilis) [1, 8]. In some countries, even pap smear and screening for chlamydia trachomatis is still performed routinely as part of the fertility evaluation [1]. If no causal factor is found after the investigation, then the diagnosis of unexplained infertility is established.

The management options offered depend on the result of the evaluation and vary from expectant management to a wide range of assisted reproductive technologies (ART). The most effective form of ART is *in vitro* fertili-

zation (IVF) with the use of autologous and, alternatively, donated oocytes. In Europe, according to the ESHRE (European Society of Human Reproduction & Embryology), during 2013, 637 550 treatment cycles were performed with autologous oocytes, and 39 689 with donated oocytes [9]. It is estimated that 3.6% of all children born in Sweden during 2013 were conceived via IVF treatment [10]. The success rate of IVF, that is to say, the pregnancy rate per oocyte retrieval varies and can reach up to between 38 and 55% [1, 11, 12]. The outcome is affected by various factors, such as female age or underlying cause of infertility [1, 11], with oocyte donation being the most efficient form of ART.

Oocyte Donation

A series of societal changes which affect the reproductive potential of individuals has occurred during the last decades in Sweden. The purpose of implementing these changes was to provide people with the same rights and opportunities to reproduce, regardless of civil status or sexual orientation. Sperm donation from identifiable donors for heterosexual couples was first permitted in 1985 and, in 2005, the law was extended to include lesbian couples. Infertility treatment with donated oocytes became available in 2003 at the University clinics [13]. Recently (2016) it was legislated that fertility treatment would be offered to single women within the Swedish public health care system [14, 15]. Lastly, oocyte cryopreservation – otherwise known as "egg freezing" – a form of fertility preservation for social or medical reasons (i.e. before chemotherapy due to cancer or other diseases, upon diagnosis of Turner syndrome, etc.), has gained wide acceptance since its introduction in 2011. Embryo donation, that is, donation of both sperm and oocytes at the same time, as well as surrogacy, is still not permitted in Sweden.

To date, oocyte donation (OD) with oocytes originating from healthy volunteer donors is performed in Sweden at the fertility clinics of the seven University hospitals, which comprise Stockholm, Uppsala, Gothenburg, Malmö, Linköping, Örebro and Umeå. Oocyte donors undergo ovarian stimulation and retrieval for the sole purpose of providing oocytes to others (oocyte sharing is not permitted). According to the latest data, 2.6% of the total IVF and ICSI annual treatment cycles during 2013 in Sweden were performed with donated oocytes (corresponding to a total of 458 started cycles of IVF with fresh and frozen embryos) and 87 infants were born during that year [10].

One cycle of oocyte donation is similar, to a large extent, to a conventional IVF cycle. It typically takes eight to twelve days of self-injection with FSH to stimulate follicle growth from the donor [16]. This is followed by transvaginal extraction of oocytes under ultrasound guidance and fertiliza-

tion with the male partner's sperm in the laboratory through conventional IVF or through ICSI. The recipient woman is simultaneously treated hormonally, preparing the uterine lining before the transferal of the fertilized embryo [16].

After 2–5 days of incubation, the recipient couple return to the fertility centre and the embryo is transferred into the recipient's uterus. A few weeks later, a pregnancy test can confirm whether a pregnancy has been established. An ultrasound examination is made around the 7^{th}–8^{th} gestational week to verify the result in case of a positive pregnancy test [16]. Pregnancy and live birth rates have been reported in the US to be as high as 66% and 56%, respectively, mainly due to multiple embryos being transferred to the recipient [11].

The Swedish legislation in practice

The oocyte donor

Infertile couples, as well as potential donors, undergo careful medical, social and psychological evaluation by the treating physician and psychologist at the treating clinics in order to be accepted for inclusion in the gamete donation program [17, 18]. The overall goal is to ensure that the prospective child will grow up within good conditions [18]. Oocyte donors have to be of legal age (at least 18 years) and must give written consent that their gametes may be used for fertilization. Donors are usually recruited voluntarily and receive no significant monetary compensation, except for travel and work expenses associated with the gamete donation; which corresponds to a flat rate of 4 000–8 000 SEK per treatment, depending on the county council. Despite the financial compensation, oocyte donors in Sweden are driven mostly by altruistic motives and empathy towards the childless couple [19]. Their motivations do not seem to be triggered by curiosity regarding their own fertility status or by financial interest to the same degree as sperm donors are [19]. The exact number of children that a donor can generate is not clearly regulated by law. Nevertheless, the county councils in Sweden have set a donation limit to a maximum of six families in order to minimize the risk of accidental consanguinity [18, 20, 21]. Gametes from a dead person may not be used in treatment. Donors can be known to the recipient couple or can be recruited by them; alternatively, they may have their identity concealed from the recipient couple. In Sweden, only 16% of donations are provided by donors who are known to the recipient couple [20]. After the treatment, the donor can obtain information from the fertility clinic about whether the donation resulted in a live birth. Finally, donors do not have any rights or responsibilities towards the child; financially, legally or emotionally [17]. Unfortunately, due to the scarcity of oocyte donors, the waiting

period from acceptance to treatment for the recipient couples varies from 6 months to 2 years throughout the country.

According to a published study, that included 82% of the recruited donors during 2005–2008, the mean age of oocyte donors in Sweden was estimated to be 31.5 ± 4.6 years (range 20–42 years) [22]. The majority (75%) of donors were in a relationship/cohabiting or married [19] and 69% of them had biological children of their own (range 1–4 children). Furthermore, a follow-up study confirmed that the women who had been accepted to the donation program were all well-adjusted and mature in their personalities; which is not surprising as they must all undergo medical and psychological evaluation. That they are well-adjusted is reassuring for the healthcare providers and the recipient couples, but even more reassuring for the child in later life [19, 22]. Lastly, the child born through gamete donation has the legal right to contact the fertility clinics in Sweden in order to gain identifying information about the "genetic" mother (oocyte donor) when he/she is sufficiently mature, that is to say, around 18 years of age [17].

The oocyte recipient

Both the child's as well as the mother's welfare and safety demand consideration and, therefore, suggestions for determining treatment eligibility have been made. According to the recommendations from the National Board of Health and Welfare in Sweden [18], recipient women should be healthy, mentally stable and of fertile age, so most clinics decline treatment to women over 40 years of age. Other recommendations include a weight limit, excluding women with severe obesity (body mass index (BMI) $\geq$ 35 kg/m^2) from receiving treatment [23] but practices differ between the various centers. Recently, the harmonization of the eligibility criteria and treatment opportunities has been attempted at a national level [8]. It should be highlighted that the participating clinics at the nationwide oocyte donation program, despite not having a standardized way of making the assessment but rather following their own clinical policy, seem nevertheless to be unanimous regarding the importance of the good health status of the oocyte recipients [18]. Currently, OD is only allowed in Sweden when medically indicated, that is, predominantly due to premature ovarian insufficiency under the age of 40 (either iatrogenic after chemo-radiotherapy and ovarian surgery, or idiopathic), Turner syndrome, declined ovarian reserve in women with functioning ovaries (otherwise known as poor responders), repeated failed IVF attempts, and maternally inherited genetic disorders [1]. The rules are not equally strict in other countries where women with natural menopause or comorbidities can also receive treatment. According to a Swedish study, the mean age of oocyte recipients was 33.7 ± 3.6 [20] and, during 2013, less than 10% of women receiving treatment in Sweden were above 40 years of age, which is by far the lowest rate for this age group in Europe [9, 10].

The maternal demographic data included in international studies on pregnancies achieved through oocyte donation are often characterized by advanced maternal age and high rates of nulliparity and multiple gestation [24, 25]. The latter intrinsic confounding factors are also known to affect gestational complications, making it difficult to assess the individual risk attributed to oocyte donation.

So far, reports on oocyte donation have been contradictory in relation to pregnancy and delivery outcomes. Previous studies have demonstrated increased rates of gestational diabetes [26-28], hypertensive diseases of pregnancy [25, 28-34], placental abnormalities [33, 35], preterm delivery [28, 35-37], increased rate of caesarean delivery [26, 30, 31, 35, 36] and higher incidence of postpartum bleeding [27, 30, 38]. Other studies, however, challenge these findings and attribute them mostly to plurality [26, 39, 40], emphasizing the need for more appropriate control groups.

Regarding perinatal outcomes, however, the reports seem to be overall encouraging [30-33, 36, 37, 41]. The prevalence of major congenital malformations is comparable to that of the general population [27]. Nonetheless, controversial reports exist regarding prematurity [28, 37], Apgar score [36, 40], birth weight [28, 36, 40, 42], rate of low birth weight (LBW) (<2500 gr) [28, 36, 37, 42, 43], rate of small for gestational age (SGA) infants [28, 37], and admission to Neonatal Intensive Care Unit [28], attributing some of the outcomes mainly to advanced maternal age, the presence of multiple pregnancies and prematurity as a consequence.

It should be stressed that Sweden represents a unique setting for study within this particular field due to the organization of the public health system. The healthcare system is decentralized to local county centers and municipalities and is mainly taxpayer funded. All citizens are entitled to care on equal terms. In particular, regarding reproductive health, the national health insurance program covers all gamete donation treatment costs, providing the opportunity to the citizens to benefit equally, regardless of their financial status [44]. Furthermore, the maternal health care system is organized within a well-developed primary care sector with standardized and free-of-charge antenatal care with good availability. The community midwife is the primary caregiver in a system of close supervision and surveillance and provides referral for obstetric assessment by physicians when potential complications are detected [45]. National and local guidelines lead to standardized health care provision [44].

Oocyte donation and inadequate placentation

Predicting the outcome of a pregnancy still represents a clinical challenge. Implantation, a critical step for the establishment of a pregnancy, requires coordination between complex molecular and cellular events, resulting in uterine growth and differentiation, blastocyst formation-adhesion and invasion, as well as placental formation [46, 47]. Successful implantation requires a receptive endometrium, a normal and functional embryo at the blastocyst stage and a synchronized dialogue between the mother and the developing embryo [48, 49]. Inadequate implantation may lead to pregnancy failure or other adverse pregnancy outcomes associated with poor placentation.

Miscarriage

Sporadic miscarriage

Sporadic miscarriage is a relatively common incident for otherwise healthy women, but becomes heart-wrenching and frustrating when it recurs. Sporadic miscarriage is defined as involuntary failure of a pregnancy before 20 weeks of gestation (dated from the last menstrual period) or below a fetal weight of 500 g [7, 50]. Overall, approximately 12–15% of all clinically recognized pregnancies end in miscarriage. It is, however, believed that the true incidence of miscarriage, even including early losses, is two to four times higher (30–60%), most of which go unnoticed [7, 51].

Recurrent Miscarriage

Historically, recurrent miscarriage (RM) (also habitual abortion or recurrent pregnancy loss) was defined as three or more consecutive spontaneous miscarriages with or without livebirth, all of which with the same biological father [52]. Nowadays, there are discrepancies in the definitions of RM adopted by different scientific committees in relation to the number and rank of miscarriages that make up the prescribed criteria. The European Society of Human Reproduction and Embryology (ESHRE) [53], as well as the Royal College of Obstetricians and Gynaecologists (RCOG) [54], define RM as the loss of three or more consecutive pregnancies. ACOG (American College of Obstetricians and Gynecologists), on the other hand, include in their definition two or three or more consecutive pregnancy losses [55]. However, the American Society for Reproductive Medicine (ASRM) Practice Committee defines RM as two or more clinical miscarriages confirmed by ultrasonography or histopathologic examination, that are not necessarily consecutive [2].

RM affects 1–3% of couples of reproductive age depending on the number of miscarriages described in the definition [56]. It is classified as early or late, according to whether the demise occurs before or after the 12th week of gestation [57]. It is furthermore divided and classified as either primary or secondary, depending on whether a livebirth occurred before the RM diagnosis [58]. There is, however, controversy about whether very early pregnancies should be included in the definition. If only clinical miscarriages are included, the prevalence is estimated at around 0.8–1.4%, whereas it is calculated to be around 2–3% if biochemical pregnancies are also accounted for; nevertheless, this rate is considerably lower than that attributed to sporadic miscarriage [59]. Kolte et al. [60] demonstrated that non-visualized pregnancy loss (biochemical pregnancy loss and failed pregnancy of unknown location combined) contribute negatively to the chance of live birth in the subsequent pregnancy; the relative risk is equivalent to the impact conferred by each additional clinical miscarriage [60]. The data, according to the authors, support the suggestion of including non-visualized pregnancy losses in the definition of RM. It remains to be seen whether this criterion will be adopted by the international scientific societies.

