Clinical evaluation of preimplantation genetic diagnosis for BRCA 1/ 2 mutations

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Clinical evaluation of preimplantation genetic diagnosis for *BRCA1/2* mutations

Inge A.P. Derks-Smeets

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“The most important questions come from people on the frontlines,
the most righteous projects demand the most rigorous science,
and no question is too big to ask.”

Dr. Mary-Claire King, the Jackson Laboratory , 2017
Dr. King identified the genomic region of *BRCA1* in 1990

Voor Elna

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Preface

Origin of this thesis

Preimplantation genetic diagnosis (PGD) for hereditary cancer predisposition syndromes was legalized in the Netherlands in 2008. Hereditary breast and ovarian cancer (HBOC) syndrome based on pathogenic mutations in the *BRCA1* or *BRCA2* gene is one of the cancer predispositions PGD is offered for since. A substantial number of couples affected by a *BRCA1/2* mutation visited the PGD outpatient clinic in Maastricht University Medical Center (Maastricht UMC+) for counseling regarding this reproductive option. When discussing PGD with these couples it became obvious that the issue whether or not to opt for PGD was rather difficult to resolve for many of them. Often, there was not a clear 'yes' or 'no', but a complex mixture of pros and cons. Questions regarding the reproductive chances of the treatment were frequently asked: "What is the chance to conceive in the first PGD attempt?" And, in case the first attempt would be unsuccessful: "How many treatments will be offered and considered sensible?" Further: "How many couples have delivered at least one child after PGD?", and, "What if we wish for more than one child?"

For women affected with a *BRCA1/2* mutation there were some additional items to consider. In 2010 a study was published suggesting a reduced ovarian reserve in *BRCA* mutation carriers, showing a lower number of oocytes after ovarian stimulation for IVF in *BRCA1* mutation carriers.¹ Although this study was the first of its kind and the number of patients on which the conclusions were drawn was very small, it raised concern regarding the reproductive fitness of this specific patient population.

In case of a female carrier the safety of ovarian stimulation for the *in vitro* fertilization (IVF) treatment involved in PGD was another important issue. Many female *BRCA1/2* mutation carriers asked whether exposure to ovarian stimulation would be harmful to their own health: "Would this treatment increase my own risk of breast cancer?" Their intention of using PGD was, among others, to prevent their child from the familial cancer predisposition, but was there a price to pay? Another, related question concerned the timing of preventive breast surgery. A substantial part of the women deciding on PGD also considered a bilateral prophylactic mastectomy. Would it be wise to remove the breasts before the start of IVF/PGD or was it safe enough to wait in order to be able to breastfeed? It was impossible to answer these questions with confidence since the oncological safety of ovarian stimulation for IVF in *BRCA1/2* mutation carriers was barely studied.²

The aforementioned topics were even more of clinical interest because of a potential connection between them. It was possible that a reduced ovarian reserve in *BRCA1/2* mutation carriers would expose this group of women to higher cumulative doses of gonadotropins, due to either elevated daily doses of gonadotropins and/or due to ovarian stimulation for a prolonged period of time. Besides, if ovarian reserve would

be diminished this might worsen the treatment outcome in female *BRCA1/2* mutation carriers and therewith increase the need for more treatment attempts. What effect would this have on breast cancer risks in these women with strongly elevated *a priori* risks?

These clinical questions formed the origin for this thesis on patient perspectives and clinical suitability of PGD for HBOC. In order to start answering the patient-centered questions, a multidisciplinary research project was set up. It concerned a multicenter, international collaboration, in which the following centers participated: Maastricht UMC+ (departments of clinical genetics, obstetrics and gynecology - unit of reproductive medicine, and internal medicine - unit of medical oncology), Maastricht University (department of epidemiology), Universitair Ziekenhuis Brussel, Brussels, Belgium (centers for reproductive medicine and medical genetics), University Medical Center Utrecht (departments of reproductive medicine and genetics), University Medical Center Groningen (departments of obstetrics and gynecology and clinical genetics), and Academic Medical Center Amsterdam (center for reproductive medicine and department of clinical genetics). Additionally, for the study in chapter 5 collaboration with the national HEBON consortium was established. Furthermore, Radboud University Medical Center Nijmegen and the Netherlands Cancer Institute, Antoni van Leeuwenhoek Hospital Amsterdam participated in the study in chapter 7.

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General introduction

Genetics

Hereditary breast and ovarian cancer (HBOC) syndrome is a genetic tumor syndrome caused by mutations in the *BRCA1* and/or *BRCA2* gene. The *BRCA1* and *BRCA2* gene were identified as tumor suppressor genes in 1994 and 1995 respectively.^{1,2} *BRCA1* (MIM 113705) is mapped to chromosome 17q21.31 and involved in several biological processes (e.g., DNA double-strand break repair by homologous recombination, regulation of gene expression, cell cycle checkpoint control, and chromatin remodeling).³ *BRCA2* (MIM 600185) is located on chromosome 13q13.1 and participates in double-strand break repair by homologous recombination via an interaction with RAD51.⁴ Hence, the *BRCA* genes play pivotal roles in the maintenance of genomic stability and therewith prevent cells from malignant transformation. The *BRCA* genes follow the so-called 'two-hit' model introduced in 1971 by Knudson, which describes the inactivation of tumor suppressor genes after loss-of-heterozygosity: one allele is eliminated by a germline mutation (i.e., the first hit), followed by the loss of the other allele due to an acquired somatic mutation (i.e., the second hit).⁵

Genetic predispositions in general account for 5-10% of all breast cancer cases. Of these, 25% can be ascribed to mutations in the *BRCA1/2* genes.^{6,7} The prevalence of *BRCA1/2* mutations is estimated at 0.25-0.50% in the general population, although some mutations may be more prevalent in specific ethnic groups.^{8,9} For instance, the founder mutations 185delAG and 5382insC in *BRCA1* and 6174delT in *BRCA2* have a combined prevalence of approximately 2.5% in Ashkenazi Jews.¹⁰

Recently, the *CHEK2* gene was introduced in clinical diagnostics as breast cancer susceptibility gene. Heterozygous carriers of the c.1100delC mutation (a founder mutation with a prevalence of approximately 1% in Western Europe) face an increased lifetime risk of breast cancer up to 35-55% in case they have at least one first-degree *CHEK2*-mutated relative with breast cancer. The risk of breast cancer for homozygous carriers is estimated at 60-80%.^{11,12}

Several other inherited cancer syndromes are also known for an increased risk of breast cancer although penetrance is lower than in HBOC. These include *PTEN* hamartoma tumor syndrome (formerly known as Cowden syndrome, *PTEN*), Li-Fraumeni syndrome (*TP53*), Peutz-Jeghers syndrome (*STK11*), and hereditary diffuse gastric cancer (*CDH1*).¹³

Clinical presentation

Female carriers of a *BRCA1* or *BRCA2* mutation face elevated risks of breast, fallopian tube, and ovarian cancer. Male mutation carriers are at increased risk of breast and prostate cancer. Female *BRCA1* mutation carriers may be susceptible for serous(-like) endometrial cancer. Additionally, both male and female *BRCA2* mutation carriers are prone for pancreatic cancer and possibly melanoma.¹⁴⁻¹⁸ Penetrance of female breast cancer is estimated to be 72% (95% confidence interval (CI) 65-79%) and 69% (95% CI 61-77%) for *BRCA1* and *BRCA2* mutation carriers respectively at the age of eighty. For ovarian cancer these figures are expected to be 44% (95% CI 36-53%) and 17% (95% CI 11-25%) for *BRCA1* and *BRCA2*, respectively.¹⁹ However, a recent analysis based on a Dutch cohort only revealed lower cumulative cancer risks, in particular for *BRCA1* mutation carriers.²⁰ This lower penetrance may be explained by lower cancer risks associated with older birth cohorts, a less severely affected family history, and founder mutations in specific gene regions. Cumulative lifetime risks to develop a contralateral breast tumor are estimated at 83% (95% CI 69-94%) for *BRCA1* and 62% (95% CI 44-80%) for *BRCA2* mutation carriers at the age of seventy.²¹

BRCA-associated tumors have a younger age of onset and exhibit different pathological characteristics than sporadic tumors. *BRCA1*-associated breast cancers are for instance often poorly differentiated (i.e., Bloom and Richardson grade 3) and triple negative (i.e., estrogen and progesterone receptor and Her2Neu negative). They demonstrate a more aggressive growth and higher mitotic index than *BRCA2*-associated and sporadic tumors.^{22,23} Nevertheless, survival after *BRCA1/2*-associated breast cancer appears to be the same when compared to survival after sporadic breast cancer.²⁴ Most *BRCA1/2*-associated ovarian cancers are of serous histological subtype and poorly differentiated.²⁵ However, the survival of *BRCA1/2*-associated ovarian cancers may be better when compared to sporadic ovarian cancer patients.²⁶ The recent introduction of PARP-inhibitors in the treatment of *BRCA1/2*-associated ovarian cancer may further improve survival.²⁷

Surveillance of the breasts with the purpose to detect malignancies in an early stage and to reduce mortality is offered to female *BRCA1/2* mutation carriers.^{28,29} In the Netherlands, clinical breast examination and magnetic resonance imaging (MRI) of the breasts is annually performed from the age of 25, combined with an annual mammography from the age of 30. Screening lasts until 60 years of age. From then female *BRCA1/2* mutation carriers are enrolled in the regular population based screening program for breast cancer.³⁰ Female *BRCA1/2* mutation carriers can also opt for risk-reducing surgery which may improve survival in carriers without a history of cancer.^{31,32}

Screening of the ovaries (i.e., ultrasonographic examination and determination of tumor marker Ca-125 in peripheral blood) has proven to be ineffective for the detection of *BRCA1/2*-associated ovarian cancer in an early stage.^{33,34} As a

consequence, *BRCA1/2* mutation carriers are advised to undergo a bilateral risk-reducing salpingo-oophorectomy after child bearing, from the age of 35-40 for *BRCA1* mutation carriers and 40-45 for *BRCA2* mutation carriers.³⁰

Reproductive options

The autosomal dominant inheritance of the *BRCA1* and *BRCA2* gene lead to a 50% risk for mutation carriers to transmit the susceptibility for HBOC to their offspring. For couples who want to prevent this increased cancer risk in their children, two strategies are available for the conception of a child genetically related to both partners: prenatal diagnosis (PD) and preimplantation genetic diagnosis (PGD).

PD for HBOC can be performed once a pregnancy is achieved. In the Netherlands, PD for HBOC is performed in a two-step approach. In the first step the gender of the fetus is determined by non-invasive prenatal testing (NIPT), available from the ninth week of gestation.³⁵ Further diagnostics is only offered in case of a female fetus. At eleven to thirteen weeks of pregnancy a chorionic villus biopsy is performed followed by genetic analysis of the obtained placental DNA material, which is identical to the fetal DNA. When test results show that the female fetus has inherited the familial *BRCA1/2* mutation the pregnancy is terminated. Before continuing to this second, invasive step it has been talked over with the prospective parents that in ongoing pregnancies the autonomy of the unborn child should be protected by avoiding prenatal disclosure of a late-onset disorder carrier status. Literature regarding PD for *BRCA1/2* mutations is scarce.^{36,37} In the Netherlands, PD for HBOC is considered rather controversial by a majority of both clinicians and *BRCA1/2* mutation carrier couples because of its late onset, incomplete penetrance and preventive and therapeutic options. As a result, PD is rarely performed for this indication.³⁸

Since a decade PGD is available for pathogenic mutations in the *BRCA1* and *BRCA2* gene.³⁹ PGD involves *in vitro* fertilization (IVF). Ovarian stimulation is performed to produce a surplus of oocytes at the same time. After several days of stimulation an oocyte pick-up is performed by transvaginal aspiration of the oocytes. Fertilization of the oocytes takes place using intracytoplasmic sperm injection (ICSI) in order to avoid contamination of the zona pellucida with residual spermatozoa.^{40,41} Up to now, most PGD clinics perform embryo biopsy in the cleavage stage, removing one or seldomly two blastomeres from the embryo for genetic analysis on the third day post-fertilization. It is expected that in the near future a growing number of PGD clinics will switch to trophectoderm biopsy, removing a number of trophectoderm cells for genetic analysis at day five or six post-fertilization.⁴² Single-cell analysis of the blastomeres is performed using polymerase chain reaction (PCR), based on haplotyping of at least two informative flanking microsatellite markers on each side of

the concerning *BRCA* locus. In case the marker analysis is not informative, a mutation-specific protocol is set-up based on the private mutation and at least one informative marker. One or two embryo(s) without the familial *BRCA1/2* mutation are transferred into the uterus the day after single-cell analysis. The chance of pregnancy per cycle with oocyte pick-up varies between PGD clinics but is approximately 25%.⁴³

PGD setting

Maastricht University Medical Center (Maastricht UMC+) is the only licensed center for PGD in the Netherlands and performs PGD since 1995. Transport PGD has been established in collaboration with the University Medical Centers Utrecht and Groningen and Academic Medical Center Amsterdam, in a collaboration called 'PGD Nederland' since 2008.