The number of previous miscarriages and maternal age at conception are considered to be two independent risk factors for subsequent miscarriage [61]. Furthermore, when spontaneous miscarriage was examined in different age strata, the proportion of pregnancies ending in spontaneous miscarriage among nulliparous increased from 8.9%, to 12.4%, to 22.7%, to 44.6% after one, two three or four miscarriages, respectively [61]. Moreover, the risk of miscarriage increases steeply after the age of 35 years, from 11.1% at 20–24 years, to 51% at 40–44 years [61]. The relationship between spontaneous miscarriage and maternal chronological age correlates with the rate of aneuploidy in oocytes. The frequency of aneuploidy discovered after cytogenetic analysis rose from 10% to 50% among women younger than age 35 compared to 43-year-olds [62]. In women above 45 years of age, almost 100% of oocytes examined presented some kind of aneuploidy [62]. This phenomenon is thought to be the result of malsegregation of single-chromatids or whole-chromosomes during meiotic division. Nowadays, new evidence has additionally associated the oocyte aneuploidy risk with maternal "biological age" as assessed by ovarian reserve testing [63, 64]; it has been observed that diminished ovarian reserve is associated with increased aneuploidy rate of gametes and resulting biopsied embryos [65]. Lastly, paternal age has also been implicated in the risk of spontaneous miscarriage [65].

The etiology of RM is established in only 50% of investigated cases while the rest remain unresolved (so-called idiopathic RM). Among the most identified causes are embryonic chromosomal abnormalities; chromosomal paternal aberrations (balanced translocations and inversions); congenital (septate uterus) or acquired uterine malformations (submucosal fibroids, intrauterine polyps); endocrinological disorders (poorly controlled diabetes melli-

tus, untreated hypothyroidism); immunologic factors; thrombophilia; and antiphospholipid syndrome (APS). Recently, certain early pregnancy units have included ovarian reserve testing (such as measurement of AMH levels) in the standard work-up in order to detect diminished ovarian reserve as a possible contributing factor [66]. It should be highlighted that, of the aforementioned factors, some are known to be causative (t ex fetal aneuploidy) while others only increase susceptibility to the condition (t ex thrombophilia). However, while idiopathic RM is thought to be a heterogeneous condition with multifactorial etiology it does include a genetic component as seen through its familiar predisposition in families affected by RM [67].

It should be added that recent biochemical and epidemiological studies have slowly shifted the interest to the endometrium, supporting the new hypothesis of disrupted endometrial selectivity of embryo quality. In other words, women who suffer from RM permit embryos of poor viability to implant inappropriately for long enough to present as a clinical, rather than as a preclinical pregnancy loss [59]. This novel concept requires further elucidation and confirmation.

The management of the condition lies in treating the abnormal agent discovered, if any. Strong evidence suggests that the use of: preimplantation genetic diagnosis (PGD) in the case of parental chromosomal aberration; myomectomy, polypectomy or septal resection in the case of uterus anomaly; and low dose aspirin (ASA) as well as low molecular weight heparin (LMWH) in the case of APS; is indicated.

Healthcare providers have so far empirically prescribed progesterone supplements, ASA and LMWH, to women with unexplained RM, even in the absence of inherited thrombophilia. However, multicenter randomized controlled trials and a Cochrane review exploring progesterone supplementation and antithrombophilic medication (ASA and/or LMWH) in women with idiopathic RM showed no beneficial effect against the condition (i.e. increase in live-birth) [68-73]. Therefore, the use of these treatments is not generally supported.

Treatment with glucocorticoids, immunotherapy with intravenous immunoglobulins or intralipid infusion, still remains controversial and requires further research before it becomes clinical praxis.

In summary, the prognosis of RM is generally favourable, even without treatment, and couples should be informed that solid evidence is lacking to support several commonly used interventions for idiopathic RM.

Recurrent miscarriage and preeclampsia

Although the exact pathophysiological mechanism remains obscure, it has been hypothesized that early pregnancy complications (such as RM) and placental dysfunction disorders (i.e. preeclampsia, stillbirth, and intrauterine

growth restriction) may share elements of the same etiology. Histopathological studies have observed similar cellular damage, such as endothelial dysfunction, among women with unexplained recurrent miscarriage, as well as among women who had previously suffered from preeclampsia, linking the two placenta-mediated conditions to each other, as well as to future vascular complications [74, 75].

The former association has also been confirmed by epidemiological studies investigating the impact of first trimester events and subsequent late obstetric complications [76]. In fact, large population-based Swedish [77], as well as Norwegian [78], studies have shown that women with three or more self-reported prior miscarriages faced a higher risk of preeclampsia in the forthcoming pregnancy compared to women without a history of prior miscarriage. This association in the Swedish study population seemed in fact stronger for preterm placental dysfunction disorders when compared with term disorders [77]. The latter observation supports the notion that complete implantation/placentation failure may result in a miscarriage, whereas a partial failure is more likely to result in late placenta-mediated pregnancy complications, such as preeclampsia.

Gestational Hypertensive Disorders

Hypertensive disorders of pregnancy, including chronic and gestational hypertension, *de novo* preeclampsia or that superimposed on chronic hypertension, and eclampsia, constitute a clinically challenging group of pregnancy complications that are responsible for a substantial proportion of maternal and neonatal morbidity and mortality [79]. Gestational hypertensive disorders (GHD) constitute a pregnancy-specific syndrome with still unknown causes. However, the placenta seems to be both a necessary and sufficient component for the development of these conditions [80]. Chronic and gestational hypertension are defined as hypertension (blood pressure $\geq$140/90) diagnosed before and after 20 weeks of gestation, respectively, measured on at least two occasions, 4–6 hours apart [81]. Preeclampsia (PE) was until recently defined as new onset hypertension on two separate occasions with significant proteinuria (>0.3 g/24 h), after the 20th gestational week in a previously normotensive woman [82]. The International Society of Hypertension in Pregnancy (ISSHP) revised the definition of preeclampsia in 2014, suggesting that a clinical diagnosis can be made, even in the absence of proteinuria, if organ-specific signs or symptoms are present instead [82]. The latter definition is in agreement with the national recommendations from the USA and Canada, as well as from Australia and New Zealand. Nonetheless, albuminuria is still a necessary diagnostic criterion according to the national guidelines of Great Britain [83] and Sweden [84]. Maternal organ-specific injury may include liver involvement, neurological complications (cerebral

or visual symptoms), pulmonary edema, hematological complications (thrombocytopenia), renal impaired function and placental insufficiency [82]. Preeclampsia is often classified depending on the time of delivery; as early onset (<34 weeks of gestation) or late onset (≥34 weeks of gestation) [82]. It can also be graded as mild or severe disease, with the latter including end-organ engagement.

Gestational hypertensive disorders are considered to be complex clinical syndromes that share clinical features (i.e. hypertension). From now on we will refer mostly to preeclampsia, as preeclampsia presents the most significant complication and it is the syndrome most studied amongst hypertensive disorders [85].

Epidemiology

Preeclampsia and gestational hypertension complicate 2–3% and 5–6% of all pregnancies, respectively [86, 87] and, together with eclampsia, remain the most common cause of maternal death in Europe [81]. It has been estimated that 10–15% of maternal mortality, 25% of stillbirths and neonatal deaths [86], 12–15% of fetal growth restricted and small for gestational age infants, as well as 15–20% of preterm births are attributable to severe preeclampsia and eclampsia [79]. Eclampsia, HELLP syndrome, pulmonary edema, renal failure, hemorrhagic stroke and liver rupture are some of the main causes of maternal mortality associated with preeclampsia [81, 88]. In Asia and Africa, hypertensive disorders account annually for 22 000–25 000 of maternal deaths, 3 800 of maternal deaths in Latin America and the Caribbean, and 150 of maternal deaths in industrialised countries [89].

Pathophysiology

The pathogenesis of preeclampsia is still debated, but the concept of impaired placental function has long since been established. In early-onset PE, placental dysfunction is considered predominant, whereas in late PE, maternal genetic, behavioural and environmental factors are thought to have a higher impact [90]. However, to date, it might be regarded as simplistic to view the disease genesis as dichotomous [90]; it is instead considered to be a mixture of maternal and placental factors in varying proportions. It has been hypothesized that the primary stage of PE is associated with poor maternal tolerance to paternal antigens found in semen/sperm (preconceptual stage) [91]. The excessive immune response results in shallow invasion of the cytotrophoblasts into the decidua. The impaired decidualization leads to incomplete remodelling of the spiral arteries with restricted blood flow. This leads to increased resistance in the uteroplacental arteries with subsequent intermittent hypoperfusion, in turn leading to oxidative stress in the placenta [91, 92]. Simultaneously, acute atherosis of the spiral arteries takes place, as

well as distal thrombosis and occlusion. This is enhanced by the release of components from the intervillous space into the maternal circulation that in turn stimulate the production of inflammatory cytokines. The last stage is characterized by systemic maternal disease, evident by the exaggerated endothelial activation and the generalized inflammatory state, leading to clinically manifested preeclampsia [92].

Risk factors

Maternal characteristics that are thought to predispose women to preeclampsia include: advanced maternal age (>40 years), obesity (BMI >35 kg/m^2), nulliparity, >10 years from last pregnancy, preeclampsia in prior pregnancy (particularly if severe or early-onset), positive family history of preeclampsia, pre-existing medical conditions (including pre-gestational diabetes mellitus, chronic hypertension or underlying renal disease), antiphospholipid syndrome, and multiple gestation [82, 88]. Several studies have recently demonstrated an increased risk of gestational hypertensive disorders, including PE among pregnancies after oocyte donation, which introduces oocyte donation as an independent risk factor for preeclampsia [31, 93-95].

Management

The effective management of preeclampsia may be divided into three categories; prevention of preeclampsia, early detection, and treatment. Optimization of diet and lifestyle and pre-pregnancy counseling is the initial approach. Furthermore, women at high risk of PE on the basis of clinical factors should be administered low-dose aspirin (ASA) (75 mg/day) at bedtime from the 12^{th} until the 36^{th} gestational week [96]. New evidence in fact supports the addition of LMWH to ASA, since it has been demonstrated that it reduced the risk of recurrent PE and ameliorated neonatal outcomes compared to ASA alone [97].

Regarding early detection of preeclampsia, recent research focuses on blood measurement of angiogenic factors in the first and early second trimesters, such as VEGF (Vascular Endothelial Growth Factor) and PlGF (Placental growth factor) in order to predict early-onset PE. PlGF, which is a member of the VEGF family, is an angiogenic, proinflammatory factor produced by trophoblast cells and has a central role in the regulation of VEGF-dependent angiogenesis [90]. PlGF has long been associated with the pathogenesis of PE and is thought to be a secondary marker for the placental dysfunction that occurs in PE [90, 98]. However, due to the heterogeneous nature of PE, it seems improbable that a single risk factor or biomarker will predict those at risk of developing preeclampsia. An algorithm combining risk factor analysis and biochemical features might become of use in the future [80, 98].

International guidelines recommend pharmacologic treatment/antihypertensive medication after the diagnosis of the condition, especially if severe hypertension is present [88]. However, the syndrome resolves only after delivery or attrition of the placenta. The decision for the optimal timing of the delivery should be based on the balance between the maternal and fetal risks of continuing the pregnancy and the neonatal risks of ending the pregnancy. The obstetrician should consider expectant management for women with preeclampsia from gestational age of fetal vitality (around gestational week 22) to gestational week 33+6 [82]. However, progressive deterioration of fetal well-being, of the maternal clinical or biochemical status or inability to control maternal blood pressure despite antihypertensive medication, might necessitate earlier delivery [88]. After gestational week 34, the recommendation is to lean towards delivery of women with severe preeclampsia when the benefits of delivery outweigh the risks of conservative management. After gestational week 37, evaluation of the benefits and risks of delivery should be considered in women with both mild and severe preeclampsia [82, 88].