In the first years, PGD was only carried out for severe monogenic, fully penetrant diseases. In 2008, there was an intense nation-wide debate in which politics, media, patient organizations, as well as the PGD center in Maastricht UMC+ were involved, addressing the legalization of PGD for hereditary cancer syndromes, including *BRCA1/2* mutations.⁴⁴ Opponents of the broadening of PGD indications warned for a slippery slope, given the late age at onset, incomplete penetrance and the availability of preventive and therapeutic options. However, supporters argued for a permissive policy with the "right" of affected couples for a free reproductive choice, referring to the serious nature of hereditary cancer syndromes and the related burden. In the end, it was decided to accept PGD for hereditary cancer syndromes taking into account several terms and conditions, recorded in the national PGD regulation.⁴⁵ One of the consequences was the installation of a national committee for the assessment of new PGD indication categories. Additionally, a yearly report of treatments performed was a prerequisite in order to guarantee transparency in PGD practice. Since the legalization of PGD for hereditary cancer predisposition syndromes HBOC evolved into one of the most often applied autosomal dominant PGD indications in the Netherlands.⁴⁶

Since 1995, over 1200 couples have been treated with PGD in the Netherlands because of an increased risk of a child with a genetic condition. More than 2500 PGD procedures have been performed and over 500 children have been born.⁴⁶

Since many years there is a clinical as well as scientific collaboration between the PGD centers of Maastricht UMC+ and Universitair Ziekenhuis Brussel (UZ Brussels), Brussels, Belgium, one of the world's leading PGD centers. Two of the studies presented in this thesis are based on joint results and provide therewith an exclusive overview of the expertise of both centers.

Aims of this thesis

The general aim of this thesis is to evaluate whether PGD is a suitable reproductive option for couples with a *BRCA1/2* mutation. The specific research questions are:

*1. Which motives and considerations are taken into account by couples carrying a *BRCA1/2* mutation, when deciding on PGD and PD?*

In **chapter 2**, the reproductive decision-making process regarding PGD and PD for *BRCA1/2* mutations is studied in a qualitative approach. Pros and cons from patients' perspectives as well as the emotional impact of the decision-making is set out.

*2. Is PGD for *BRCA1/2* mutations a suitable reproductive option, based on the first clinical experiences?*

Chapter 3 provides an overview of the combined clinical experience with PGD for *BRCA1/2* mutations in the Netherlands and UZ Brussels. The first years since the start of the program are evaluated. The genetic, gynecological, and oncological aspects of the procedure are described and outcome data of PGD treatments performed are provided. Additionally, the results of patients follow-up are presented. **Chapter 4** outlines the development of universal single-cell PGD tests for mutations in the *BRCA1* and *BRCA2* gene.

*3. Is ovarian stimulation for IVF with or without PGD safe for female *BRCA1/2* mutation carriers in terms of breast cancer risks?*

The influence of ovarian stimulation for IVF with or without PGD on the risk of primary breast cancer in female *BRCA1/2* mutation carriers is studied using a nationwide cohort in **chapter 5**.

*4. Do female *BRCA1/2* mutation carriers have a reduced ovarian reserve?*

This topic is addressed in two separate studies, using different outcome parameters. **Chapter 6** reports on a retrospective study using the number of mature oocytes in response to ovarian stimulation for IVF/PGD as a proxy variable for ovarian reserve status in *BRCA1/2* mutation carriers. **Chapter 7** presents a prospective study in which anti-Müllerian hormone levels are measured to evaluate ovarian reserve status in *BRCA1/2* mutation carriers.

Finally, **chapter 8** is a general discussion reflecting on the study results and placing them into perspective. Furthermore, recommendations for clinical practice and future scientific research are provided.

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Part I

PGD for *BRCA1/2* mutations

Chapter 2

Decision-making on preimplantation genetic diagnosis and prenatal diagnosis: a challenge for couples with hereditary breast and ovarian cancer

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Abstract

Study question

How do couples with a *BRCA1/2* mutation decide on preimplantation genetic diagnosis (PGD) and prenatal diagnosis (PD) for hereditary breast and ovarian cancer (HBOC) syndrome?

Summary answer

BRCA couples primarily classify PGD and/or PD as reproductive options based on the perceived severity of HBOC and moral considerations, and consequently weigh the few important advantages of PGD against numerous smaller disadvantages.

What is known already

Awareness of PGD is generally low among persons at high risk for hereditary cancers. Most persons with HBOC are in favor of offering PGD for *BRCA1/2* mutations, although only a minority would consider this option for themselves. Studies exploring the motivations for using or refraining from PGD among well-informed *BRCA* mutation carriers of reproductive age are lacking. We studied the reproductive decision-making process by interviewing a group of well-informed, reproductive aged couples carrying a *BRCA1/2* mutation, regarding their decisional motives and considerations.

Study design, size, duration

This exploratory, qualitative study investigated the motives and considerations taken into account by couples with a *BRCA1/2* mutation and who have received extensive counseling on PGD and PD and have made a well-informed decision regarding these options. Eighteen couples took part in focus group and dyadic interviews between January and September 2012.

Participants/materials, setting, methods

Semi-structured focus groups were conducted containing two to four couples, assembled based on the reproductive method the couple had chosen: PGD ($n = 6$ couples) or conception without testing ($n = 8$ couples). Couples who had chosen PD for *BRCA* ($n = 4$) were interviewed dyadically. Two of the women, of whom one had chosen PGD and the other had chosen no testing, had a history of breast cancer.

Main results and the role of chance

None of the couples who opted for PGD or conception without testing found the use of PD, with possible pregnancy termination, acceptable. PD users chose this method because of decisive, mainly practical reasons (natural conception, high chance of favorable outcome). Motives and considerations regarding PGD largely overlapped between PGD users, PD users, and non-users, all mentioning some significant advantages (e.g., protecting the child and family from the mutation) and many smaller disadvantages (e.g., the necessity of *in vitro* fertilization (IVF), low chance of pregnancy by IVF/PGD). For female mutation carriers, the safety of hormonal stimulation and the time required for PGD before undergoing prophylactic surgeries were important factors in the decision. Non-users expressed doubts about the moral justness of their decision afterwards and emphasized the impact the decision still had on their lives.

Limitations, reason for caution

The interviewed couples were at different stages in their chosen trajectory, up to three years after completion. This may have led to recall bias of original motives and considerations.

Couples who did not actively seek information about PGD were excluded. Therefore the results may not be readily generalizable to all *BRCA* couples.

Wider implications of the findings

The perceived severity of HBOC and, for female mutation carriers, the safety of hormonal stimulation and the time frames for PGD planning before prophylactic surgeries are essential items *BRCA* couples consider in reproductive decision-making. The emotional impact of this decision should not be underestimated; especially non-users may experience feelings of doubt or guilt up to several years afterwards. PGD counseling with tailored information addressing these items and decisional support in order to guarantee well-informed decision-making is needed.

Trial registration number

Not applicable.

Introduction

Hereditary breast and ovarian cancer (HBOC) syndrome is an autosomal dominant predisposition caused by a mutation in breast cancer genes, *BRCA1* and *BRCA2*. Female mutation carriers face risks of 57% (*BRCA1*) and 49% (*BRCA2*) for breast cancer and 40% (*BRCA1*) and 18% (*BRCA2*) for ovarian cancer by the age of 70.¹ In contrast, Dutch women without a *BRCA* mutation have a lifetime risk of 12.7% and 1.3% for breast and ovarian cancer, respectively.² Among women worldwide, breast cancer is the most common malignancy and primary cause of cancer mortality. Around 5–10% of all breast cancer cases and over 30% of breast cancer diagnoses under the age of 30 are attributable to a *BRCA1/2* mutation.^{3,4} Breast and ovarian cancer related to *BRCA* mutations is associated with a relatively early age of onset. Female mutation carriers are given the option of periodic screening and/or prophylactic surgery of breasts and/or ovaries to decrease morbidity and mortality.⁵

Persons with a *BRCA* mutation have a 50% prospect of passing on the susceptibility for HBOC to their offspring. Preimplantation genetic diagnosis (PGD) and prenatal diagnosis (PD) are available reproductive options to prevent this. With PD, non-invasive fetal sex determination is performed at nine weeks of pregnancy and, in case of a female, this is followed by chorionic villus sampling with the intention to terminate the pregnancy if the fetus is affected. With the relatively new technique of PGD, *in vitro* fertilized (IVF) embryos are genetically diagnosed before implantation and only unaffected embryos are transferred to the uterus. However, the use of the aforementioned techniques, especially PD, for HBOC raises ethical concerns given the reduced penetrance of HBOC, its onset at adult age, and the availability of preventive and therapeutic options.^{6,7} These characteristics may explain the generally low acceptability of PD for *BRCA* among persons affected with HBOC.^{7,8} To date, studies exploring the motivations regarding PD uptake among well-informed *BRCA* mutation carriers of reproductive age are lacking.

For PGD, a physically demanding *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) treatment is necessary regardless of the couple's fertility. Moreover, the chance of conception with IVF/ICSI is limited even among normally fertile couples given the pregnancy rate of 28.7% per aspiration in Europe.⁹ This rate decreases even further when PGD is added due to the reduction of eligible embryos for transfer when excluding those with the genetic condition.

In the Netherlands, PGD was introduced in 1995 and, after nationwide political and ethical discussions, approved for late onset inherited cancer predisposition syndromes in 2008. Nowadays, HBOC is one of the most frequent indications for PGD in Maastricht University Medical Center (Maastricht UMC+), the only licensed PGD center in the Netherlands. PD for HBOC is available on a case-by-case base in several University Medical Centers. In the Netherlands, PGD and PD treatments are covered by the health insurance system. The female exclusion criteria for a PGD treatment are

specified as following: age >40, BMI >30 and FSH level > 15mIU/ml. Both PGD and PD are available for *BRCA* in many European countries as well as in the USA.^{6,10-13}

In 2003, the European Society of Human Reproduction and Embryology (ESHRE) ethics taskforce argued that PGD was acceptable for adult onset and multifactorial diseases such as HBOC and other cancer predispositions, despite uncertainties about prospective improvements in preventive and therapeutic options.¹⁴

Opinion surveys among persons affected by HBOC show that the majority, after being informed about PGD, is in favor of offering PGD for *BRCA1/2* mutations, although only a minority would consider this option for themselves.^{8,10,12,13,15-20} However, the aforementioned studies were not designed to explore the process from hypothetical acceptability or PGD intention to actual PGD use, since they frequently lacked a focus on *BRCA* mutation carriers of reproductive age and included persons with diverse levels of knowledge regarding PGD. The few studies available on attitudes and motives regarding PGD among couples who were well-informed (i.e., who had had an informative PGD consultation) or who had experience with PGD have been carried out in the general PGD population.^{21,22,23} Nevertheless, motives may be dependent on the genetic condition PGD is considered for. In-depth studies regarding the motives and considerations taken into account by couples carrying a *BRCA* mutation are needed, in order to gain insight into the aspects influencing reproductive decision-making in this population. This knowledge can be valuable for the optimization of patient decision support for a growing group of couples facing this quandary.

This study therefore aims to provide an integral qualitative account of the decision-making process among couples carrying a *BRCA1/2* mutation, who seriously considered PGD as a reproductive option. Motives and considerations for opting for or against PGD as well as the reproductive alternatives (PD and conception without testing) were addressed. Furthermore, PGD users, PD users, and non-users were asked to reflect on the reproductive option chosen.

Materials and methods

Recruitment of couples

Couples carrying a *BRCA1/2* mutation were eligible for participation if they had received standardized counseling on their reproductive options by an expert in reproductive genetics between 2008 and 2012 at the PGD center of Maastricht UMC+, and had made a final decision whether or not to use PGD or PD. During counseling, verbal and written information was provided about the PGD procedure (including IVF/ICSI, embryo biopsy, chance of pregnancy, risk of misdiagnosis and health of children born after PGD). In addition, PD was discussed, consisting of non-invasive fetal sex determination, followed by chorionic villus sampling in case of a female fetus

and termination of pregnancy (TOP) in case of an affected female fetus. Inclusion criteria for the study were at least eighteen years of age and a full understanding of the Dutch language. Exclusion criteria were presence of one or more medical reasons to reject the couple from PGD, severe physical or psychological illness, presence of more than one indication for PGD, divorce, and foreign place of residency.

Out of a total of 69 potential couples, 47 couples were selected and invited to participate by letter. Purposive sampling²⁴ was conducted in order to include at least four couples from each reproductive choice (PGD, PD, and conception without testing) with variable demographic factors (i.e., sex of the mutation carrier, *BRCA1* and *BRCA2* mutations, asymptomatic mutation carriers, and breast cancer survivors). Based on an expected participation rate of 25%, 47 out of 69 eligible couples were selected. After informed consent, couples were contacted by telephone to schedule the interviews. Reasons for non-participation were collected (Table 2.1).

Table 2.1 Reasons for non-participation

Reason	n (couples)
Not interested	7
No response to the invitation	5
Unwillingness to participate in an interview	5
Unwillingness to look back at the decision made to conceive without testing (with or without unsuccessful PGD attempt in the past)	5
Divorce	2
Lack of time	1

n, number of couples; PGD, preimplantation genetic diagnosis

Procedure

A semi-structured topic guide was developed to direct both the focus group and the dyadic interviews, focussing on perception of the (dis)advantages of PGD and PD and the most decisive reasons for making the final reproductive decision. The topic guide was pretested in a personal interview, which was included in the analyses since no adjustments were made following. Focus groups were conducted containing two to four couples (n = four to eight persons), assembled based on the reproductive method the couple had chosen and subsequently used after counseling (PGD or conception without testing) in order to avoid disconcerting discussions within groups. All participants who were assembled in a focus group were offered a dyadic interview if they preferred this but none made use of this alternative. Focus group interviews are an effective qualitative research method to explore and clarify individuals' experiences, perceptions, and beliefs concerning a certain topic.²⁵ Couples who had chosen PD for HBOC were scheduled in dyadic interviews (i.e., an interview including both partners). This was done because of the delicate character of the subject and to avoid participants being confronted with couples who had experienced different pregnancy outcomes after PD. Focus groups were held at geographically convenient

and neutral locations throughout the Netherlands, whereas dyadic interviews were held at the couples' homes. During the focus groups, the moderator, trained by an expert on (group) interviewing techniques, was accompanied by an assistant who took observational notes. Interviews were conducted between January and September 2012 and lasted between 80 and 100 min. Before initiation of the interviews, participants completed a questionnaire on demographic parameters, personal reproductive and oncologic history, and family history.

Data preparation and analysis

All interviews were audio-taped and transcribed verbatim. Data analysis was performed using the software program Nvivo 9.0. Grounded theory approach was used allowing codes, concepts and categories to emerge from the data.²⁶ Open coding of the data was followed by axial coding, organizing the data into segments based on keywords and concepts to form categories and identify major themes. For reliability reasons, data were coded by two independent researchers with consultation of a third independent researcher in case of discordance. Since no new major themes emerged in the final interviews, saturation of themes was suggested.

Ethical approval

The procedures were approved by the local medical ethics committee of Maastricht UMC+.

Results

Couples' characteristics

Of the 47 invited couples, 22 were willing to participate. The overall response rate was 46.8%: 39.1% for PGD, 66.7% for PD, and 50.0% for non-users. Four willing couples were not interviewed because saturation of themes had been achieved. Thus, eighteen couples participated in the interviews (17 males and 18 females). One female partner of a male mutation carrier participated alone since her partner found the topic too difficult to discuss. This personal interview acted as a pre-test, but did not substantially deviate from the dyadic interviews. Other reasons for non-participation are summarized in Table 2.1.

Four focus groups were conducted, two among couples who decided to use PGD (three and two couples, respectively) and two among couples who decided not to use PGD or PD (three and four couples, respectively). Furthermore, five dyadic and one personal interviews were conducted; four dyadic interviews were among couples who

opted for PD for HBOC (of whom one couple had initially chosen PGD but converted to PD after an unexpected natural conception, and one couple who converted their choice to PGD after a TOP), one was with a PGD couple (dyadic interview because of logistic reasons) and there was the aforementioned pre-test (personal interview) with the female partner of a couple who chose no testing (Figure 2.1). Counseling took place between six months and four years prior to the interviews and although all couples had made a reproductive decision, participants were in different stages of enactment of their reproductive decision at the time of the interview (Figure 2.1). The couples' characteristics are summarized in Tables 2.2 and 2.3.

Table 2.2 Couples' characteristics

	Reproductive choice (initial use)		
	PGD (n = 6)	PD (n = 4)	No testing (n = 8 ^a)
Partner at risk (M/F)	3/3	1/3	2/6
History of breast cancer ^b (M/F)	0/1	0/0	0/1
Gene mutation			
<i>BRCA1</i> (M/F)	1/1	0/3	2/4
<i>BRCA2</i> (M/F)	2/2	1/0	0/2
Mean age (years) at time of the interview (SD)			
Male	33.5 (3.3)	33.5 (4.5)	32.9 (5.6)
Female	31.8 (2.2)	32.3 (1.9)	31.6 (2.3)
Education			
Education middle (M/F)	2/0	1/1	3/1
Education high (M/F)	4/6	3/3	4/7
Religious (Christianity) (M/F)	1/3	0/0	1/2
Not religious (M/F)	5/3	4/4	6/6
Time interval (months) between counseling and interview (SD)	22 (18.6)	33 (18.4)	31 (9.8)

PGD, preimplantation genetic diagnosis; PD, prenatal diagnosis; n, number of couples; M, male; F, female; *BRCA1*, breast cancer gene 1; *BRCA2*, breast cancer gene 2; SD, standard deviation

^a For one of these couples, only the female partner participated in the interview

^b Both women were treated for breast cancer before PGD counseling

General results

All participants but one indicated that they wanted a child biologically related to both partners. Reproductive decisions such as remaining childless, adoption, or use of donor gametes were only considered briefly, if at all. Most couples saw PGD and conception without testing as the only reproductive options. A minority of couples considered PD as a third option; all these couples ultimately decided to use PD. Before PGD counseling, the majority of couples, including those refraining from PGD, indicated that they intended to opt for PGD.

Figure 2.1 Couples' decisional process from PGD counseling until the interview
Dotted arrow = change of reproductive choice
n, number of couples; PGD, preimplantation genetic diagnosis; PD, prenatal diagnosis;
TOP, termination of pregnancy

There was a large overlap in motives and considerations to opt for or refrain from PGD mentioned by the participants who decided in favor of PGD and those who opted for PD or conception without testing. All three categories of couples mentioned a small number of important advantages and a larger number of less important disadvantages of PGD. Motives and considerations in the reproductive choices could be classified as physical, psychological, social, ethical/moral, and practical (Table 2.4). In the results, we distinguish moral from ethical considerations, by defining moral considerations as individual internal principles regarding a person's ideals and right or wrong conduct, and by defining ethical considerations as social or external rules of conduct in respect to human actions.²⁷

Table 2.3 Couples' reproductive history at the time of interview

Reproductive history	Couples (n = 18)	Couple codes	Time interval (months) counseling - interview
Before reproductive counseling			
Infertility (IVF/ICSI indication)	3	3-7-14	3-38-38
≥1 child(ren) without testing	3	1-5-8	8-41-30
≥1 miscarriage(s)	1	1	8
After reproductive counselling			
Preparation phase PGD	3	1-2-3	8-6-3
Experience PGD			
1 PGD attempt, 0 live births	1	17 ^a	26
2 PGD attempts, 1 live birth	1	12	42
2 PGD attempts, 1 live birth, 1 ongoing pregnancy	1	16	44
3 PGD attempts, 0 live births	1	11	27
Preparation phase PD (trying to conceive)	1	13	20
Experience PD			
1 PD attempt, 1 live birth of unaffected female	1	14	38
1 PD attempt, 1 TOP of affected female	1	17 ^a	26
2 PD attempts, 2 live births of males	1	15	39
Preparation phase no testing (trying to conceive)	3	4-7-10	16-38-18
Experience no testing			
≥1 child(ren) without testing	5	5-6-8-9-18	41-36-30-28-41

n, number of couples; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection; PGD, preimplantation genetic diagnosis; PD, prenatal diagnosis; TOP, termination of pregnancy

^a Couple converted their choice from PD to PGD (after TOP)

Motives and considerations to opt for PGD

The most frequently mentioned motive in considering PGD was, in all categories of couples, protecting the future child from the *BRCA* mutation. In this context, the majority of couples primarily indicated they wanted to protect their child from the physical and psychological impact of the *BRCA* mutation, i.e., the risk of cancer and the quandary of whether or not to opt for genetic testing, prophylactic surgeries, and/or reproductive options. One father said: "For me that was the most important thing. I don't want to burden my child with a little time-delayed bomb." (C6, conception without testing). Often, female participants illustrated their comments with personal experiences: "My mother died because of cancer, I am a mutation carrier myself. My breasts are removed... Therefore, I don't want my child to experience the same things that I did." (C17, PD). Some women specifically pointed out that radical surgery should not be classified as a good preventive measure for breast and/or ovarian cancer and that they felt a strong need to protect a potential daughter from this choice between two evils: "They say that nowadays there are good preventive measures. Well, if you classify this as a good preventive measure... when you, as a 27 or 28 year old woman, have to let them amputate your breasts... This I think, you cannot classify as a good measure, that's just nonsense." (C12, PGD).