Following a preeclamptic pregnancy, women face higher long-term risk of presenting with cardiovascular disease, such as chronic hypertension, ischemic heart disease, stroke and venous thromboembolism later in life [99, 100]. They should therefore be advised about the importance of preventive measures such as the adoption of a healthy lifestyle and, together with their general health practitioner, plan an annual follow-up of their blood pressure and metabolic profile [88, 99, 101].

The long-term effects of preeclampsia seem to be extended, even on the health of the offspring of preeclamptic mothers. When children who were born after preeclamptic pregnancies were followed up in childhood and puberty, higher systolic and diastolic blood pressure was observed [100]. Furthermore, a higher risk of stroke among the offspring from pregnancies complicated with severe preeclampsia was also demonstrated [102]. The latter findings provide evidence that *in utero* exposure to PE might lead to vascular dysfunction that persists even in later life [103].

Preeclampsia and impaired angiogenesis

In normal pregnancies, the remodelling of the maternal spiral arteries of the uterus permits a drastic increase in the blood flow necessary to provide nutrients for fetal growth. Dysregulation of the vascular development of the placenta (anti-angiogenesis) is regarded to be the pathogenetic mechanism underlying an array of pregnancy complications such as infertility, recurrent pregnancy loss, preeclampsia, fetal growth restriction and stillbirth [104-106]. Furthermore, it has been reported that preeclamptic women may present with up-regulated placental antiangiogenic factors that disrupt the ma-

ternal endothelium, leading to an antiangiogenic state which in turn can result in clinical signs of preeclampsia [88]. Therefore, recent studies have focused on the association between gestational vascular diseases and polymorphisms in genes related to angiogenesis [105, 106] (i.e. the growth of new blood vessels from pre-existing ones) and vasoconstriction, such as VEGF [107, 108], p53 protein, endothelial nitric oxide synthase (eNOS) [109] and histidine-rich glycoprotein (HRG).

The Histidine-rich glycoprotein

Histidine–rich glycoprotein (HRG), a single polypeptide chain protein, is an endogenous regulator of angiogenesis, circulating at high concentration in plasma. The protein, first isolated in 1972, is synthesized in liver parenchymal cells and it is either transported as a free protein in plasma or stored in the a-granules of platelets [110] and released after thrombin stimulation [111]. Furthermore, HRG is produced and secreted by preimplantation embryos [112]. The human *HRG* gene is mapped at chromosome 3 in position 3q28-29 (reassigned in position 3q27 after the complete determination of the human genome sequence) and consists of seven exons and six introns [113]. The *HRG* gene encodes a 507 amino acid long multi-domain protein that has an approximate molecular mass of 75 kDa [114]. The HRG plasma concentration during the neonatal period is low and it increases gradually with age. Interestingly, during pregnancy, HRG levels decline gradually at the beginning of the second trimester of pregnancy, diminishing by approximately 50% at parturition [115], and returning to normal within two weeks after delivery [116]. It is assumed that estrogens are responsible for this change [117].

Based on sequence analysis and spectroscopic studies, it has been shown that HRG protein consists of three main domains: two amino-terminal domains [N_1(residues 1-112) and N_2(113-229)], a central histidine-rich region (HRR) (330-389) flanked by two proline-rich regions [PRR_1(255-314) and PRR_2(398-439)], and a C-terminal domain (C)(440-507) [118]. It is now widely accepted that the minimal active domain corresponding to the amino acid sequence 330–364 in the HRR domain is mainly responsible for the antiangiogenic properties of the HRG protein [119].

HRG contains six disulfide bonds [118], it is heavily glycosylated, and a recent study suggests that it might even be phosphorylated [120] (Figure 1). Of the six disulfide bonds, four are intradomain and two interdomain linking the HRR region to the PRR_2 domain in the intact protein. It has been suggested that the disulfide bridges are involved in maintaining the native folding, attenuating susceptibility to modifications, such as dispersion upon proteolytic digestion, that could as a result alter the function of the protein [117, 121].

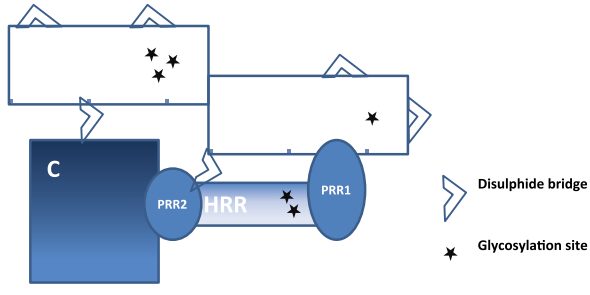

Figure 1. HRG structure. Adapted by Jones et al. [116]

HRG Functions

HRG is involved in several distinct biological functions; among them regulation of blood coagulation, fibrinolysis, chemotaxis and focal adhesion of endothelial cells, cytoskeletal organization during vessel formation, immune complex formation as well as apoptosis [117]. Supporting the importance of these biological functions for fertility regulation is the abundance of HRG throughout the female reproductive tract (i.e. in follicular fluid, endometrium, fallopian tube and in myometrium) where the oocyte develops, is fertilized and later implants [122]. HRG seems to act as an adaptor molecule, interacting with a wide range of ligands including divalent metal ions (Ca^{2+}, Zn^{2+}), glycosaminoglycans such as heparin/heparan sulphate, fibrinogen, plasmin and plasminogen, thrombospondin (TSP), VEGF, and members of the fibroblast growth factor (FGF) family [116]. It should be noted that HRG exhibits both pro- and anti-angiogenic properties; the effect is potentiated through interactions with various ligands, its multi-domain structure and the activities of its proteolytically-released fragments, notably the histidine-rich region [123], making it an important intermediary in angiogenesis.

HRG in Reproduction

A single nucleotide polymorphism (SNP) is a variation in a single nucleotide that occurs in a specific position in the genome corresponding to at least one percent of the population (>1%) [124, 125]. A SNP can be located within the coding region of a gene, within the gene's regulatory sequences or in an intergenic region that does not affect the expression of a gene [124].

At least ten naturally occurring HRG single-nucleotide polymorphisms (SNPs) have been identified [126]; however, the biological mechanisms behind fertility and HRG have not been fully investigated. So far, two HRG SNPs have been associated with the interaction of the processes of relevance for fertility. One widely studied SNP is the C633T, in which cytosine (C) is replaced by thymidine (T) (exon 5) at position 633 in the mRNA sequence denoted C633T (also known as rs9898). The cytosine replacement results in the change of amino acid from proline to serine at position 204 in the protein (in some publications this amino acid is denoted as occupying position 186, corresponding to the protein without its signal peptide) [127]. This amino acid shift results in the creation of an extra site for N-glycosylation at amino acid position 202 [116] and the production of two variants of HRG: isoform 1 (Pro, 75 kDa); and isoform 2 (Ser, 77 kDa) [113].

Lindgren et al. [128] have described that there is an increased prevalence of the HRG 633T SNP among women with recurrent miscarriage who have never had children. Nordqvist et al. described an association between the HRG C633T SNP and pregnancy success rates in IVF [122]. Furthermore, they reported an association with ovarian response during IVF stimulation [129]. Lastly, Lindgren et al. [130] have also demonstrated that treatment with peptides corresponding to the C633T polymorphism affects the proliferation and migration of human endometrial endothelial cells and promotes tube formation, leading to capillary-like structures. The authors conclude that HRG, by regulating angiogenesis, might be favourable for adequate implantation and placentation [130].

In another polymorphism of the gene, HRG A1042G SNP, an adenine (A) nucleotide is replaced by a guanine (G), which results in a change from histidine to arginine at amino-acid position 340 in the histidine-rich region (HRR) [131]. It should be reiterated that this is the region mainly responsible for the anti-angiogenic capacity of HRG [123].

Motivated by the gap identified in the knowledge, we decided to undertake a project to dig deeper into these implantation defects, aiming to explore whether angiogenesis-related models are associated with pathologic conditions arising from defective implantation or impaired development of the placenta.

AIMS

The specific aims of this thesis are:

I To examine whether the Histidine-rich glycoprotein A1042G polymorphism is associated with recurrent miscarriage.

II To investigate whether singleton pregnancies following medically motivated oocyte donation are associated with adverse obstetric outcomes compared to pregnancies conceived with autologous oocytes and whether outcomes differ depending on treatment indication.

III To study whether neonatal outcomes differ among infants conceived after oocyte donation *vs* after non-donor IVF and spontaneously conceived pregnancies.

IV To explore whether preeclampsia and gestational hypertensive disorders are associated with the C633T HRG polymorphism known to be of relevance for regulation of implantation and placentation.

Materials and Methods

Overview of the studies

Table 1. Overview of the studies

Study	Study design	Participants	Variables studied
I	Retrospective case-control study	186 women with RM and 380 controls	HRG A1042G SNP carriership
II	Retrospective cohort study	Singleton pregnant women, of which 76 after OD, 63 after non-donor IVF and 150 after spontaneous conception	Various obstetric outcomes originating from MBR
III	Retrospective cohort study	Singleton pregnant women, of which 76 after OD, 63 after non-donor IVF and 149 after spontaneous conception	Various neonatal outcomes originating from MBR
IV	Retrospective nested case-control study	96 women with hypertensive disorders of pregnancy and 200 controls	HRG C633T SNP carriership

Paper I

Study Design

The study was designed as a retrospective case-control study. Participation was voluntary and without compensation. All included women were able to communicate in Swedish and gave their consent after confirming that they had read and understood the written information provided. Participating women answered standardized questions on reproductive history and underwent a brief health examination, including measurements of weight and height. Information was also obtained from their medical records. All women in the cases group, upon diagnosis with recurrent miscarriage, and all targeted controls, according to local routine guidelines, were evaluated in relation to thyroid function. Because ethnicity distribution did not differ between groups, all of the analyses performed were in an ethnically mixed group. Apart from their obstetric record, cases and controls fulfilled the same inclusion and exclusion criteria.

Study population

Cases were recruited from three University Hospitals in Sweden. Women with a diagnosis of three or more verified consecutive miscarriages in the

first or second trimester of pregnancy during the years 1989–2009 were identified in the out-patient registers of the included clinics and invited to participate in the study. Women with known risk factors for recurrent miscarriage, such as Systemic Lupus Erythematosus (SLE), diabetes mellitus type 1, severe thrombophilia and major chromosomal aberrations, were excluded. A total of 186 cases were included, of which 43 suffered from primary miscarriage (defined as no children either before or after diagnosis). In order to study recurrent miscarriage as a reason for sub-fertility, women with primary recurrent miscarriage were also compared to controls (subgroup analysis).

The control participants ($n = 380$) were randomly chosen from the Uppsala University Hospital biobank of pregnant women when attending the second trimester routine ultrasound scan. None of the women had a history of miscarriage or were treated with anti-thrombophilic medication and 75% had at least two spontaneous pregnancies, including the on-going pregnancy, resulting in a term (≥ 37 weeks) birth of a live infant.

Blood sampling and SNP analysis

Blood samples were collected, plasma and buffy coat were separated and genomic DNA was extracted. The samples were genotyped for the HRG A1042G SNP (rs2228243) using the TaqMan Genotyping Assay.

Statistical analysis

Demographic and clinical characteristics were compared between controls and cases (both the entire study population and those with primary RM) as well as between genotype groups using Chi-square test, Student's *t*-test and the Mann-Whitney *U* test. Afterwards, a logistic regression analysis was performed, examining the association between HRG A1042G SNP and recurrent miscarriage (entire study population and subgroup of cases with primary RM). Finally, a logistic regression model including factors with possible associations with exposure and outcome ($p<0.25$), such as age, pre-pregnancy smoking, BMI, thyroid disease and genotype, was composed.

Details of Ethics Approval

The study was approved by the Regional Ethics Committee of the Medical Faculty of Uppsala University Hospital, Uppsala and the Ethical Committee of Karolinska Institutet, Stockholm (2009/177-32 and 2006/1545-31/4).