Table 2.4 Motives considered regarding PGD, PD, and no testing for HBOC

<i>Preimplantation genetic diagnosis – Prenatal diagnosis – No testing</i>		
	Motives to choose (n)	Motives to refrain (n)
Physical	Protecting the child from mutation (13) Protecting potential daughter from radical prophylactic surgeries (7) Additional medical check-ups woman (3)	Potential influence of ovarian stimulation on cancer risks (10) Potential effects on child's health due to biopsy in embryonic stage (9) Physical strain of IVF treatment (5)
Psychological	Avoidance of feelings of guilt towards child (15) <u>Avoidance of TOP (7)</u> Reassurance from beginning of pregnancy (6) Preventing mutation in both males and females (3) <u>Avoidance of stress and tension associated with PD (1)</u> Participation in a remarkable process (1) Reassuring feeling of simulating nature by selecting the highest quality embryo (1) Preservation of romance and control regarding pregnancy (14) <u>Faith in future medical developments regarding HBOC (10)</u>	Loss of romance and control regarding pregnancy (14) Psychological strain emerging from success-related uncertainties during trajectory (11) Dilemma in case of unsuccessfulness (8) Tired of medical procedures regarding BRCA (6) Inevitability of involving direct environment (6) Despite complex procedure, no guarantee for a healthy child (5) Emotional influence of hormone injections (4) Necessity of IVF when normally fertile (4) Fear of disappointment (3) Potential impact on relationship (3) Male mutation carrier's feelings of guilt towards partner undergoing procedure (3) Daily reminder of the seriousness of the predisposition during treatment (1) Fear of negative reactions from environment (5)
Social	Wiping out mutation in family line (12) Protecting child from reproductive dilemma (8) Pioneering for (younger) family members (1) <u>Confidence in capability to guide/support child with mutation through personal experiences (4)</u>	
Moral/ethical	Moral duty to protect the child (9) <u>Nature of condition (i.e. late onset, incomplete penetrance, preventive possibilities) (9)</u>	Nature of condition (i.e. late onset, incomplete penetrance, preventive possibilities) (9) Disposal of affected (male) embryos (7) Interference in a natural process/playing for God (4) Treatment was or could not be considered for previous child(ren) (2)
Practical	PGD only minor addition in case of IVF or ICSI indication (4) Good accessibility and reimbursement of treatment (3) Relatively high chance of success (8)	Relatively low chance of successful pregnancy (14) Frequent hospital appointments (13) Relatively long duration of trajectory (8) Difficult integration in timely planning of prophylactic surgeries (5) Desire for (large) family less achievable (3) Necessity to collect blood from near family members (2)

PGD, preimplantation genetic diagnosis; PD, prenatal diagnosis; HBOC, hereditary breast and ovarian cancer; n, number of couples that considered this motive (non-correlated to decisiveness); IVF, *in vitro* fertilization; TOP, termination of pregnancy; BRCA, breast cancer gene; ICSI, intracytoplasmic sperm injection

The two female breast cancer survivors emphasized the physical and emotional severity of their disease, e.g.: “What I have been through, that’s just really horrible, yes horrible, you know. I mean my surgeries and all... and the moment you have to undergo the chemotherapy, well, that is something you wish no one ever has to go through.” (C8, conception without testing). A majority of couples expressed the desire to not only protect their own children, but to completely wipe out the *BRCA* mutation in the family line. For instance: “I strongly feel that I want to stop it with me.” (C17, PD).

Half of the couples believed it was their moral duty to protect their future child(ren) from suffering, given the fact that they are aware of the risk and the reproductive options to avoid it: “I couldn’t feel at ease with consciously burdening my child with this.” (C13, PD). Avoidance of feelings of guilt towards future child(ren), accompanied by a fear of immense future regret when choosing the ‘easier way’, was frequently mentioned by couples of all three categories as a motive in favor of PGD or PD: “What I was afraid of myself, or still am actually, are those feelings of guilt. They might not be so relevant now, but in about twenty or thirty years when my child would go for a DNA test... Imagine it will be positive, then I would have to relive this all over again. And then, I would tell myself: it’s your own fault and you could have prevented this...” (C6, conception without testing). Another woman expressed her concern that her son might go through the same reproductive dilemma as she did in case he turns out to be a *BRCA* mutation carrier: “Sometimes I look at my son and think: ‘Will you end up in the same sticky situation with your partner as we did, just because we may have chosen the easy way out?’” (C18, conception without testing).

Motives and considerations to refrain from PGD

Couples in all three categories mentioned many motives to refrain from PGD, which could be subdivided into general motives, *BRCA*-related motives, and motives that are only of relevance to female mutation carriers.

General motives concerned the physical and psychological burden of an IVF treatment, especially for fertile couples: “To me it felt very serious, needing an IVF treatment while we are normally fertile.” (C14, PD). The necessity to convert conception into a medical process and losing the sense of romance and control as a couple were a major drawback. Male mutation carriers expressed feelings of guilt towards their partner: “I would especially regret that I am the source of the evil in this case and you (i.e., the female partner) would have to go through all this hormone misery...” (C5, conception without testing). Couples who already had children before PGD for HBOC became available felt a moral drawback when considering PGD for a next child. Additionally, many couples feared the dilemma of what to do in case PGD would turn out to be unsuccessful. For some couples, especially those who had a desire for a large(r) family, this was a decisive reason to refrain from PGD: “Preferably,

we would like to have two children. But what are the chances that we eventually would get two children through PGD?” (C15, PD). Moreover, almost half of the couples said that ethical motives regarding selection in general had influenced their decision-making process, as well as the disposal of (male) affected embryos. As one participant expressed: “We talked about it a lot, and then I slowly began to realize that there would also be embryos which will be, well, discarded. And although they are affected, they are still embryos and therefore children, if you look at it that way. I’ve never been able to shake that off...” (C18, conception without testing). Additionally, practical issues like the relatively low chance of pregnancy, the frequent hospital appointments, the need to involve family members for the genetic preparation, and the long duration of the PGD trajectory played a role.

Whereas half of the couples indicated that the (very) high perceived severity of HBOC was an important reason to opt for PGD or PD, the other half stated that they had taken the nature of the condition into consideration and decided not to interfere in the reproductive process. One female non-carrier said: “We went thinking... What if? It’s fifty-fifty... Maybe it’s a boy, that would be positive. If it’s a girl, she only has a 50% chance of being a mutation carrier. Well, in case she inherits the mutation, there is a chance she won’t fall ill at all. And if she does, there may be good therapeutic options. That was our consideration, and we keep reminding ourselves of that.” (C18, conception without testing). While half of the couples felt moral drawbacks from selection in general, a substantial portion of the remaining couples had difficulties with accepting methods such as PGD and PD for HBOC because of the reduced penetrance and late onset character of the condition and the preventive and therapeutic options available. Not all female mutation carriers experienced their genetic predisposition as a burden to the extent that they wanted to prevent transmission of their mutation by means of PGD or PD: “It’s not like it makes you unhappy or something like that.” (C6, conception without testing), and “The amputation of my breasts you know, it all sounds very intense but I am not really that upset about it.” (C10, conception without testing). Other mentioned *BRCA*-related motives to refrain from PGD were the fact that using PGD would not guarantee a child free of breast and ovarian cancer due to the non-genetic background risk, confidence in being able to guide and support a child in case he/she inherits the mutation, and faith in future medical developments. As one father said: “It makes you start thinking... Imagine you would have a girl, yet another thirty years along the road medical science will look completely different. Who knows if they don’t have a vaccine for breast cancer by then?” (C7, conception without testing).

For female mutation carriers uncertainty regarding a potential influence of ovarian stimulation on the cancer risk was a very important aspect: “That’s actually your biggest concern, right? That you bring a child into this world and then you fall ill yourself, due to the hormones...” (C2, female breast cancer survivor, PGD). In addition to this, most female mutation carriers were very aware of the fact that their time

window was limited due to the need for prophylactic surgeries: “If afterwards you still need preventive breast surgery and subsequently your ovaries have to be removed... and you don’t want to do all that on the same day... So then you start to calculate and eventually we became aware of the fact that maybe we should already be initiating the PGD trajectory while we were not even that occupied with the matter of having children yet.” (C12, PGD). Moreover, the necessity of medical interference once more, next to all procedures female mutation carriers had gone through already, was mentioned as a disadvantage of PGD.

PGD versus PD

A minority of seven couples stated they would not opt for PD because of religious and/or ethical objections against TOP in general. Eleven couples, however, had made a personal reflection on the acceptability of TOP for HBOC. All six couples who opted for PGD clearly indicated perceiving a moral difference between embryo selection and TOP specifying that termination is a too drastic measure to avoid HBOC: “It depends on the consideration of selection which I think is still okay. But when taking my own life as an example, terminating a pregnancy is simply not justified.” (C12, PGD).

The four couples who found PD for HBOC acceptable in fact chose this method. All four couples indicated that for them PGD was the most ideal option from a moral point of view as well. However, the relatively low chance of pregnancy by PGD, mostly in combination with the duration of the trajectory, directed their choice to PD. Furthermore, they appreciated the possibility of conceiving naturally without medical intervention: “Getting pregnant this way is a natural process like it is for other couples. You know, I have had my breast surgery and one day I will have to remove my ovaries... Sometimes you just want to be normal.” (C17, PD, TOP affected girl). The PD couples all judged the 75% chance of a good outcome as fairly high. When explicitly discussing the possibility of conceiving an affected girl and the necessity of TOP, the couples said they felt prepared and had confidence in standing by their choice. One couple said: “Termination of pregnancy in case of an affected girl would obviously be a massive burden for us. However, I would prefer that instead of having to tell my daughter she might be a mutation carrier.” (C13, planning to use PD after conception). However, the only PD couple who experienced TOP because of HBOC converted to PGD for their second attempt to fulfil their child wish, indicating that in spite of having no regrets about this first endeavour, they could not emotionally cope with another TOP. They additionally specified that after this experience, the disadvantages of PGD had diminished in their perception.

Other advantages of PD compared with PGD mentioned by the PD couples were the absence of the need to inform others about their attempts to conceive, which for PGD is necessary given the genetic preparations involving family members, and the possibility to control their own planning. The couples who already experienced PD

perceived the two consecutive diagnostic steps as beneficial, like they had two chances to receive a good result: “The possibility of the sex determination in blood was a kind of a trigger for us... That could prevent us from the necessity of chorionic villus sampling, at only eight or nine weeks of pregnancy. At that point we would already know what sex we would be dealing with.” (C15, PD, two sons). Another couple said: “It just became really burdensome when we found out it was a girl. We did not expect that at all. (...) That tough decision suddenly became much more imminent and I was really concerned by that. But well, we still had a 50% chance...” (C14, PD, one unaffected girl).

All four PD couples took the fact that PD did not prevent HBOC in males into consideration in their decision-making. One couple initially had difficulties with the impossibility to avoid male mutation carriers by PD: “At first we struggled with the fact that in case of a boy no additional diagnostics would be carried out. We preferred a child without *BRCA* mutation, to put an end to this... But since termination of pregnancy is such a drastic measure we felt at ease to do it this way.” (C17, PD, TOP affected girl). Besides the risk of TOP, the weeks of uncertainty when waiting for the PD results were mentioned as a major disadvantage of PD. One male said: “You only know after several weeks, it takes so long... For me that is the most prominent disadvantage.” (C13, PD, trying to conceive). The same couple regretted the fact that their chance of having a girl was no longer 50/50 but dropped to 1/3, since both boys and girls have a 50% risk of carrying the *BRCA* mutation but only a girl with the mutation will be medically aborted in the Netherlands.

Emotional impact of reproductive decision-making

None of the couples regretted the choice they made. However, several couples said that becoming parents had changed their perspectives on pregnancy and parenthood. One woman who underwent PD said: “Only since I was pregnant myself I can really estimate the value of a pregnancy. Before that time I could not have imagined. I simply thought ‘if it’s not okay we’ll terminate and try again.’” (C14, unaffected girl after PD). At the time of the interview, this couple had the intention to re-use PD for a second child. However, a few months later the couple was pregnant and informed the researcher that they had decided to continue this pregnancy of a second daughter without invasive diagnostics for HBOC. They did not feel capable of terminating the pregnancy in case of an unfavorable result and were confident in being able to guide and support a daughter with HBOC.

The couples who chose for PGD did not regret that choice, but indicated that although they had prepared themselves for the physical burden and the practical impact of the treatment, they had been unable to anticipate on the psychological strains: “The waiting during the actual treatment... during those two weeks of hormonal stimulation, if you are even able to manage that, until the moment of embryo transfer

and the pregnancy test two weeks later. The tension... I never could have prepared myself for that.” (C12). In addition, in some cases, the IVF/PGD treatment had had more impact on their spousal relationship than previously imagined. A male said: “There were many moments when you (i.e., the partner) were troubled and you couldn’t really express yourself or I didn’t really understand and then I could clearly feel the tension between us.” (C11). During the treatment, the dilemma of how to proceed in case PGD would not succeed eventually arose. Since many couples perceived PGD as the most ideal option, they feared that the choice between remaining childless and choosing another option which might not (completely) protect the child from HBOC would cause an emotional load: “It’s a real drawback that once you have completed the trajectory it might not have been successful. What are you going to do then? Are you still going for the natural way? Well, that will obviously cause an emotional burden.” (C11). Couples agreed that using PGD to conceive a first child made it ethically difficult to make a different choice for a second child. However, when PGD was not successful for any child, the conversion to conception without testing seemed to be much easier to make: “Our desire to become parents has only increased since our PGD experience. In case PGD remains unsuccessful, we will try to conceive the natural way. Ultimately, we have done whatever was possible. That was very important for us.” (C11).

The PD couples felt at ease with the decisions made. Two out of three couples experiencing PD said they had been unsure about the extent to which they should involve their social network in the procedure; they needed support, but feared disturbing advice and social judgements: “At that point, you don’t want to hear any arguments in favor of a different decision. You only want to hear that your decision is the only right one to make.” (C17). Some couples experienced difficulties in explaining their choice to their social surrounding: “It is much easier to explain your choice for PGD to your social network than your choice to terminate the pregnancy in case of an unfavorable outcome. (...) It felt like we were among the very few who make a decision like this.” (C15).

Half of the couples who had chosen for conception without testing expressed their doubts about the moral justness of their decision, even when the decision was made a few years ago and the couple had completed their family in the meantime: “And now you do hope that she doesn’t have it. That is something you start to think about... We did make the right choice, didn’t we?” (C9) and “But still, if it turns out that my second daughter would have it, while I did have this choice for her... I think I would go to pieces at that moment. I would always keep thinking; what if I had..., I wish I had, maybe...” (C8). Several couples emphasized that the reproductive decision-making process they went through still had a major impact on their lives: “It is só hard not to know whether we have made the right choice, I really can’t say... But I still dwell on that on a daily basis.” (C18). Many of these couples said they felt uncomfortable when confronted with the decision made. This is confirmed by the fact that unwillingness to

look back at the decision made was one of the main reasons not to participate in the study among non-users.

Discussion

This study provides a qualitative assessment of the motives and considerations that well-informed couples carrying a *BRCA1/2* mutation take into account when deciding on PGD and PD. Perceived (dis)advantages and reasons to opt for or refrain from these reproductive methods were explored and satisfaction with the choice made was assessed during semi-structured (focus group) interviews.

The most important factor taken into account was the perceived severity of HBOC, which was generally based on personal and familial experience with cancer and sacrifices to be made for preventive measures. Half of the couples perceived that living with HBOC was serious enough to outweigh disadvantages of PGD and/or PD; the others did not. All couples who opted for PGD clearly indicated perceiving a moral difference between embryo selection and the termination of a pregnancy, specifying that they considered PD as a too drastic measure to avoid HBOC. In contrast, all couples who found PD for HBOC acceptable actually chose this option, despite the fact that all these couples had a preference for PGD from a moral point of view. Some significant practical and psychological aspects directed their final choice towards PD, showing that the possibility of avoiding the risk of TOP by choosing PGD could not outweigh the negative aspects. This corresponds with findings from previous studies.^{28,29} Several previous studies indicated that experience with TOP for a genetic disorder influences the acceptance of PGD, in particular for women.^{29,30} This was also the case for the interviewed couple that experienced TOP after PD and subsequently opted for PGD, indicating that they did not want to terminate another pregnancy.

The same motives and considerations played a role for couples opting for PGD and couples refraining from PGD. The PGD couples mentioned numerous negative aspects of PGD, but indicated that the main advantage, 'preventing transmission of the *BRCA* mutation, both for their own child as well as future generations', outweighed the accumulated disadvantages. In the previous literature this advantage is usually separated from the benefit of protecting the child from possible physical and mental suffering.^{13,20,22,31,32} The majority of motives to refrain from PGD, such as limited success rates, duration of the trajectory, procedural and human risks and safety, correspond to those reflected in previous studies.^{13,31} Moreover, in the specific context of HBOC, we found in concurrence with Dekeuwer and Bateman²⁰ that female *BRCA* mutation carriers worry about the unknown influence of hormonal stimulation needed for IVF on their breast cancer risk. Several studies suggest an association,

although inconsistent, between IVF medication and an increased breast cancer risk in both the general female population³³⁻³⁵ and in women with HBOC.³⁶⁻³⁹

Women carrying a *BRCA* mutation have to cope with many decisions and life events in a short period of time (i.e., DNA testing, coping with an unfavorable test result, decision-making on possible medical interventions as well as reproductive decision-making). Since female *BRCA* mutation carriers are generally advised to undergo a risk-reducing salpingo-oophorectomy from their mid-thirties, the timeframe in which they can have offspring is tight. As a result, female carriers may feel forced to cope with complex reproductive issues at a (much) younger age than they might have wanted to.^{16,17,20,40}

Several couples who eventually decided in favor of conception without testing for *BRCA* expressed feelings of doubt or even guilt afterwards, and feared the moment if it turns out that their child(ren) have inherited the *BRCA* mutation. These feelings are not uncommon among parents with a genetic susceptibility.^{41,42} The mere possibility of PGD and PD can cause an emotional burden once people become aware and choose to refrain from it, known as the technological imperative. This aspect should not be neglected in reproductive counseling. Couples choosing for a natural pregnancy without testing might be as much in need for emotional support during and even after this trajectory, as PGD or PD users may be. This group must not be forgotten.

Study strengths and limitations

This is the first study on motives and considerations regarding PGD and PD use in well-informed *BRCA1/2* mutation carrying couples of reproductive age. A substantial diversity of responses was attained by including PGD users, PD users, and non-users, male and female asymptomatic mutation carriers, as well as female breast cancer survivors, and their partners. Interviews were assembled according to the reproductive option chosen, in order to guarantee a safe environment in which one could express and discuss feelings and opinions openly and without judgement. We believe it therefore gives a rich and in-depth overview of reproductive motives.

All couples had made a reproductive decision, but the fact that the couples were at different stages in their chosen reproductive trajectory may have led to a coloured perception of experiences and outcomes, as well as recall-bias of motives and considerations. Our design excluded couples who did not actively seek information about PGD, or *a priori* decided to refrain from having their own, genetically related, children.

Conclusions and recommendations

Reproductive decision-making regarding PGD and PD has proven to be a very complex and stressful process for couples with HBOC. We found that the process was mainly guided by the couples' perceived seriousness of the predisposition as well as their moral views regarding selection. The safety of IVF and the compatibility of the PGD planning process with prophylactic surgeries were essential factors for female mutation carriers. For some couples, the emotional impact of the decision was substantial and long-lasting. Non-users could be confronted with feelings of doubt or guilt up to years after the decision has been made.

Reproductive counseling requires highly skilled professionals who are able to guide couples in a challenging process of reconciliation with a wide variety of moral considerations and emotions regarding their reproductive wishes. Knowledge of the condition-specific reproductive motives may motivate the adaptation of current best practice guidelines by means of further tailoring of counseling practices, e.g., by providing additional decision support in the form of a patient decision aid.⁴³ Such a decision aid should be offered complementary to counseling.

In addition, the emotional burden which is experienced by non-users after they have made their decision to refrain requires more attention. Emotional support, during the decision-making process as well as afterwards, should be actively offered to all couples, including those refraining from PGD and PD. Further research regarding the longterm consequences of the reproductive decision on emotional well-being is required.

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Chapter 3

Hereditary breast and ovarian cancer and reproduction: an observational study on the suitability of preimplantation genetic diagnosis for both asymptomatic carriers and breast cancer survivors

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Abstract

Preimplantation genetic diagnosis (PGD) is a reproductive option for *BRCA1/2* mutation carriers wishing to avoid transmission of the predisposition for hereditary breast and ovarian cancer (HBOC) syndrome to their offspring. Embryos obtained by *in vitro* fertilization with intracytoplasmic sperm injection (IVF/ICSI) are tested for the presence of the mutation. Only *BRCA* mutation negative embryos are transferred into the uterus. The suitability and outcome of PGD for HBOC are evaluated in an observational cohort study on treatments carried out in two of Western Europe's largest PGD centers from 2006 until 2012. Male mutation carriers, asymptomatic female mutation carriers and breast cancer survivors were eligible. If available, PGD on embryos cryopreserved before chemotherapy was possible. Generic PGD polymerase chain reaction (PCR) tests were developed based on haplotyping, if necessary combined with mutation detection. Seventy couples underwent PGD for *BRCA1/2*. 42/71 carriers (59.2%) were female, six (14.3%) of whom have had breast cancer prior to PGD. In total, 145 PGD cycles were performed. 720 embryos were tested, identifying 294 (40.8%) as *BRCA* mutation negative. Of fresh IVF/PGD cycles, 23.9% resulted in a clinical pregnancy. Three cycles involved PGD on embryos cryopreserved before chemotherapy; two of these women delivered a healthy child. Overall, 38 children were liveborn. Two *BRCA1* mutation carriers were diagnosed with breast cancer shortly after PGD treatment, despite negative screening prior to PGD. PGD for HBOC proved to be suitable, yielding good pregnancy rates for asymptomatic mutation carriers as well as breast cancer survivors. Because of two cases of breast cancer shortly after treatment, maternal safety of IVF (with or without PGD) in female mutation carriers needs further evaluation.

Purpose

Hereditary breast and ovarian cancer (HBOC) syndrome is an autosomal dominant cancer predisposition syndrome caused by mutations in tumor suppressor genes Breast Cancer 1 (*BRCA1*, 17q21.31, MIM 113705) or Breast Cancer 2 (*BRCA2*, 13q13.1, MIM 600185). Female mutation carriers have strongly increased risks for both breast and ovarian cancer, estimated at 57 and 49% for breast cancer and 40 and 18% for ovarian cancer for *BRCA1* and *BRCA2*, respectively, at the age of 70.¹ In comparison, women in the United Kingdom in general have a 12.5% lifetime risk for invasive breast cancer and 1.9% for invasive ovarian cancer.^{2,3} The prevalence of germline *BRCA1/2* mutations is estimated at 0.1–1.0% in the general population, making HBOC one of the more prevalent autosomal dominant genetic disorders.^{4,5}

Carriers of a *BRCA1/2* mutation have a 50% risk of passing this predisposition to their offspring. There are several reproductive options to circumvent this, but only two lead to a child genetically related to both partners: prenatal diagnosis and preimplantation genetic diagnosis (PGD). Prenatal diagnosis on the one hand involves genetic testing of a fetus for the presence of a familial *BRCA1/2* mutation during pregnancy, followed by pregnancy termination in case of an unfavorable result. Although applied on a small scale, reports regarding clinical experience with prenatal diagnosis for HBOC are not available in the literature to date. PGD on the other hand involves *in vitro* fertilization (IVF) with intracytoplasmic sperm injection (ICSI), followed by genetic testing of the embryos for the presence of a familial *BRCA1/2* mutation before intrauterine transfer. PGD has been successfully applied since 1990 for an expanding list of monogenic disorders and chromosomal abnormalities.⁶ In 2003, the Ethics Taskforce of the European Society of Human Reproduction and Embryology stated that it is acceptable to perform PGD for late onset and multifactorial diseases, including HBOC.⁷

In 2005, a survey amongst *BRCA1/2* mutation carriers was carried out to investigate the public attitude towards PGD for HBOC, an important step in the legalisation of PGD for hereditary cancer syndromes in the United Kingdom.^{8,9} This study and other opinion surveys have shown that most *BRCA* mutation carriers consider PGD for HBOC as an acceptable reproductive option, although only a minority of them would consider using PGD personally.^{9,10} However, appliance of both prenatal diagnosis as well as PGD for HBOC remains controversial, considering the reduced penetrance of the condition, its late onset, and availability of prophylactic and therapeutic options.¹¹ Previous research has shown that safety of ovarian stimulation for IVF is an important consideration for female *BRCA* mutation carriers when deciding on PGD.¹² This topic has not extensively been studied in female *BRCA1/2* mutation carriers to date, although one case–control study did not find a significant adverse effect on the incidence of breast cancer.¹³ Up to now, some case reports and small case series have

been reported on the clinical experience with PGD for HBOC, with Jasper and colleagues as the first to report a pregnancy in 2008.¹⁴⁻¹⁸

In 2006, PGD for HBOC was started at the Universitair Ziekenhuis Brussel, Brussels, Belgium (hereafter named center A) and in 2008 at Maastricht University Medical Center, Maastricht, the Netherlands (hereafter center B), two large centers for PGD in Western Europe.¹⁹ In this study, we aim to determine the suitability of this treatment, for both asymptomatic male and female *BRCA1/2* mutation carriers as well as *BRCA* mutation positive female breast cancer survivors, in terms of genetic results, pregnancy rates, and successful deliveries. Additionally, we report on cancer outcome of female mutation carriers.