Papers II and III

Study Population

This study was carried out at a national level and was a component of the greater multicentre study on gamete donation [20]. The design was that of a retrospective cohort study. Participation was voluntary and without compensation. All included women were able to communicate in Swedish and gave their consent after being informed about the study and their rights as participants. Consecutive couples who received treatment with donated oocytes at the seven University fertility clinics in Sweden were approached regarding participation during the period 2005–2008. The Index group comprised women from the whole of Sweden who later gave birth to a singleton following treatment with donated oocytes given that the treatment indications were also available (Figure 2, Appendix).

In order to evaluate the outcome, two control groups were used;

a) Control group A comprised nulliparous women with spontaneously conceived pregnancies, singleton deliveries and no history of subfertility found in the medical register. Women in Control group A were matched to the Index group in regard to age in three categories, ≤29, 30–35, ≥36 years, at a ratio of 2:1. Apart from the study design eligibility criteria, Control group A was otherwise randomly chosen.

b) Control group B comprised women undergoing IVF treatment with their own gametes due to couple infertility who later conceived with singleton pregnancies at the seven University hospitals in Sweden. Control group B participants were not matched to the Index group in regards to age (Figure 3, Appendix).

In Paper II, Control group A consisted of 150 women, whereas in Paper III, this group consisted of 149 participants after excluding one woman who experienced stillbirth during the third trimester. Control Group B and the Index group consisted of 63 and 76 women, respectively, in both Papers II and III.

Data were retrieved from the Swedish Medical Birth Register (MBR), a validated Swedish population-based register held by the Swedish National Board of Health and Welfare. The MBR includes information regarding prenatal, delivery and neonatal care [132, 133]. Additional medical information for the oocyte recipients originated from their treatment protocol after the scrutinizing of the medical records at each centre.

Statistical analysis

Demographic and clinical characteristics were compared between women in the Index group and Control groups A and B, using Student's *t*-test, the Mann-Whitney *U* test, and Chi-square test, respectively. Thereafter, the

association between the various obstetric and neonatal outcomes and the status of the index/control group was studied. The comparisons were carried out with the use of Chi-square or Fisher's exact test, as well as with a single and a multiple logistic regression model after adjusting for relevant covariates, that is to say, maternal age, BMI, gestational length and either nicotine use and presence of chronic medical conditions (Paper II) or delivery by caesarean section (Paper III). Finally, it should be added that after performing a sensitivity analysis concerning parity in our population, its influence on obstetric outcomes was assessed to be low and thus parity was not included as a confounding factor in the multivariate regression model. Women who conceived spontaneously (Control group A) or by conventional IVF (Control group B) were considered to be the reference category. The odds ratios (ORs) and corresponding 95% confidence intervals (95% CIs) were calculated. Lastly, in order to investigate the effect of treatment indication on the most common obstetric and neonatal outcomes, a subgroup analysis was performed within the Index group and compared to Control group A.

Ethics Approval

The study was approved by the Regional Ethical Review Board in Linköping, Sweden (Nr M29-05, T113-07 and Nr 2012/289-32).

Paper IV

Study design and study population

The study was designed as a retrospective nested case-control study. The study population originates from the Uppsala BASIC Biobank. In the "Biology, Affect, Stress, Imaging and Cognition in pregnancy and the puerperium" (BASIC) cohort, all women able to adequately communicate in Swedish who attended the second trimester ultrasound scan at the Uppsala University Hospital were approached to participate. The women were given oral as well as written information, after which written consent was obtained. The participation rate was estimated to be around 22% [134]. Blood samples were collected during inclusion as well as upon delivery. Furthermore, medical information relating to health status, current medication and past reproductive history were retrieved retrospectively from their medical records at the Department of Obstetrics and Gynecology at Uppsala University Hospital. For the current study design, the study population included 200 healthy controls having conceived spontaneously with singletons and who delivered uneventfully and 96 cases with singleton deliveries who had been diagnosed with hypertensive disorders of pregnancy (i.e. chronic and gestational hypertension, preeclampsia, eclampsia or HELLP syndrome).

Blood sampling and SNP analysis

Blood samples were collected from all participating women, plasma and buffy coat were separated and genomic DNA was extracted. The samples were genotyped for the HRG C633T SNP (rs9898) using the TaqMan Genotyping Assay.

Statistical analysis

Demographic and clinical characteristics were compared between controls and cases, as well as between genotype groups using Chi-square test, Student's *t*-test and the Mann-Whitney *U* test. Afterwards, a logistic regression analysis was performed, examining the association between HRG C633T SNP and gestational hypertensive disorders. Finally, a multivariate logistic regression model including maternal age, maternal BMI, parity and genotype was composed. The odds ratios (ORs) and corresponding 95% confidence intervals (95% CIs) were calculated.

Ethics Approval

The study was approved by the Regional Ethics Committee of the Medical Faculty of Uppsala University Hospital, Uppsala (D-Nr 2012/254).

Results

Paper I

Demographic data, clinical characteristics and HRG genotype

Regarding background characteristics, women with recurrent miscarriage had a higher prevalence of pre-pregnancy smoking, hypothyroidism and a higher BMI but did not differ in age compared to controls. Interestingly, heterozygous (A/G) carriers of HRG A1042G SNP were overrepresented among controls compared to cases (34.7% *vs* 26.3%; $p<0.05$). The difference was even more apparent (34.7% *vs* 16.3%, $p<0.05$) when investigating the controls versus the subgroup of cases with primary recurrent miscarriage. Regarding demographics in this subgroup, age, BMI and smoking rate appeared similar between the groups with the exception of hypothyroidism, which was significantly lower among controls.

When investigating the distribution of genotype across the study population, homozygous and heterozygous carriers of the A1042G genotype had otherwise similar clinical characteristics with the exception of hypothyroidism, which was more prevalent among homozygous G/G carriers.

Genotype and risk of recurrent miscarriage

When comparing cases and controls, both in the entire study population as well as in the subgroup of cases, it was found that heterozygous A/G carriers had the lowest likelihood of recurrent miscarriage [(OR 0.67, 95% CI 0.46–0.99) and (OR 0.37, CI 95% 0.16–0.84) respectively] compared to A/A or G/G carriers; the association remained significant even after adjustment for the covariates mentioned above (Table 2).

Table 2. Potential risk factors associated with recurrent miscarriage in a subgroup analysis (cases include only women with primary recurrent miscarriage).

	Unadjusted OR (95% CI)	Adjusted OR[a](95% CI)
Age		
≤35 years	1	1
≥36 years	1.31 (0.63–2.71)	1.40 (0.65–2.96)
BMI, kg/m^2		
≤30	1	1
≥31	1.86 (0.77–4.48)	1.99 (0.80–4.95)
Smoking		
No	1	1
Yes	2.19 (0.98–4.89)	2.37 (1.04–5.44)*
Hypothyroidism		
No	1	1
Yes	4.97 (1.76–14.02)**	4.63 (1.61–13.36) **
HRG A1042G genotype		
A/A or G/G	1	1
A/G	0.37 (0.16–0.84)*	0.36 (0.15–0.84)*

BMI, body mass index; Pre-pregnancy smokers for cases, or smoker at first visit to the prenatal center in gestational week 10 for controls; HRG A1042G genotype refers to either heterozygous carriers (A/G) or homozygous (A/A) or (G/G) carriers
[a]Adjusted for age, BMI, smoking and thyroid disease
*p<0.05, **p<0.01

Papers II and III

Background characteristics

Oocyte recipients were more likely to be older, non-smokers and have higher BMI compared to women from Control groups A and B. However, if age stratification was performed, no significant differences with regard to age were noted due to initial matching according to study design. Index and Control groups did not differ regarding nulliparity. Chronic medical conditions had a similar prevalence between the groups with the exception of hypothyroidism among oocyte recipients, 40% of which could be attributed to women with Turner syndrome, probably as a result of careful pre-pregnancy

screening for Turner syndrome in Sweden [135] (7 oocyte recipients *vs* 1 in Control group A and 0 in Control group B).

Recipients of donated oocytes received treatment commonly due to premature ovarian failure or "poor responder" status [136] (48.7%), Turner syndrome (13.2%) and after bilateral oophorectomy or post-chemotherapy (11.8%).

Obstetric Outcomes

Women in the Index group (oocyte recipients) had a higher likelihood of hypertensive disorders [aOR 2.84, 95% CI (1.04–7.81)], oligohydramnios [aOR 12.74, 95% CI (1.24–130.49)], postpartum haemorrhage [aOR 7.11, 95% CI (2.02–24.97)] and retained placenta [aOR 6.71, 95% CI (1.58–28.40)] when compared to Control group A, after adjusting for relevant covariates. Similar trends, though not statistically significant, were observed when comparing women in the Index group to those in Control group B. Caesarean delivery [aOR 2.95, 95% CI (1.52–5.71); aOR 5.20, 95% CI (2.21–12.22)] and induction of labour [aOR 3.00, 95% CI (1.39–6.44); aOR 2.80, 95% CI (1.10–7.08)] occurred more frequently in the Index group, compared to Control group A and B, respectively. No associations were found with regard to pre-gestational and gestational diabetes mellitus, placenta praevia and placental abruption, expected SGA or LGA infant as diagnosed by antenatal ultrasonography, polyhydramnios, the use of epidural analgesia or nitrous oxide use during active labour, and obstetrical lacerations to the 3rd or 4th grade (data not shown). When taking into account the cause of oocyte donation treatment, a higher likelihood of intervention was observed (i.e. induction and caesarean section rate) among women with diminished ovarian reserve compared to all other OD indications, while the risk of hypertensive disorders did not differ between groups after adjustment (Table 3).

Two of the obstetric outcomes (i.e. induction of labor and CS) were further analyzed and the results observed are reported in the thesis but not included on the published articles: induction of labor (n = 33) was performed among oocyte recipients, mainly due to PE (6/33, 18%), pre-labor rupture of membranes (5/33, 15%), oligohydramnios (6/33, 18%), postterm pregnancy (4/33, 12%) and SGA diagnosis antenatally (1/33, 3%). Among women who became pregnant through oocyte donation undergoing labor induction, in the end, 52% (17/33) were delivered through emergency CS (data not shown), most of whom (11/17) had received a diagnosis related to functional dystocia/uterine inertia. Furthermore, in our study, among oocyte recipients who underwent non-emergency CS (n = 18), 28% (5/18) were due to breach presentation, 17% (3/18) were due to some kind of pelvic disproportion/abnormality, 12% were due to prior uterine operation (2/18) and 6% (1/18) were due to placenta previa (data not shown). Among those undergo-

ing emergency CS (n = 24), 21% (5/24) had exhibited acute fetal distress during vaginal delivery, 45.5% (11/24) had presented with uterine inertia, 12.5% (3/24) had failed to progress after labor induction and 21% (5/24) also suffered from PE/Eclampsia (not necessarily that being the only reason responsible for undergoing CS) (data not shown). Lastly, we observed that 7/10 (70%) women with TS were delivered with CS.

Table 3. Delivery-related outcomes for women treated with donated oocytes (Index group) compared to women with no history of infertility (Control group A) and women treated with non-donor IVF (Control group B)

Outcome	Index group *n/N* (%)	Control group A *n/N* (%)	AOR (95% CI)	Control group B *n/N* (%)	AOR (95% CI)
Normal Delivery	21/76 (27.6%)	90/150 (60%)	0.30 (0.15–0.60)	39/63 (61.9%)	0.29 (0.13–0.65)
Instrumental delivery[a]	13/58 (22.4%)	20/142 (14.1%)	1.95 (0.75–5.09)	12/53 (22.6%)	0.86 (0.29–2.55)
Cesarean Section (CS)	42/76 (55.3%)	39/150 (26%)	2.95 (1.52–5.71)	12/63 (19%)	5.20 (2.21–12.22)
Non emergency CS	18/76 (23.7%)	8/150 (5.3%)	5.13 (2.00–13.17)	10/63 (15.9%)	1.82 (0.71–4.66)
Emergency CS[a]	24/58 (41.4%)	31/142 (21.8%)	1.93(0.88–4.22)	2/53 (3.8%)	15.98 (3.27–78.23)
Uterine Inertia[a]	13/58 (22.4%)	32/142 (22.5%)	0.88 (0.36–2.16)	4/53 (7.5%)	3.67 (0.92–14.66)
Induction of labor[a]	33/58 (56.9%)	31/142 (21.8%)	3.00 (1.39–6.44)	12/53 (22.6%)	2.80 (1.10–7.08)
Hypertensive disorders of pregnancy	12/76 (15.8%)	9/150 (6%)	2.84 (1.04–7.81)	6/63 (9.5%)	1.66 (0.54–5.08)
Fetal distress[a]	16/58 (27.6%)	21/142 (14.8%)	1.96 (0.78–4.97)	5/53 (9.4%)	2.86 (0.83–9.86)
Maternal hospitalization post-partum≥3d	59/75 (78.7%)	86/148 (58.1%)	1.14 (0.51–2.58)	35/63 (55.6%)	1.74 (0.70–4.34)[b]
Post partum hemorrhage	12/76 (15.8%)	7/150 (4.7%)	7.11(2.02–24.97)	6/63 (6.5%)	3.67(1.03–13.03)[c]
Retained placenta[d]	8/34 (23.5%)	4/111 (3.6%)	6.71 (1.58–28.40)	4/51 (7.8%)	2.98 (0.73–12.18)

The outcomes are reported as prevalence (*n*/*N*, %) or Adjusted Odds Ratios (aOR) and corresponding 95% confidence intervals (CI)
Outcomes are adjusted for maternal age (<35, ≥35 yrs), BMI (<25 or ≥25kg/m^2), gestational length, chronic condition (yes/no), Nicotine use (yes/no).
[a]Excluding all non-emergency Cesarean section(CS)
[b]Adjusted for usual covariates, CS and Hypertensive disorders
[c]Adjusted for usual covariates and CS
[d]Excluding all CS

Neonatal outcomes

The risk of being born preterm was higher among infants conceived with donated oocytes compared to infants resulting from other modes of conception (17.1% in the Index group *vs* 8.1% in Control group A and 4.8% in Control group B). Infants conceived through oocyte donation had a lower

median birthweight (3.238±840 *vs* 3.495±693; *p*=0.011) and birth length (50±3.5 *vs* 51±3; *p*=0.013) compared to infants spontaneously conceived. Similar trends were observed when compared to infants conceived through autologous IVF; however, the results did not reach statistical significance. No differences were noted regarding birth weight and length among term infants. Infants from the Index group compared to Control group A were classified more frequently as small for gestational age (8% *vs* 2%) and more often had an Apgar score of below 7 at 5 minutes (6.7% *vs* 0.7%). The occurrence of neonatal asphyxia (7.9% *vs* 0%) and low birth weight (<2500gr) (10.5% *vs* 1.6%) were more prevalent in neonates from the Index group compared to those in Control group B. No difference was noted in the study population regarding the prevalence of congenital malformations, neonatal hospital stay, neonatal jaundice and hypoglycaemia, LGA diagnosis, and ten-minute Apgar score. Lastly, infants born to mothers after oocyte donation with indication other than diminished ovarian reserve had a lower median birth weight and length and were more often small for gestational age compared to neonates whose mother was diagnosed with diminished ovarian reserve or had been conceived naturally (Table 4).

Table 4. Neonatal outcomes of women treated with donated oocytes (Index group) compared to women with no history of infertility (Control group A) and women treated with non-donor IVF (Control group B)

Outcome	Index group		Control group A		Control group B	
	Median (IQR)	Range	Median (IQR)	Range	Median (IQR)	Range
Gestational length, weeks	40(4)	28-42	40(3)	28-43	39(2.3)	36-42
Head circumference, cm	35(3)	25-38	35(2)	25-40	35(3)	30-38
Birth length, cm	50(3.5)	39-54	51(3)	32-56	50(3.3)	44-56
Birth length term infants, cm	50(4)	45-54	51(3)	42-56	50(3)	44-56
Birthweight, grams	3238(840)	1105-4910	3495(693)	730-5800	3545(785)	1985-5420
Birthweight term infants, grams	3380(795)	2284-4910	3512(668)	2200-5800	3585(743)	1985-5420

Table 4(continued).

Outcome	Index group	Control group A		Control group B	
	n/*N*(%)	*n*/*N*(%)	aOR [a] (95% CI)	*n*/*N*(%)	aOR (95% CI)
Perinatal death (<7 d after birth)	1/76	0/149		0/63	
Congenital malformation[a]	4/72 (5.6%)	5/139 (3.6%)	1.37 (0.23–8.14)	4/60 (6.7%)	0.32 (0.05–2.05)
5 min AS[a] <7	5/75 (6.7%)	1/149 (0.7%)	7.01 (0.40–123.43)	1/63 (1.6%)	1.24 (0.11–14.18)
10 min AS <7	1/75 (1.3%)	0	-	0	-
Asphyxia[a]	6/76 (7.9%)	4/149 (2.7%)	2.49 (0.52–12.00)	0	-
Preterm delivery (<37w)[b]	13/76 (17.1%)	12/149 (8.1%)*	2.24 (0.87–5.81)	3/63 (4.8%)	4.35 (1.08–17.52)*
LGA[b]	1/75 (1.3%)	4/149 (2.7%)	0.32 (0.03–3.46)	3/63 (4.8%)	0.09(0.01-1.06)
SGA[b]	6/75 (8.0%)	3/149 (2.0%)	3.93 (0.59–19.69)	1/63 (1.6%)	1.70 (0.16–17.72)
Low birthweight[a] (<2500 gr)	8/76 (10.5%)	8/149 (5.4%)	0.65 (0.10–4.30)	1/63 (1.6%)	0.50 (0.02–13.81)
Jaundice[a]	2/76 (2.6%)	3/149 (2.0%)	1.62 (0.23–11.68)	4/63 (6.3%)	0.32 (0.04–2.44)
Hypoglycemia[a]	3/76 (3.9%)	4/149 (2.7%)	1.74 (0.25–12.03)	3/63 (4.8%)	0.44 (0.07–2.90)
Male gender of the child[a]	39/76 (51.3%)	71/149 (47.7%)	0.85 (0.45–1.60)	31/63 (49.2%)	0.70 (0.32–1.52)
Infant Hospital stay, ≥3d[a]	27/68 (39.7%)	39/139 (27.7%)	1.13 (0.56–2.31)	18/58 (31.0%)	0.94 (0.38–2.30)

The outcomes are reported as median value and Interquartile Range (IQR), prevalence (*n*/*N*, %) or Adjusted Odds Ratios (aOR) and corresponding 95% confidence intervals (CI)

[a] Adjusted for maternal age when giving birth (< or ≥ 35yrs), maternal BMI at first antenatal visit (< or ≥25 kg/m^2), gestational age (continuous variable) and caesarean section (no/yes)

[b] Adjusted for maternal age when giving birth (< or ≥ 35yrs), maternal BMI at first antenatal visit (< or ≥25 kg/m^2) and caesarean section (no/yes)

Paper IV

Demographic data, clinical characteristics and HRG genotype

Women diagnosed with gestational hypertensive disorders were older (32.5±7 *vs* 31±6), with higher BMI (25.97±6.91 *vs* 23.55±5.18) and they delivered earlier (39±2 *vs* 40±1) and their infants had lower birthweight (3450±873 *vs* 3680±658) and length (50±4 *vs* 51±3). No differences were noted regarding nicotine use, parity, overall health status of the participants and intrapartum or postpartum complications. Regarding the above obstetric

and neonatal outcomes, no differences were noted in regard to HRG C633T genotype carriership among controls.

Genotype and risk of gestational hypertensive disorders

The genotypic distribution of the HRG C633T SNP on the study population was investigated; homozygous C/C genotype was more prevalent among controls compared to cases (39.6% *vs* 52.5%; p=0.037). Afterwards, the association between the genotype and the presence of gestational hypertensive disorders was studied with a logistic regression model. The odds of hypertensive disorders were higher among heterozygous C/T or homozygous T/T *vs* homozygous C/C carriers [OR 1.69, 95% CI (1.03–2.77)]. The association remained significant even after adjustment for maternal age, BMI and parity (Table 5).

Table 5. Logistic regression model for gestation hypertensive disorders associated with HRG C633T genotype

	Homozygous C/C	**Heterozygous C/T or Homozygous T/T**
Gestational Hypertensive Disorders [Unadjusted OR (95% CI)]	1	OR1.69 (1.03–2.77)
Gestational Hypertensive Disorders [Adjusted OR (95% CI)]	1	aOR 1.74 (1.03–2.96)

The multivariate model is adjusted the following covariates: maternal age (completed years upon delivery), maternal BMI (during the first antenatal visit on the prenatal centre during the 10th gestational week) and parity (primiparity *vs* multiparity)

Discussion

Papers I and IV

Methodological considerations

The studies in Papers I and IV were designed as case-control studies. A case-control study is a particularly suitable design to initially investigate an association between a risk factor (i.e. genotype-polymorphism) and a disease or outcome of rare occurrence (i.e. recurrent miscarriage or gestational hypertensive disorders). To our knowledge, these were the first studies to examine the possible association between HRG polymorphisms (i.e. A1042G and C633T SNPs) and conditions resulting from implantation defects. The study design can therefore be considered as appropriate and efficient.

Case-control studies have the ability to assess a hypothesis but do not usually establish causation. It should, however, be noted that both our studies are based on the robust and widely accepted a priori hypothesis of impaired trophoblast invasion and inadequate placentation that has been confirmed consistently by several studies in this field.

The major limitation of case-control studies is the risk of recall bias because the information is collected by self-report. Although that possibility cannot be entirely excluded, especially for the confounders, the risk is estimated to be nevertheless low, as the reported outcome is based on a definite diagnosis and the genotype was not known prior to study inclusion (Paper I). In Paper IV, all necessary background and clinical information was extracted from the participants' medical records retrospectively, eliminating the risk of recall bias.

Another important point of this study design is that the study participants selected should be at similar risk of developing the rare outcome of interest. In Papers I and IV, cases and controls stem from the same source population (the BASIC cohort) (Study IV) or the same geographic area and are ethnically homogenous (Study I).

In the multivariable logistic regression analysis, in order to estimate the risk more accurately, we adjusted for known confounders to the outcome, such as maternal age, BMI and smoking (Paper I) or parity (Paper IV). It can be debated whether these factors are real confounders in a more modern epidemiological concept [137]. Hypothyroidism (both overt and subclinical) is now considered as an independent risk factor, both for infertility and RPL

[2] (Paper I). Age, BMI and smoking have been consistently reported as risk factors for reproductive diseases. One would reasonably therefore wonder whether these covariates could affect the inheritance pattern or carriership of this set of alleles (genotype) in an individual. Nonetheless, it cannot be ruled out that, through epigenetic mechanisms and regulation of gene transcription, they could modify the consequent expression of a phenotype. Epigenetic mechanisms, such as DNA methylation, are dynamically regulated by lifestyle and environmental factors such as age-BMI [138] or smoking [124]. Placental epigenetic variation has recently emerged as a candidate mediator of environmental effect on the function of the placenta and as a significant regulator of pregnancy outcome [139]. Because the exact mechanisms remain incompletely understood, further studies in epigenetic epidemiology are needed.

Considerations on genetic association studies

A polymorphism corresponds to a variant present in the population in a relatively high frequency (>1%) [124]. A variant by itself is not sufficient to cause a disease, although it may contribute to susceptibility to a disease or a variation in the functional properties of a protein. Most reproductive diseases seem to represent complex genetic disorders; it is thought that no simple correspondence between genotype and phenotype exists [124] and both genetic and environmental factors contribute to the susceptibility risk. Complex diseases occur as the result of numerous common variants at different loci which individually have a small effect but collectively contribute to an individual's susceptibility to disease [106]. Association studies are therefore used to test the associations of a phenotype and a single SNP, multiple SNPs and/or haplotypes based on the common-disease common-variant hypothesis. The goal of population association studies is to identify patterns of polymorphisms that vary systematically between individuals with different disease states and could therefore represent the effects of either risk-enhancing or protective alleles [140].