Methods

Patients

Observational cohort study on PGD cycles performed for *BRCA1/2* mutations from the onset in 2006 until 1-1-2012. Couples of whom at least one partner was known to have a *BRCA1/2* mutation were referred for PGD counseling to our centers. We provided them with verbal and written information regarding the PGD procedure (including IVF and ICSI, embryo biopsy, single cell analysis, chance of pregnancy, and risk of misdiagnosis). We considered female age >40 years and female body mass index (BMI) >30 kg/m² as relative contra-indications for PGD, whereas female age >43 years and female BMI >35 kg/m² were absolute contra-indications.

Gynecological screening procedures

We performed gynecological and andrological examination, including sperm analysis, female hormonal assessment, and virology tests of both partners to ensure suitability of the couple for IVF/ICSI treatment. In cases where embryos were harvested by IVF/ICSI prior to chemotherapy, appliance of PGD on these cryopreserved embryos was possible.

Oncological screening procedures

We screened female mutation carriers without a bilateral prophylactic mastectomy in the past for the presence of occult breast cancer before admission to the PGD program. In addition to annual screening procedures, at least a magnetic resonance imaging (MRI) of the breasts was performed prior to the start of PGD.^{20,21} *BRCA* mutation positive women with a history of breast cancer were eligible for PGD, if they had been free of malignant disease for at least two years after their oncological

treatment. Depending on age and familial phenotype, we screened female mutation carriers for the presence of occult ovarian carcinoma prior to admission to the PGD program by gynecological and ultrasound examination and CA-125 determination in blood.

PGD procedures

Prior to the introduction of the PGD program, we obtained medical ethical approval of the institutional review boards at both centers. All couples gave their informed consent before PGD was started. We performed IVF and PGD according to international guidelines^{22,23} and used ICSI for fertilization to avoid contamination of the zona pellucida with spermatozoa, which may disturb the PGD analysis. We biopsied obtained embryos three days after fertilization. Single cell analysis of the blastomeres was performed using polymerase chain reaction (PCR), based on haplotyping of at least two informative flanking microsatellite markers on each side of the *BRCA1/2* loci. In a minority of cases, this generic test was not informative. In these cases we set up a mutation-specific protocol, based on identifying the private mutation in combination with at least one informative marker (Table 3.1).^{15,24}

Table 3.1 PGD strategies for *BRCA1/2* mutations

	Center A ^a	Center B ^b
Indirect testing <i>BRCA1</i> mutations ^c	BRCA1STR24CA, BRCA1STR20TG, BRCA1STR16GA, BRCA1STR4, BRCA1STR21CA, D17S2249, D17S1323, D17S855	D17S932, BRCA1_dis24AC, D17S950, D17S1814, D17S800, D17S1787
Indirect testing <i>BRCA2</i> mutations ^c	BRCA2STR19TG, BRCA2STR20GT, BRCA2STR18AC, D13S260, D13S171	D13S171, D13S1695, BRCA2_dist18AC, D13S267, D13S289, D13S260, D13S1698, BRCA2STR19
Alkaline lysis buffer	50 mM DTT, 200 mM NaOH	50 mM DTT, 200 mM NaOH
Freezing post tubing	30' -20°	30' -20°C
Decontamination	UV-C	UV-C
Polymerase	Qiagen Multiplex PCR KIT	Qiagen Multiplex PCR KIT
Split for multiplex PCR	No	No
Genetic analyser	ABI3730xl	ABI3730xl

PGD, preimplantation genetic diagnosis; *BRCA1*, breast cancer gene 1; *BRCA2*, breast cancer gene 2; PCR, polymerase chain reaction

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^c Many of the markers have not been published; the primer sequences were designed in-house and are available upon request. In case indirect testing was not possible due to either non-informativity of markers or availability of family members, mutation-specific tests were developed including the typical familial mutation combined with at least two markers

After single cell analysis, we classified the embryos as affected (*BRCA1/2* mutation present), unaffected (*BRCA1/2* mutation absent), abnormal (abnormal genotype, e.g., haploidy or triploidy), or no diagnosis (no test result or inconclusive *BRCA1/2* status). Subsequently, one or two unaffected embryos were transferred into the uterus at day four or five post-fertilization. The number of transferred embryos depended on embryo quality, female age, number of previous unsuccessful PGD attempts, and the couples' preference for transferring only one embryo. Supernumerary unaffected embryos of sufficient quality were cryopreserved and transferred in a subsequent cycle after thawing (defined as 'frozen/thawed embryo transfer cycle, FET').²⁵ FETs were included in the survey, if they followed a fresh IVF/PGD cycle during the study period, and in case the embryo transfer was performed before 1-10-2012.

Pregnancy rates are reported as positive hCG tests as well as clinical pregnancy rates. The clinical pregnancy rate was diagnosed according to the standard definition, i.e., a pregnancy diagnosed by transvaginal ultrasonographic visualisation of one or more gestational sacs or definite clinical signs of pregnancy, including ectopic pregnancy. A delivery was defined as the birth of one or more fetuses after at least twenty completed weeks of gestational age.²⁵

Couples were given the option of prenatal diagnosis to confirm PGD outcome. Follow-up of pregnancies and children was carried out at center A as described earlier²⁶ and at center B using a questionnaire. At the end of the study time (i.e., 1-10-2012), all female *BRCA1/2* mutation carriers were contacted by telephone and asked for their health status, including diagnosis of breast cancer since the last PGD treatment and prophylactic surgeries performed in the meantime.

Ethical statement

This study complies with current laws in The Netherlands and Belgium. Medical ethical approval of the institutional review boards was obtained before the start of PGD at both centers. Participants gave their informed consent before they were enrolled in the program.

Statistical analysis

Statistical analyses were performed using SPSS 18.0.0. Data are presented as mean and standard deviation (for continuous variables) or number of cases and percentages (for categorical variables).

Results

Patients

Seventy couples underwent PGD for HBOC. In one couple, the male and female partner were both a *BRCA1* mutation carrier. Of 71 mutation carriers, 42 were female (59.2%). Of the female mutation carriers, 28 (66.7%) had a *BRCA1* mutation and fourteen (33.3%) had a *BRCA2* mutation. Of 29 male mutation carriers, 21 (72.4%) had a *BRCA1* mutation and eight (27.6%) had a *BRCA2* mutation. Over a quarter of female mutation carriers (11/42, 26.2%) had undergone a bilateral prophylactic mastectomy before PGD. Six out of 42 female mutation carriers (14.3%) had a history of breast cancer (Table 3.2).

Table 3.2 Couples' characteristics

	n = 70	
Nulliparity prior to PGD	60	(85.7%)
At-risk person		
Male	28	(40.0%)
Female	41	(58.6%)
Both partners	1	(1.4%) ^a
Mutation		
<i>BRCA1</i>	48	(68.6%)
<i>BRCA2</i>	22	(31.4%)
Mean female age in years (SD)	29.5	(3.6)
Female mutation carriers (SD)	29.6	(3.7)
Mean female BMI (SD)	23.1	(3.4)
Female mutation carriers with bilateral prophylactic mastectomy	11	(26.2%)
Female mutation carriers with history of breast cancer	6	(14.3%)

n, number of couples; PGD, preimplantation genetic diagnosis; *BRCA1*, breast cancer gene 1; *BRCA2*, breast cancer gene 2; SD, standard deviation; BMI, Body Mass Index (kg/m²)

^a Both partners were *BRCA1* mutation carrier, only unaffected embryos were eligible for embryo transfer

Outcome

In total, 145 PGD cycles were carried out (Table 3.3). Three of these cycles involved PGD on embryos cryopreserved before chemotherapy because of breast cancer. Overall, 720 embryos were tested for a familial *BRCA1/2* mutation, identifying 294 (40.8%) as unaffected, 311 (43.2%) as affected, 70 (9.7%) as abnormal, and 45 (6.3%) as having no diagnosis. In 87 out of 142 fresh IVF/PGD cycles (61.3%), one or two embryos were transferred, resulting in 37 positive hCG tests and 34 clinical pregnancies. Clinical pregnancy rates were 23.9% per cycle started and 39.1% per embryo transfer. Subsequently to these fresh IVF/PGD cycles, 34 FETs were performed, resulting in ten positive hCG tests and nine clinical pregnancies (clinical pregnancy rate 26.5% per embryo transfer, Table 3.3).

Table 3.3 Reproductive outcome of PGD for HBOC (n = 70 couples)

	IVF/PGD cycles	PGD on embryos cryopreserved before chemotherapy
PGD treatments started	142	3
Mean treatments started per couple (SD)	2.1 (1.3)	1
Biopsied embryos	720	
Unaffected	294 (40.8%)	
Affected	311 (43.2%)	
Abnormal	70 (9.7%)	
No diagnosis	45 (6.3%)	
Ovarian stimulations to embryo transfer ^a	87 (61.3%)	n/a
Positive hCG tests	37	n/a
% per oocyte pick-up	30.3%	
% per embryo transfer	42.5%	
Clinical pregnancies	34	
% per oocyte pick-up	27.9%	
% per embryo transfer	39.1%	
FET	34	3
Positive hCG tests after FET	10	2
% per FET after IVF/PGD	29.4%	
% per FET of embryo(s) cryopreserved before chemotherapy		66.7%
Clinical pregnancies after FET	9	2
% per FET after IVF/PGD	26.5%	
% per FET of embryo(s) cryopreserved before chemotherapy		66.7%
Pregnancies (total)		49
Pregnancies ongoing ≥ 12 weeks		36
Lost to follow-up < 12 weeks		1
Deliveries (≥ 20 weeks)		36
Singletons		31
Twins		5 (10 children)
Lost to follow-up ≥ 20 weeks		0

PGD, preimplantation genetic diagnosis; HBOC, hereditary breast and ovarian cancer; n, number of couples; IVF, *in vitro* fertilization; SD, standard deviation; hCG, human chorionic gonadotropin; FET, frozen/thawed embryo transfer cycle; n/a, not applicable

^a One couple, who underwent two IVF/PGD cycles, requested cryopreservation of unaffected embryos because of risk-reducing salpingo-oophorectomy. In five other IVF/PGD cycles unaffected embryos were cryopreserved to postpone embryo transfer for different reasons (ovarian hyperstimulation syndrome (n = 2), insufficient endometrial buildup (n = 2), and delay of PGD results (n = 1))

Three out of six women with a history of breast cancer had harvested embryos prior to chemotherapy and underwent PGD on these embryos. Two of them delivered a healthy child after PGD. Subsequently, two of these three women were denied a fresh ovarian stimulation for PGD because of a diminished ovarian reserve after chemotherapy. The third woman and the three women who did not cryopreserve embryos were treated in one or more fresh IVF/PGD cycles. One of them delivered a healthy child (Table 3.4).

Table 3.4 Characteristics of women with a history of breast cancer before PGD treatment

	Center A ^a		Center B ^b			Patient F
	Patient A ^c	Patient B	Patient C	Patient D	Patient E	
Reproductive history	None	None	None	None	2005 healthy daughter, 2007 miscarriage, 2007 molar pregnancy	None
Gynecological history	None	None	None	2007 unilateral salpingo- oophorectomy (inflammation)	2007 dilatation and curettage	None
Gene mutation	<i>BRCA1</i>	<i>BRCA1</i>	<i>BRCA1</i>	<i>BRCA2</i>	<i>BRCA1</i>	<i>BRCA2</i>
Age at breast cancer diagnosis	31	29	26	33	33	32
Oncological treatment						
Surgery	Mastectomy	Mastectomy	Skin sparing mastectomy	Mastectomy	Modified radical mastectomy	Lumpectomy
Chemotherapy	Yes	Yes	Yes	Yes	Yes	No
Irradiation	No	Yes	Yes	No	Yes	Yes
Contralateral prophylactic mastectomy	No	Yes	Yes	Yes	Yes	No
Embryos cryopreserved before chemotherapy	Yes	No	Yes	Yes	Yes	n/a
Disease free interval before first PGD cycle (years)	2.5	4.0	2.0	3.8	2.8	4.5
PGD on embryos cryopreserved before chemotherapy	No	n/a	Yes	Yes	Yes	n/a
Outcome	n/a	n/a	Healthy son born	Not pregnant	Healthy daughter born	n/a
IVF/PGD after recovery from cancer	Yes	Yes	Yes	No (denied because of chemotherapy induced infertility)	No (denied because of chemotherapy induced infertility)	Yes
Outcome	No embryo transfer	Not pregnant	Ectopic pregnancy	n/a	n/a	Healthy son born

PGD, preimplantation genetic diagnosis; *BRCA1*, breast cancer gene 1; *BRCA2*, breast cancer gene 2; n/a, not applicable; IVF, *in vitro* fertilization.

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^c Same patient as patient G in Table 3.5. This patient was diagnosed with breast cancer and underwent one IVF cycle prior to chemotherapy to cryopreserve embryos (n = 6). After recovery, she chose for a new ovarian stimulation for IVF/PGD instead of using her cryopreserved embryos first. After IVF/PGD, she was diagnosed with contralateral breast cancer and needed chemotherapy again (see Table 3.5). She underwent two IVF cycles to cryopreserve embryos (n = 1). Besides, a laparoscopic unilateral oophorectomy took place to cryopreserve the ovary. After recovery of the second breast cancer, she conceived spontaneously and gave birth to a twin

Table 3.5 Overview of women who were diagnosed with breast cancer after PGD treatment

	Patient G ^a Center A ^b <i>BRCA 1</i>	Patient H Center B ^c <i>BRCA 1</i>
PGD center		
Gene mutation		
Oncological history prior to PGD	Diagnosed with breast cancer in 2006: invasive ductal carcinoma, T2mN0M0, triple negative. Treatment consisted of mastectomy and chemotherapy	None
Reproductive history prior to PGD cycle after which breast cancer was diagnosed	One IVF cycle for fertility preservation prior to chemotherapy in 2006 (6 embryos). It was the patient's choice to undergo a new ovarian stimulation for PGD instead of using the cryopreserved embryos first	None
Age at breast cancer diagnosis after PGD	34	28
Last breast screening before PGD	One month before PGD, MRI: no abnormalities	Two months before PGD, MRI: no abnormalities
Number of IVF/PGD cycles	1	1
Outcome IVF/PGD cycle	No embryo transfer	Not pregnant
Breast cancer diagnosis	Two months after PGD, MRI	Three months after PGD, MRI
Pathology	Invasive ductal carcinoma, T1cN0M0, triple negative	Invasive ductal carcinoma, T1bN1aM0, triple negative
Treatment		
Oncological surgery	Mastectomy, SNP	Lumpectomy, axillary lymph node dissection
Systemic therapy	Yes	Yes
Irradiation	No	Yes
Contralateral prophylactic mastectomy	n/a	Planned
Current status	No evidence of disease, delivered twin after spontaneous conception (see legend Table 3.4)	No evidence of disease, wishes to continue PGD after recovery of prophylactic surgery

PGD, preimplantation genetic diagnosis; *BRCA1*, breast cancer gene 1; IVF, *in vitro* fertilization; MRI, magnetic resonance imaging; SNP, sentinel node procedure; n/a, not applicable

^a Same patient as patient A in Table 3.4

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^c Maastricht University Medical Center, Maastricht, the Netherlands

In center A, four couples pregnant after PGD for *BRCA1* requested prenatal diagnosis to confirm PGD diagnosis. In two cases, a chorionic villus biopsy was performed (one in a twin pregnancy), amniocentesis in the other two. All results were *BRCA* mutation negative, confirming PGD outcome. None of the pregnant couples treated in center B opted for prenatal diagnosis to confirm PGD diagnosis.

Out of a total of 45 clinical pregnancies, 36 (80.0%) proceeded to birth. The other nine resulted in a miscarriage or concerned ectopic pregnancies. Of 41 children (31 singletons and five twins), 38 (92.7%) were born alive. One singleton pregnancy was terminated at 23 weeks of gestation because of multiple congenital malformations based on a de novo chromosomal abnormality (deletion 3q26.2 and duplication 15q11.2). Two other children were stillborn: one member of twins died in utero at 24 weeks of gestation for unknown reasons, the other sibling was born alive at 33 weeks. One member of another twin died in utero due to abruption of the placenta at 35 weeks of gestation. The other sibling was born alive. One singleton pregnancy was complicated by premature labor at 26 weeks of gestation. At the end of study time, at age 2.5 years, the girl born was doing well.

Follow-up of female *BRCA1/2* mutation carriers

Two *BRCA1* mutation carriers (one in each PGD center) were diagnosed with early stage triple negative breast cancer within two, respectively, three months after their first ovarian stimulation for IVF/PGD, despite having a negative breast screening shortly before (Table 3.5). One of them had a history of contralateral breast cancer. Both women did not become pregnant after PGD.

One female mutation carrier did not want to be contacted to check on her medical condition after PGD for personal reasons; all other female mutation carriers were contacted. None of them had been diagnosed with breast cancer after PGD. Mean exposed follow-up time (from ovarian stimulation until end of follow-up or until bilateral prophylactic mastectomy) was 27.5 months (range 2–68 months).

Conclusions

This study establishes the clinical suitability of PGD for *BRCA1/2* mutations in both asymptomatic mutation carriers and *BRCA1/2* mutation positive female breast cancer survivors, either in a fresh IVF/PGD cycle as well as on embryos harvested before chemotherapy. A series of 145 consecutive PGD cycles are presented, the first large series of PGD for HBOC. When compared to the outcome of PGD for autosomal dominant disorders as reported by the European Society of Human Reproduction and Embryology PGD consortium, our clinical pregnancy rates are in line with these data (39.1 versus 26.7% per embryo transfer respectively).⁶ Two factors known to influence

reproductive outcome in *BRCA1/2* mutation carriers were present in our series: on the one hand, the women included in our survey were younger than those reported by the PGD consortium (29.6 versus 34 years), which is a favorable factor for reproductive outcome. On the other hand, it was hypothesised that *BRCA1/2* mutations may unfavorably reduce ovarian reserve due to accumulated DNA damage secondary to inadequate DNA repair.²⁷ In total, 49 pregnancies were established resulting in the birth of 31 singletons and five twins. The observation of two perinatal deaths and one pregnancy termination because of major malformations in our cohort of 41 children is presumed to be an coincidence; PGD is not associated with an increased risk for perinatal deaths or major congenital malformations.²⁶ However, the health of children born after PGD (for HBOC) needs to be subject to further research and longer follow-up.

Analysis of the blastomeres for the presence of the familial *BRCA1/2* mutation reflected the suspected 50/50 distribution of unaffected (41%) versus affected (43%) embryos. Almost 10% of the embryos showed fertilization abnormalities (e.g., haploidy or triploidy), which is not an uncommon finding in preimplantation embryos. We presume that the diagnostic accuracy of PGD analyses based on PCR is high: an earlier study in one of our centers reported a false-negative rate of 0.5% in surplus embryos.²⁸ This is in accordance to the reported misdiagnosis rate of 0.4% in pregnancies established after PGD for monogenic disorders detected by PCR analysis.²⁹ However, since only few of the pregnant couples opted for prenatal diagnosis, definitive confirmation of PGD diagnosis was not possible in the majority of cases. Because genetic testing for adult-onset disorders in childhood is ethically controversial and therefore discouraged,³⁰ postnatal testing after PGD for HBOC was not performed.

In addition to the suitability of PGD for HBOC following a fresh IVF/PGD cycle, we also demonstrated that PGD on embryos harvested prior to chemotherapy is an applicable option: two out of three women treated delivered a healthy child. These results stress the importance of timely counseling regarding fertility preserving options available for young women with breast cancer.^{31,32} This is important not only to retain an option to reproduce in case oncological treatment would cause infertility, but also because PGD can be applied on harvested embryos in case of *BRCA1/2* mutation carrier status. Known *BRCA* mutation status at the moment of fertility preservation is not a prerequisite, provided that ICSI is used for fertilization to keep the possibility of PCR analysis. When cryopreserved embryos of sufficient quality are available, it is preferable to use these first for PGD. This can save the patient a new ovarian stimulation, which may be less successful in case of a diminished ovarian reserve after oncological treatment.

Two women in our cohort were diagnosed with breast cancer after their first IVF/PGD cycle. One of these women had a history of contralateral breast cancer. Both women were carrier of a *BRCA1* mutation. *BRCA1*-associated tumors are characterised by a higher proportion of interval tumors and a younger age and more often an

unfavorable size at diagnosis, when compared with *BRCA2*-associated tumors. Besides, invasive *BRCA1*-associated breast tumors are often high grade and rapidly growing.^{33,34} Whilst a possible linkage between IVF treatment and breast cancer risk has extensively been studied in the general population,³⁵ safety of IVF with regards to the risk for breast cancer has not been systematically studied in female *BRCA1/2* mutation carriers. Gonadotropin use for IVF results in a rise in estrogens. Several observations suggest an influence of (prolonged) exposure to estrogens on incidence of *BRCA1/2*-associated breast cancers, although approximately 80% of *BRCA1*-associated tumors are oestrogen and progesterone receptor negative.³⁶ Kotsopoulos and colleagues conducted a matched case-control study to examine the influence of fertility medications for IVF treatment on breast cancer risk in *BRCA1/2* mutation carriers. They were able to include 26 carriers with a history of gonadotropin use, sixteen of whom were diagnosed with breast cancer (multivariate odds ratio 2.32, 95% CI 0.91–5.95, $p = 0.08$). The sample size of the study may have been too limited however to detect a significant adverse effect of gonadotropin use on breast cancer risk in *BRCA1/2* mutation carriers.¹³ One study reported an association between fertility treatment and an increased risk for breast cancer in women with a positive family history for breast cancer (relative risk 1.4, 95% CI 1.0–1.9),³⁷ whilst others did not find fertility (treatment) and breast cancer to be associated in these women.^{38,39} It is possible, yet unproven that administering gonadotropins may have led to an acceleration in growth of pre-existing, but not yet detectable, tumors in the two affected *BRCA1* mutation carriers in our cohort. Therefore, we stress the importance of screening of the breasts before admission to IVF/PGD treatment, as well as after treatments. Larger studies are needed to elucidate whether our observation is just a coincidental finding in a population with a high *a priori* risk for breast cancer, or whether a causal relationship exists.

This study has some limitations. Firstly, the sample size is relatively small. Secondly, reliability of PGD diagnosis could not be confirmed: it was ethically impossible to test *BRCA* mutation status of the children born after PGD, due to the late onset character of the predisposition and the children's autonomy. Finally, our study was not primarily designed to assess maternal safety of IVF in female mutation carriers.

Recommendations

This survey shows that PGD for HBOC is an established and suitable technique with good reproductive outcome, which should be offered as part of a comprehensive approach to the counseling and treatment of all *BRCA1/2* mutated patients. It is important that medical professionals involved in the care for *BRCA1/2* mutation carriers are aware of this reproductive option, in order to inform patients timely and to refer them, at request, to a specialized PGD center. In case of a newly diagnosed breast cancer in a woman of reproductive age, it is essential to be aware of the

possibility of PGD if and when *BRCA1/2* mutation carriership would turn out. Given the complex medical history of female mutation carriers and our observation of two breast cancer cases after PGD treatment, a multidisciplinary approach is a prerequisite in PGD practice. In addition, oncological screening of female mutation carriers before admission to the treatment as well as careful follow-up is required.