The next step, after an association is observed, is to evaluate whether causation exists. In order to assess the causal nature of an observed association, the Bradford Hill criteria should be examined, such as specificity, temporality, consistency, plausibility and coherence. However, in our case, the demonstration of specificity can be challenging because the conditions of recurrent miscarriage and preeclampsia are considered multifactorial [141]. This increases the need for Genome Wide Association Studies (GWAS) in order to identify susceptible DNA variations associated with defective implantation conditions [106]. GWAS should then be followed by functional studies in order to confirm the results and elucidate the pathophysiology by making use of the genomics for gene transcription/gene translation/protein-protein interactions. One should, however, not forget that epigenetic modifi-

cation, including imprinting and gene-gene as well as gene-environment interactions (such as those due to age, diet, smoking, and obesity), may also contribute to the clinical phenotype [106], making it much more complex than the genetic study of a disease.

Population stratification, and the careful definition of inclusion criteria and sample size, are important issues to be concerned with in human genetic surveys, which may otherwise result in estimation errors. One should therefore always be cautious in the interpretation of the results because a positive association is not always proof of a direct causation, but might be a sign of linkage disequilibrium (where alleles between two or more loci are correlated), population stratification due to ethnic admixture or might even have occurred by random chance.

Significance of the results in the general context

In Paper I, we have demonstrated that heterozygous women for the HRG A1042G SNP seldom suffer from recurrent pregnancy loss. The protective effect of heterozygocity was more apparent when comparing fertile women to women who had never had children, either before or after RM diagnosis. The association demonstrated here remained significant even after adjusting for covariates with possible influence on the outcome, such as age, BMI, smoking and hypothyroidism. The findings are consistent with the theory of "heterozygote advantage" observed even in fertility settings [142, 143], apart from disease resistance models (such as those observed in the relationship between sickle cell anemia and malaria). Heterozygous carriers display a selective advantage in reproductive fitness over homozygous dominant and recessive carriers, which partly explains the genetic variability in natural populations.

In Paper IV it has been shown that heterozygous C/T and homozygous T/T carriers suffer more often from gestational hypertensive disorders compared to homozygous C/C carriers. The inheritance of the T-allele enhances the risk of gestational hypertensive disorders and the risk persists even after adjustment for maternal age, BMI and parity. This finding is in agreement with previous studies demonstrating the T-minor allele (homozygous T/T genotype) to be less beneficial, both regarding fertility [122, 129, 144], and regarding the development of recurrent miscarriage [128].

Although both reproductive conditions, that is to say, recurrent miscarriage and gestational hypertensive disorders, are considered to be complex diseases, both SNPs can potentially, when combined with other biomarkers and together with risk factors, be used in algorithms to predict individual susceptibility for the conditions and for pre-conceptual genetic counseling of the reproductive couples.

Proposed mechanism of HRG affecting fertility

HRG is a complex molecule, involved in the regulation of several biological processes through its ability to interact with many ligands. It can act as an adaptor molecule, changing its properties based on the components of the microenvironment or dependent on proteolytic cleavage [127].

Although the exact tridimensional structure of the entire HRG molecule still remains unknown, by X-ray crystallography examination it has been described as a cystatine-like fold of a five-stranded anti-parallel β-sheet, twisted around a five-turn α-helix [121]. This structure is stabilized by the six disulfide bonds that protect the molecule from dispersing upon proteolytic digestion [117]. One disulfide bond bridging a glutathione adduct at Cys 185 (N_2 domain) within the HRR/PRR region in the intact HRG molecule (Cys 407) seems to be of particular importance. The redox modification of this susceptible disulfide bond (Cys 185–Cys 407), together with plasmin cleavage of HRG, releases the HRR region, the only antiangiogenic fragment of HRG. The HRR region exhibits an inhibitory effect on blood vessel formation *in vivo* and *in vitro* by interrupting VEGF-induced endothelial cell motility [119].

In a simplified model, HRG functions would be summarized as follows. At sites of tissue injury and angiogenesis, the full-length HRG protein interacts with heparan sulfate and heparin, inhibiting the antithrombin-heparin interaction and limiting the anticoagulant activity of antithrombin. Thus, HRG exhibits pro-thrombotic potential [127]. Furthermore, HRG promotes angiogenesis indirectly by interacting with thrombospondin (TSP) and blocking the interaction of the TSP-CD 36 receptor on the endothelial cells. By inhibiting the otherwise antiangiogenic effect of TSP, HRG enhances the migratory potential of cells and promotes angiogenesis. However, when HRG interacts with plasmin the opposite effects are observed; the HRR/PRR fragment is proteolytically released and antiangiogenesis is promoted. It is also plausible that by reducing the levels of intact HRG, the ability of the full-length protein to promote angiogenesis through its interaction with thrombospondin will be reduced. Therefore, it is likely that the cleavage of HRG might negatively regulate angiogenesis by several mechanisms simultaneously [118] and thus modulate the ability of HRG to bind to various ligands, possibly by altering the conformation of the molecule and/or disrupting the ligand binding sites [145] regulating its biological function [121]. The presence of heparin, Zn^{2+}, or acidic pH was found to protect HRG from plasmin cleavage [145]. Therefore whether HRG is pro- or anti-angiogenic may be highly contextual, depending on the presence of heparin, Zn^{2+} or pH variations.

Although we do not know the exact mechanism by which HRG A1042G and C633T SNP affect implantation and placentation, they seem nevertheless to have clinical significance. The amino acid substitution (arginine in-

stead of histidine) in HRG A1042G SNP is located in the antiangiogenic domain, whereas the serine substitution (HRG C633T SNP) results in an extra glycosylation site close to the disulfide bridge between the N_2 domain and the HRR/PRR fragment of the HRG protein. Thus it could be postulated that the added glycosylation alters the stability of the disulfide bond and could potentially affect the release of the HRR/PRR peptide [144, 146]. Taking into account that this fragment is associated with HRG's antiangiogenic properties, the HRG polymorphisms might affect the mechanism by which HRG regulates angiogenesis in fertility processes [144, 146].

Papers II and III

Methodological considerations

Studies II and III were designed as observational retrospective cohort studies. Both studies are part of a prospective cohort on gamete donation conducted on a national level [19, 20]. Cohort studies are considered suitable for studying exposure to rare risk factors such as pregnancies after oocyte donation, which correspond to only 0.1% of children born annually in Sweden [10]. Furthermore, in cohort studies, a wide range of outcomes, such as obstetric (Paper II) and neonatal outcomes (Paper III) can be studied. Another strength of this study design is the reduced recall bias due to direct retrieval of all clinical information from the patients' medical records or national register. Furthermore, there is limited risk of selection bias, because participants originate from the whole of Sweden and probably share the same demographics. Therefore, the study design selected is considered appropriate.

However, one would wonder about the choice of the size of the study population. Because both studies were a sequel of the national study on gamete donation [19, 20], it was decided that Index and Control group B participants who fulfilled the inclusion criteria originating from the original cohort would constitute the study population (Papers II and III). Regarding Control group A, the size of the population was selected based on statistical criteria; after the completion of mathematical simulations, it has been estimated that, in most settings, an exposed/unexposed ratio corresponding to 1:1–2 is to be preferred due to bias minimization with simultaneous maintenance of good precision in the estimation of various outcomes [147]. In fact, a recent publication with a larger study population and greater power originating from Sweden confirms most of our findings [148], providing evidence that our study sample seems to be representative of the population in which the results will be generalized.

Parameters such as donor age, paternal age, ART method (conventional IVF or ICSI), as well as whether the pregnancy resulted from a cryo-

preserved or fresh embryo, could not be retrieved and were thus not taken into account. It should, however, be noted that other large series did not find donor age to have a significant association with perinatal outcome, attributing it instead to the homogeneity of the oocyte donor population (i.e. the majority of donors in Sweden being younger than 35 years, healthy and with proven fertility) [22, 41]. The rest of the factors mentioned above have dubious effect and have not been proven to affect the outcome before [148-150].

Pregnancies among women with Turner syndrome (TS) carry substantial risks, regardless of whether conception is natural or after oocyte donation [135, 151]. Possible complications include obstructive nephropathy, hypertension and preeclampsia, low birth weight infants, fetal growth restriction and preterm delivery among women with Turner syndrome (mosaic and non-mosaic) [135, 151, 152]. However the most serious concern remains the risk of cardiac insufficiency, aortic root dissection and sudden death [153]. Preconception evaluation by a multidisciplinary team of specialists is routinely performed in Sweden [135]. In our study it was observed that a higher proportion of oocyte recipients were diagnosed with TS than may be expected (13% instead of 10%) [148]. That might be explained by the legislative change permitting oocyte donation in January 2003 where a higher number of TS women had accumulated on the waiting lists. Given that TS diagnosis is one of the most prevalent among oocyte recipients and despite the obstetrical and perinatal risks that women with TS face, we decided to include them in the study population anyway, thereby limiting the risk of selection bias. When we repeated the statistical analysis after excluding women diagnosed with TS from our Index group, the caesarean section rate remained unaltered [35/66 (53%) instead of 42/76 (55%)] (data not shown). Regarding gestational hypertensive disorders (GHD), a slightly lower prevalence was observed when women diagnosed with TS were omitted [13.6% (9/66) instead of 15.8% (12/76)] (data not shown); however, the estimated risk of suffering from gestational hypertensive disorders remained largely unchanged, albeit slightly underpowered, when compared with natural conception pregnancies [OR 2.47, 95% CI (0.93–6.55), p=0.068] (data not shown).

Lastly, cross-border reproductive care (CBRC) that is to say, couples travelling abroad for fertility treatment in other countries (e.g. Finland, Russia, Denmark, Spain, Greece) due to financial or legal issues, is a growing worldwide phenomenon. One should therefore not forget that due to CBRC it is difficult to accurately calculate the number of children born annually in Sweden after oocyte donation [154]. Our conclusions can thus be applied on a larger proportion of the population strengthening the need for establishing guidelines on pregnancy surveillance.

Importance of the findings

Our analysis has provided evidence that oocyte donation is associated with hypertensive disorders of pregnancy, oligohydramnios, induction of labor, delivery by cesarean section, retained placenta and post-partum hemorrhage. Furthermore, it was observed that a higher prevalence of infants conceived through oocyte donation were born preterm compared to autologous conception. The association between oocyte donation and gestational hypertensive disorders remained significant even after adjustment for maternal age, maternal BMI, nicotine use, gestational length and maternal chronic diseases.

Since the initiation of the study, several more studies than the ones constituting the background for this research and reported in the introduction have been published. Among them are included reviews and meta-analyses presenting more robust results. We can therefore examine the most important findings one by one.

Gestational Hypertensive Disorders and Preeclampsia

Our results were confirmed considerably by a study conducted in Sweden based on the ART national register performed during a lengthier time period with a greater number of study participants [148].

Systematic reviews and meta analyses have confirmed the association between PE and OD. Independently, whether the control groups comprised women who became pregnant through IVF with autologous oocytes or through natural conception, oocyte recipients faced a higher risk of suffering from PE and gestational hypertensive disorders. According to other oocyte donation studies, the prevalence of gestational hypertensive disorders and preeclampsia among oocyte recipients is as high as 35% and 10%, respectively [94]. The risk of the development of PE in singleton pregnancies through oocyte donation compared to pregnancies through natural conception and autologous IVF was estimated to lie around OR 2.1–6.60 [155, 156] and OR 2.24–4.60, respectively [34, 93, 94, 155-157]. The odds of gestational hypertensive disorders developing among singleton pregnancies after oocyte donation compared to autologous IVF pregnancies have been calculated to be around OR 2.30–3.92 [34, 93, 94, 156, 157].

Systematic reviews and meta-analyses combining existing studies and increasing their estimate precision and statistical power have confirmed the association between PE and OD, concluding that neither multiple gestation nor patient age explain the variability of the effect of oocyte donation on preeclampsia [93, 94, 148, 155, 158]. The latter overturns the original myth that maternal demographics and multifetal gestations were to blame for the adverse outcomes observed in OD pregnancies, shifting the interest to other explanatory mechanisms.

Cesarean Section

Regarding the high prevalence of cesarean section (CS), our study findings were confirmed by the oocyte donation study based on the Swedish ART register. A more detailed report on the reasons CS was performed can be found in the Results section. Although it had been replicated in many older studies that the higher prevalence of CS could reflect maternal preference or the physician's inclination to perform CS on a greatly anticipated pregnancy [27, 159], that does not seem to be the case in our study. Furthermore, the high rate of CS observed (i.e. 70%) among women with TS in our study is also in agreement with the findings from the Nordic study on Turner syndrome (i.e., an 82% CS rate reported). In agreement with Hagman et al. [135], most common indications in both studies were breech presentation or cephalopelvic disproportion, probably associated with the short stature of these women [152].

Induction of labor

A list of the conditions responsible for induction of labor can be found on the Results section. A diagnosis related to uterine inertia/functional dystocia was quite usual (i.e. 65%) among oocyte recipients delivering with emergency CS after unsuccessful induction of labor. The latter could support the hypothesis of a biological effect of ageing on uterine performance during labor, implying that there might be a uterine, apart from the already known ovarian, component associated with ageing after all [160]. Age-related changes are known to occur, affecting the reproductive potential of the uterus and suggesting that there is a reduced endometrial receptivity due to altered sex steroid levels [153] and impaired myometrial contractility [160, 161], thus leading to dysfunctional labor.

Postpartum hemorrhage

We identified a higher proportion of women suffering from postpartum hemorrhage, confirmed by Nejdet et al. and Storgaard et al. [148, 156], despite adjusting for operative delivery (CS). Moreover, a higher rate of retained placenta was observed among women who were pregnant with donated oocytes compared to those with autologous oocytes. Although the exact mechanism is still unclear, placental implantation abnormalities and/or immune-mediated pathology cannot be excluded [162].

Neonatal outcomes

Infants conceived through oocyte donation had higher odds of being born preterm, being small for gestational age (SGA) and having Apgar scores below of 7 at 5 minutes compared to spontaneously conceived infants. The higher risk of OD infants being born preterm and/or SGA were also confirmed in the meta-analyses by Jeve et al. and Storgaard et al. [94, 156].

Furthermore, donor oocyte infants had lower mean birthweight and length compared to infants who were naturally conceived; the differences, however, disappeared when studying term infants only. Similar trends were observed when OD infants were compared to autologous IVF infants, but the results did not reach statistical significance. The latter observation was also confirmed in earlier meta-analyses [94, 163] as well as in a large US national registry study [37]. It is still unclear, though, how birthweight is affected by mode of conception. Malchau et al. [28], attempting to investigate this variation, adjusted infant birthweight for the presence of preeclampsia. They discovered that the risk of preterm birth and low birthweight improved, but did not disappear. It was therefore suggested that PE was not the only explanation for the more adverse perinatal outcome in OD pregnancies [28].

Moreover, in the meta-analysis conducted by Adams et al. [163], as well as in the US population-based cohort study [37], an association between low birthweight and preterm delivery was found. The latter finding is in accordance with our study findings, that is to say, that birthweight differed as a whole but not among term infants when examined by mode of conception. Once gestational age is accounted for, it seems that the effect of the donated oocytes on infant birthweight becomes "restrained" [37]. One should, however, keep in mind that the risk should be interpreted differently depending on whether preterm delivery is indicated (iatrogenic) as opposed to spontaneous. It should also be added that the former findings might be partly associated with the increased rate of CS which is usually performed before the 40^{th} gestational week.

In addition, it should be added that SGA diagnosis in Sweden is defined as birthweight below 2 standard deviations from the mean birthweight for gestational age [164], according to the sex-specific Swedish fetal growth curve, rather than lower than the 10^{th} percentile as it is used in most European and American studies. In this way, only the most severe cases were selected in our study.

Placenta pathology in egg donor pregnancies

A pregnancy after oocyte donation differs from a pregnancy with autologous oocytes in that the fetus is completely foreign (allogeneic *vs* semi-allogeneic fetus) [165]. According to the immunologic theory, the immune maternal recognition of fetal antigens presented by trophoblast or fetal cells on the fetal-maternal interface [165] results in a heightened maternal immunologic response which also plays an important role in the pathogenesis of PE [31]. OD pregnancies show a greater HLA antigen mismatch in peripheral blood compared to IVF with autologous oocytes or spontaneously conceived pregnancies [166-168]. This immunological dissimilarity leads to hyperactivation of T-helper cells (predominance of Th_2 immunity *vs* Th_1) during the first trimester, regarded as a specific reaction for OD pregnancies compared to

autologous pregnancies [169]. The immunological reactions are then transformed into histological changes. Placental histological studies have demonstrated that chronic inflammatory lesions are considered to be part of placental pathology induced by the maternal immune system to the completely allogeneic fetus [170]. In histological and immunohistochemical studies by Gundogan et al. [171] and Schonkeren et al. [170], placentas of OD and non-donor IVF pregnancies were examined. Gondogan et al. demonstrated dense fibrinoid deposition accompanied by diffuse severe chronic deciduitis in the basal plate of OD placentas, as well as increased infiltration by mononuclear cells [171]. The latter represent a form of immune-mediated placental pathology, which is thought to be a unique sign of donated oocyte conception [171]. Because it bears some resemblance to the model of solid organ transplantation, it has been postulated that the pattern is representative of a type of host-versus-graft reaction or, alternatively, an effort to inhibit rejection, or it could represent the fetus rejecting the mother [28]. Another type of placental injury [170] described was inflammatory lesions on the chorionic plate which were strongly associated with oocyte donation, but negatively correlated to the presence of PE [170]. The cellular composition of the infiltrate, examined by immunohistochemistry, revealed immunosuppressive macrophage cells of maternal origin [170]. It is still unclear whether these inflammatory reactions are a result of a destructive or a protective process against paternal antigens present in the fetal tissue [170, 171].

Similar vascular and inflammatory abnormalities (such as decidual arteriopathy and placental infarction) to the ones described in OD pregnancies were seen even in preeclamptic models [172, 173].

Regarding OD treatment indication, Keegan et al. [174] and Pados et al. [175] demonstrated that young oocyte recipients exhibited the highest rates of gestational hypertension and preeclampsia. It was thus suggested that this indicates a possible relationship between diminished ovarian function and hypertensive disorders. The underlying pathophysiological mechanism is still unclear but a hypothesis has been proposed. Kelkar et al. demonstrated the existence of antibodies against the zona pellucida and granullosa cells among patients with premature ovarian failure [176]. Interference of these antibodies with trophoblast-maternal interactions at the endometrial frontier was suggested to lead to suboptimal trophoblast invasion such as the one occurring in preeclampsia [172]. That and other immunologic factors in young patients with diminished ovarian reserve may magnify the aforementioned obstetric risks [174, 175]. The association, however, was not as clear in our population; the SGA rate, as well as rate of post-partum hemorrhage were higher, whereas birthweight and length were lower among infants born from OD mothers with non-diminished ovarian reserve. Regarding the occurrence of hypertensive disorders, the risk was assessed to be lower among POI women *vs* all other oocyte recipients after adjustment for various co-

variates. Nevertheless, we cannot rule out whether the high proportion of women diagnosed with Turner syndrome in our sample affects the observations, as most outcomes do not show any statistical difference after excluding those women from the Index group.

Clinical recommendations

To ART personnel and obstetricians

Oocyte donation has proved to be an excellent method of enabling patients to achieve pregnancy and that is evident by the increasing number of OD cycles performed annually; in the USA alone, 10.5% of fresh and frozen IVF cycles in 2013 were performed with donated oocytes [11]. Health care providers involved in fertility treatments, as well as in the follow up of a pregnancy, have an obligation to provide, to the best of their ability, accurate information, not only about the expected pregnancy, but also about the expected health of their infant when counseling reproductive couples about treatment choices.

After 2000 there was a greater awareness of the need to reduce the number of embryos transferred due to adverse perinatal outcomes, mainly due to preterm deliveries. In Sweden, since 2001, single embryo transfer (SET) is the norm [177]. Strict SET has been practiced in most (91%) OD cases [148] and a low rate of multiple birth deliveries (2.8%) has been reported [148]. However, in the USA, during 2013, only 23.6% of transfers were performed with a single embryo [11] and 34% of OD pregnancies were multifetal [11]. In a recent meta-analysis, the rate of gestational hypertensive disorders and preeclampsia were reported to be as high as 39% and 21%, respectively, in multifetal pregnancies. It is crucial therefore to reduce the number of embryos transferred and implanted per cycle [26, 37].

Oocyte donation is nowadays considered an independent risk factor for preeclampsia [31, 93, 94, 178]. Women undergoing OD treatment should therefore be counseled preconceptionally about the increased risks during an OD pregnancy. Health and lifestyle interventions are recommended (e.g. smoking cessation, maintaining normal body weight, etc.). Although the obstetric risk is independent of age or multiple pregnancy, the harmful effects are multiplied in women of advanced age (>40–45 years) and for those carrying twins or triplets [26]. Future recommendations, such as preventive prophylaxis with low dose ASA (75 mg/day) when more than one risk factor is present in order to inhibit preeclampsia and its consequences should be evaluated [157, 179]. Furthermore, serial ultrasound growth scans for SGA detection at an earlier stage might favorably influence the outcome. Lastly, preventive measures to avoid postpartum hemorrhaging such as active management of the third stage of labor and uterotonic agents should be applied.

Conclusions

- HRG genetic variations seem to be of importance in placental dysfunction disorders.
- HRG A1042G genotype is associated with recurrent miscarriage; it was shown that heterozygous A/G carriers compared to homozygous A/A or G/G carriers presented a reproductive advantage.
- HRG C633T genotype seems to influence the risk of gestational hypertensive disorders; it was shown that the carriership of T-allele heightened the risk of the condition.
- The HRG variations could be included in an algorithm along with other clinical variables in order to evaluate the individual risk against the conditions of RM and GHD.
- Pregnancies after oocyte donation have an increased prevalence of gestational hypertensive disorders.
- Infants conceived through oocyte donation have elevated odds of preterm delivery, as well as being born with lower mean birthweight and length if delivered preterm.
- Careful pre-pregnancy counseling should be offered to all couples planning to conceive with donated oocytes. Nevertheless, the neonatal outcomes seem to be favourable, especially if the infant is born at term.

Future Perspectives

A number of questions remain to be resolved about the mechanisms that are central to the establishment of a normal pregnancy. Recent research focuses on the defects of implantation and early placental development which perpetuate throughout pregnancy and later manifest in pregnancy loss, preeclampsia, and/or preterm birth.

Genome wide association studies (GWAS) and/or exome sequencing could be performed in large, well-defined populations in order to construct a blueprint of the molecular signalling network coordinating implantation. Candidate genetic variants could therefore be identified and susceptibility loci predisposing to these complex diseases can be revealed. The results could then be validated with the help of proteomics and metabolomics.

Furthermore, with the use of gene expression analysis it would be interesting to see whether other potential mediators of angiogenesis are aberrantly expressed on placentas from women pregnant after oocyte donation compared to placentas from non-donor IVF and spontaneous pregnancies. All the latter data could then be used to understand the underlying regulatory pathways that are involved in oocyte donation pregnancies. The immunological adaptation to the genetically foreign fetus (i.e. an allograft), as well as the fetus' competency to escape rejection by the maternal immune system might be finally explained. The conclusions drawn will hopefully one day be applied in the model of human tissue transplantations.

One should, however, not forget that the puzzle will only be complete after the epigenetic modifications of the placenta cells have been experimentally tested by environmental influence.

Hopefully, these approaches may lead to the improvement of the outcome of natural pregnancies and pregnancies conceived via *in vitro* fertilization, possibly up to and including the identification and testing of potential drug targets for the prevention and treatment of placental disease.

Summary in Swedish-Sammanfattning på svenska

Introduktion

Infertilitet är vanligt förekommande hos kvinnor i åldrarna 15 till 44 år och mer än 12% av alla par i reproduktiv ålder är ofrivilligt barnlösa. För att åstadkomma och fullfölja en graviditet krävs det samverkan mellan ett flertal mycket komplexa biologiska system annars komplikationer kan uppstå. Infertilitet, upprepade missfall och graviditetskomplikationer såsom preeklampsi anses alla kunna relatera till inadekvat infästning av embryot (implantation) och dysfunktionell utveckling av placenta (placentation). Fem procent av alla förstföderskor drabbas av preeklampsi med/utan tillväxthämning där risk för maternell såväl som fetal morbiditet och mortalitet föreligger. Liknande komplikationer har också nyligen rapporterats för kvinnor som är gravida med donerade ägg.

Regleringen av implantation och placentation är intrikat och de bakomliggande mekanismerna är ofullständigt kartlagda. Angiogena respektive antiangiogena faktorers betydelse för dessa mekanismer har diskuterats. Inadekvat reglering av de olika stegen i embryogenesen leder till tidiga missfall, vidare ger dysfunktionell implantation och placentation försämrad förutsättning för syre- och näringstransport till fostret vilket är en förutsättning för att en graviditet ska kunna uppkomma och fortgå.

Histidine-Rich Glykoprotein (HRG) är ett plasma glykoprotein som interagerar med ett flertal olika ligander, tex heparin och heparansulfat, plasmin och plasminogen samt thrombospondin. Det är av betydelse i olika biologiska processer där det reglerar cell adhesion, migration och proliferation. Det har nu kunnat visas att HRG finns i kvinnans reproduktiva organ och att det har en inverkan på blodkärlsnybildningen (angiogenes) i placenta i samband med en graviditet. Vidare har det visats att HRG är av betydelse för om ett humant embryo ska kunna fästa in till endometriet eller inte. Bärarskap av en specifik ”single nucleotide polymorphism” (SNP) i HRG hos kvinnan eller mannen verkar associera med graviditetsutfall vid assisterad befruktning (pilotstudie). Två specifika HRG SNPs (benämnda HRG C633T SNP och HRG A1042G SNP) är av intresse då nukleotidskiftet på DNA-nivå verkar medföra ändringar till den tredimensionella formen av proteinet och troligen till dess funktion.

Sedan 2003 i de fall där barnlösa par saknar funktionsdugliga äggceller är *in vitro* fertilisering (IVF) med donerade ägg tillåten i Sverige. Den ursprungliga indikationen för äggdonation var ovarial insufficiens. På senare år globalt har indikationer baserat på hög ålder, nedsatt ovarial reserv, sekundär infertilitet beroende på tex behandling av malignitet tidigare i livet, upprepade misslyckade IVF-försök pga nedsatt äggkvalitet och risk för nedärvning av allvarlig genetisk sjukdom tillkommit. Ett möjligt samband med ökad risk för graviditetskomplikationer vid äggdonation föreligger. Orsaken till detta är okänd men inadekvat implantation har diskuterats. De graviditeter som lyckas där ett ägg donerats, skiljer sig från en normal spontangraviditet på många sätt. Embryot som återförs till kvinnan bär på ett fetalt genom som är helt främmande för kvinnan och komplexa immunologiska interaktioner mellan kvinnan och fostret måste hanteras.

Syfte

Övergripande syfte med avhandlingen var att undersöka genetiska och epidemiologiska aspekter av gynekologiska tillstånd som kan anses bero på inadekvat implantation och placentation.

Studie I

Syftet med denna studie var att undersöka hypotesen om polymorfier av HRG (SNPs) som är av betydelse för reglering av angiogenes och koagulation associerar till habituella aborter (upprepade missfall) vilket kan anses bero på bristfällig implantation och placentation. Utfallet är habituell abort relaterat till HRG A1042G genotyp. Inom ramen för ett samarbete mellan Universitetssjukhusen i Uppsala och Stockholm (Danderyd, Karolinska Solna och Huddinge) har kvinnor med habituella aborter inkluderats i samband med att de sökte till gynekolog mottagning för utredning av besvären. Totalt har 180 kvinnor med habituell abort diagnos inkluderats som fall och 386 kvinnor med spontana graviditeter utan missfall i anamnesen utgjorde kontrollgruppen. Blodprov insamlades från varje kvinna och DNA extraherades och genotypades (SNP studie). DNA från varje kvinna i studien preparerades på Kvinnoklinikens forskningslaboratorium i Uppsala av ansvarig BMA samt doktoranden. Det utfördes statistisk bearbetning baserat på bärarskap av HRG A1042G genotyp och diagnoserna av intresse som utfall. Dataanalys visade att heterozygoterna (A/G bärare) drabbades mer sällan av upprepade missfall jämfört med homozygoterna (A/A och G/G bärare). Det negativa sambandet var ännu starkare bland barnlösa kvinnor som drabbades av upprepade missfall.

Studie II och III

Målet med de två studierna var att undersöka om det finns medicinska skillnader när det gäller graviditetskomplikationer samt perinatala och neonatala utfall hos kvinnor som genomgår äggdonation jämfört med konventionell IVF- och/eller spontant gravida kvinnor. Studien genomfördes som en multicenter registerstudie där samtliga svenska infertilitetscentra som får bedriva äggdonation dvs Sahlgrenska Göteborg, Akademiska Uppsala, US Linköping, KI/Huddinge Stockholm, US Örebro, IVF-kliniken Umeå och US Malmö ingick. Alla par som sökte till dessa kliniker under åren 2005-2008 tillfrågades om ett deltagande i studien. Inom ramen för studien har 76 par som blivit gravida med donerade ägg inkluderats. 63 IVF-gravida par och 150 spontangravida par från hela landet har dessutom inkluderats som kontroller. Relevant information gällande medicinsk bakgrundsinformation hämtades från Medicinska födelseregistret (MFR) och deras medicinska journaler. Dataanalysen visade att det är betydligt vanligare med förlossningsinduktion och kejsarsnitt efter äggdonation jmf med naturlig eller konventionell assisterad befruktning. Dessutom äggdonationsgravida kvinnor drabbas oftare av graviditets hypertensiva sjukdomar, oligohydramnios, kvarhållen placenta och postpartum blödning jämförd med spontant gravida. De barn i studien som blir till efter äggdonation föds oftare prematurt och har lägre födelsevikt och längd jmf med barn efter spontant graviditet. Skillnaderna blir mindre tydliga bland fullgångna barn. Liknande trender gällande negativa maternella samt perinatala utfall ses också efter jämförelse av äggdonationsgravida mot konventionell IVF gravida kvinnor.

Studie IV

Målsättningen med denna studie var att utreda om polymorfier av HRG (SNPs) är associerade till graviditets hypertensiva sjukdomar såsom preeklampsi, eklampsi samt graviditetshypertoni, som alla kan anses bero på implantationsdefekter. Utfallet var hypertensiva sjukdomar relaterade till HRG C633T genotyp. Prover som samlades in på Kvinnokliniken, Akademiska sjukhuset i Uppsala inom ramen för ”BASIC studien/biobanken” användes. Alla gravida kvinnor tillhörande Akademiska sjukhusets upptagningsområde tillfrågades om ett deltagande när de kom för sitt rutinultraljud i graviditetsvecka 16-18. Kvinnorna följdes därefter longitudinellt under graviditeten, där medicinskt relevant information hämtades från journalen och fördes in i SPSS datafil för statistisk bearbetning. Ett blodprov togs under graviditet och förvarades i en biobank. DNA från varje studiedeltagare preparerades på forskningslaboratorium, KK, UAS av ansvarig BMA samt doktoranden. I studien inkluderades 96 kvinnor som drabbades av graviditets hypertensiva sjukdomar (i.e. preeklampsi, eklampsi och graviditetshypertoni) samt 200 friska kontroller med okomplicerade fullgångna gravidi-

teter. Resultatet tyder på att bärarskap av T-allel innebär större risk för hypertensiva sjukdomar; C/T och T/T bärare löper högre risk att drabbas av hypertoni under graviditet jämförd med C/C bärare.

Signifikans

Vår förhoppning är att våra studier har bidragit till ökad förståelse av den komplexa genetiska bakgrunden av sjukdomar associerade till implantationsdefekter. Dessutom kan avhandlingen få en direkt klinisk tillämpning då det kan omforma rutinerna gällande mödravårdskontroller av äggdonationsgravida i Sverige, såsom ställningstagande till ASA (acetylsalicylsyra)-profylax från tidig graviditet, utökad blodtrycksmonitorering, ultraljudskontroll med Doppler i arteria uterina och umbilicalis samt bedömning av fostertillväxt och fostervattenmängd under graviditet.

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Appendix

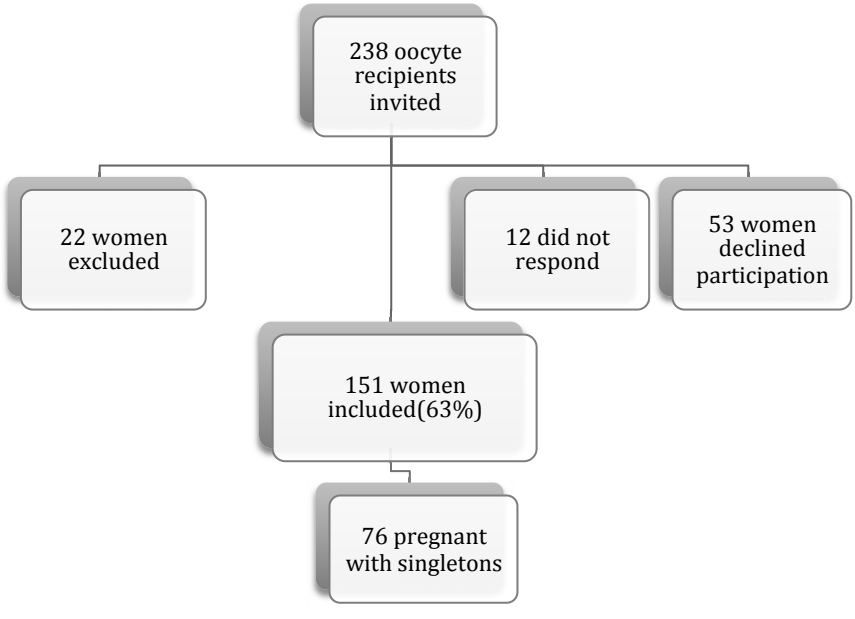

Figure 2. Flow chart of study participants, Index group (Study II and II)

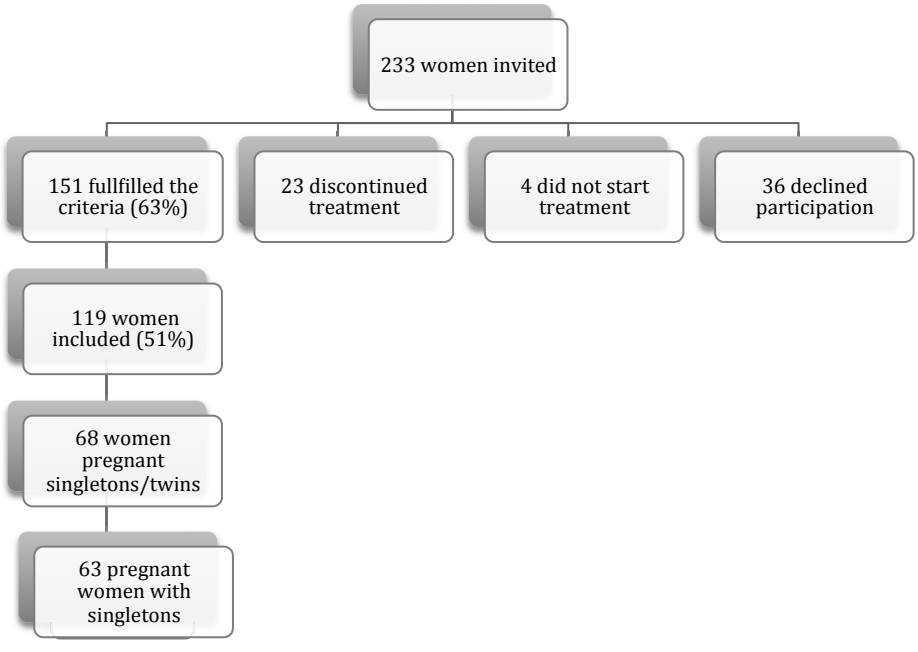

Figure 3. Flow chart of study participants, Control group B (Study II and III)

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