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Chapter 4

PGD for hereditary breast and ovarian cancer: the route to universal tests for *BRCA1* and *BRCA2* mutation carriers

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Abstract

Preimplantation genetic diagnosis (PGD) is a method of testing *in vitro* embryos as an alternative to prenatal diagnosis with possible termination of pregnancy in case of an affected child. Recently, PGD for hereditary breast and ovarian cancer caused by *BRCA1* and *BRCA2* mutations has found its way in specialized labs. We describe the route to universal single-cell PGD tests for carriers of *BRCA1/2* mutations. Originally, mutation-specific protocols with one or two markers were set up and changed when new couples were not informative. This route of changing protocols was finalized after two years with universal tests for both *BRCA1* and *BRCA2* mutation carriers based on haplotyping of, respectively, six (*BRCA1*) and eight (*BRCA2*) microsatellite markers in a multiplex polymerase chain reaction (PCR). Using all protocols, thirty couples had a total of 47 PGD cycles performed. Eight cycles were cancelled upon *in vitro* fertilization (IVF) treatment due to hypostimulation. Of the remaining 39 cycles, a total of 261 embryos were biopsied and a genetic diagnosis was obtained in 244 (93%). In 34 of the 39 cycles (84.6%) an embryo transfer was possible and resulted in eight pregnancies, leading to a fetal heart beat per oocyte pick-up of 20.5% and a fetal heart beat per embryonic transfer of 23.5%. The preparation time and costs for set-up and validation of tests are minimized. The informativity of microsatellite markers used in the universal PGD-PCR tests is based on CEPH and deCODE pedigrees, making the tests applicable in 90% of couples coming from these populations.

Introduction

In the Netherlands, the lifetime risk for women to develop breast cancer is one in eight, leading to a diagnosis of breast cancer in about 12,000 women each year. Breast cancer is thereby the most prevalent form of cancer in women in the Netherlands. In about 5–10% of the total hereditary breast and ovarian cancer (HBOC) patients, the mode of inheritance is autosomal dominant with incomplete penetrance.¹ The majority of this heritability is explained by mutations in genes *BRCA1* (MIM: 113705, Genbank: U14680) and *BRCA2* (MIM: 600185, Genbank: U43746), located on chromosomes 17q21 and 13q12.3, respectively. Both BRCA proteins act as tumor suppressor genes and have, in this role, been shown to function in transcriptional regulation, DNA repair, DNA recombination, and cell-cycle checkpoint control, thereby explaining how heterozygous loss of these genes can contribute to cancer initiation and progression.² Since the discovery of the two *BRCA* genes in 1994³ and 1995,⁴ >1,500 different mutations for the *BRCA1* gene and >1,200 mutations for the *BRCA2* gene have been identified in patients worldwide (Human Gene Mutation Database: www.hgmd.cf.ac.uk). Several founder mutations have been identified in specific ethnic populations as the Ashkenazi Jews⁵ or in specific regions/countries, such as Norway,⁶ Poland,⁷ and China.⁸ Even though in the Netherlands there have been some regional founder mutations,⁹ the majority of mutations detected in *BRCA1* and *BRCA2* genes represent private mutations.

Preimplantation genetic diagnosis (PGD) was introduced into the clinic at the Maastricht University Medical Center (Maastricht UMC+), Maastricht, the Netherlands, in 1995.¹⁰ The introduction of PGD for HBOC resulted in lots of debate and discussion due to the late onset, incomplete penetrance, and availability of preventive and therapeutic options. PGD for HBOC is now permitted in some countries as the UK,¹¹ Israel,¹² Belgium,¹³ and the Netherlands. Due to the variety of mutations carried by the different PGD applicants, mainly mutation specific tests or tests with few markers are described. As we experienced the continuing need for protocol adjustments, we aimed at designing universal PGD protocols for *BRCA1/2* mutation carriers. The methodology applied in these universal protocols is based on genetic linkage, using highly informative microsatellite markers in the close vicinity of the *BRCA1* and *BRCA2* genes that are likely inherited together during meiosis. Using this methodology, the need to incorporate the specific familial mutation is omitted; however, at least two informative family members (meiosis) are needed to definitely determine the 'risk' haplotype. These universal protocols reduce the patients' waiting time as well as set-up and validation costs.

Patients and methods

PGD couples and counseling

Thirty couples applied for a PGD procedure for HBOC between 2009 and 2011. Verbal and written information regarding the procedure, including *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), the risks and complications of IVF, PGD, the risk of misdiagnosis in PGD, the success rate of the treatment, and the health of children born after PGD was provided by a clinical geneticist or PGD physician and/or a gynecologist. The safety of ovarian stimulation for IVF treatment in *BRCA* mutation positive women was discussed and counselors explained that current knowledge does not suggest a significant increased risk for breast cancer in these women.¹⁴ Fifteen couples had a *BRCA1* mutation and fifteen couples a *BRCA2* mutation. In 60% (9/15) of both *BRCA1* and *BRCA2* couples the female carrier was the index case (Table 4.1). The average age of the women at the start of the first cycle was 30.5 years for *BRCA1* mutation carriers and 30.7 years for *BRCA2* mutation carriers. Three of the seventeen women had had breast cancer before starting with the PGD procedure, all others had undergone presymptomatic testing because of an affected relative.

Design of the PGD protocols

Microsatellite markers in or in the near vicinity of the *BRCA1* and *BRCA2* gene loci were explored using free accessible databases (i.e., NCBI; <http://www.ncbi.nlm.nih.gov>, UCSC; <http://genome.ucsc.edu>). This exploration of markers provided a list of approximately 25–30 microsatellite markers flanking the *BRCA1* and *BRCA2* loci (10–15 on either side of the locus). An overview of the developed protocols is summarized in Figure 4.1. Positions of markers relative to the *BRCA1/2* loci are depicted in Figure 4.2. During the process of protocol set-up and validation, the final selection of markers for the universal protocols was made based on heterozygosity / informativity of the markers, redundancy with others markers, competition with other markers in the multiplex polymerase chain reaction (PCR), and percentages of allelic drop-out (ADO). For the universal *BRCA1* protocol, six informative markers were selected: three proximal, one intragenic, and two distal to the *BRCA1* locus (Figure 4.2, universal markers underlined). The genetic distance between the outer markers is 2.1 cM according to Genethon and 2.14 cM according to Marshfield genetic maps (deCODE not available). The average heterozygosity of markers in the universal protocol is 0.77. For the *BRCA2* protocol, eight informative markers were selected: four proximal and four distal to the *BRCA2* locus (Figure 4.2). The genetic distance between the outer markers is 4.65 cM according to deCODE, 5.4 cM according to Genethon and 3.36 cM according to Marshfield genetic maps. The average heterozygosity of the markers in the universal protocol is 0.74.

Table 4.1 PGD cycle information per couple

BRCA1 couples	Protocol according to Fig 4.1	Female age (years)	Carrier	Mutation	Amino-acid change	Exon	PGD cycle number	Total embryos	Genotype embryos			Transfer	Pregnant	
									Not affected	Affected	Aberrant			
1	1	31	Male	IVS13+4123ins6081			1	3	3	0	0	2	No	
2	Uni	34	Male	c.3748G>T	p.Glu1250X	11	1	6	2	4	0	2	No	
	Uni						2	13	5	7	1 (h)	0	No	
3	1	30	Female	c.68_69del	p.Glu23ValfsX17	2	1	12	4	3	4 (nc)	1	2x FET	
													No	
													(1 embryo)	
4	Uni		Female	c.5266dup ^a	p.Glu1756ProfsX74	20	2	11	5	4	1 (t) + 1 (nc)	0	2	Yes
	Uni	34					1	8	3	3	1 (h)	1	2 (FET)	No
	Uni						2	9	0	8	1 (h)	0	0	No
5	Uni	28	Female	c.191G>A	p.Cys64Tyr	5	1	7	1	3	2 (h)	1	1	No
6	Uni	28	Female	c.2197_2201del	p.Glu733ThrfsX5	11	1	6	2	0	2 (h) +	0	2	Yes
											1 (nc) + 1 (r)			
7	Uni	26	Female	c.2338C>T	p.Gln780X	11	1	5	2	3	0	0	2	No
	Uni						2	4	0	3	1 (h)	0	0	No
8	Uni	27	Female	c.2080del	p.Ser694AlafsX7	11	1		Cancel > foll ^a					
	Uni						2	8	2	3	3 (h)	0	1	No
9	Uni	28	Female	c.2685_2686del	p.Pro897LysfsX7	11	1	10	5	2	2 (h)	1	2 (FET)	No
10	Uni	34	Male	c.2685_2686del	p.Pro897LysfsX7	11	1	9	4	4	1 (t)	0	2	Yes, miscarriage
	Uni		Female	c.1319del [#]	p.Leu440X		2	5	1	2	1 (h)	1	1	No
11	Uni	36					1	6	2	4	0	0	2	Yes
12	4	29	Male	c.3748G>T*	p.Glu1250X*	11	1		Cancel < foll ^b					
13	5	33	Male	c.514C>T**	p.Gln172X**	8	1		Cancel < foll ^b					
14	Uni	31	Female	c.843_846del	p.Ser282TyrfsX15	11	1	2	1	0	1 (h)	0	0	No
15	Uni	29	Male	c.5277+1G>A			1	10	3	3	1 (h)	3	1	No

BRCA1, breast cancer gene 1; PGD, preimplantation genetic diagnosis; n, number of; uni, universal protocol; h, haploid; t, triploid; nc, non-conclusive; r, recombination; FET, frozen/thawed embryo transfer cycle

*/** Mutation specific protocols (see Table 4.2 for details)

[#] Thawed cycles

^a Cancel due to hyperstimulation

^b Cancel due to poor response

Table 4.1 (continued)

BRCA2 couples	Protocol according to Fig 4.1	Female age (years)	Carrier	Mutation	Amino-acid change	Exon	PGD cycle number	Total n embryos	Genotype embryos			Transfer	Pregnant	
									Not affected	Affected	Aberrant			
												result		
1	3	37	Female	c.7419_7420del***	p.Cys2473X***	14	1	5	2	2	0	1	2	No
2	3	30	Male	c.7419_7420del***	p.Cys2473X***	14	1	2	2	0	0	0	1	No
3	3					2			Cancel > foll ^a					
3	3	31	Male	c.7419_7420del***	p.Cys2473X***	14	1	4	2	2	0	0	2	Yes
3	3					1 (2 nd child)	14	14	5	7	1 (h) + 1 (t)	0	1	Yes, after FET
4	2	29	Female	c.7618-2A>G		1	7	7	2	5	0	0	1	No
5	Uni	31	Female	c.582G>A	p.Trp194X	7	2	12	7	3	1 (h)	1	1	Yes
6	Uni					1			Cancel < foll ^b					
6	2	28	Male	c.5213_5216del	p.Thr1738IlefsX2	11	2	3	1	1	1 (h)	0	1	No
7	Uni					1	9	1	1	7	0	1	1	No
7	2	25	Female	c.7976+3del2		2	7	7	4	2	0	1	2	No
8	2	35	Male	c.1310_1313del	p.Lys437IlefsX22	10	1	4	2	1	0	1	2	No
8	Uni					1	2	0	2	1	1 (h)	0	0	No
9	Uni	29	Female	c.3847_3848del	p.Val1283LysfsX2	11	2	5	3	1	1 (h)	0	1	Yes, abortion
10	2	33	Female	c.462_463del	p.Asp156X	5	3	7	2	3	1 (h)	1	1	No
	Uni					1			Cancel < foll ^b					
	Uni					5	1	5	3	1	1 (nc)	0	1	Yes, abortion
	Uni					2	7	7	0	4 (1r)	2 (h) + 1 (t)	0	0	No
11	Uni	25	Male	c.4533del	p.Glu1511AspfsX32	11	3	6	2	4	0	0	1	No
12	Uni	33	Female	c.6275_6276del	p.Leu2092ProfsX7	11	2	7	3	3	1 (nc)	0	1	No
13	Uni	29	Female	c.9672dup	p.Tyr3225IlefsX30	27	1	5	3	2	0	0	1	No
14	Uni	29	Male	c.462_463del	p.Asp156X		1	2	1	0	0	1	1	No
	Uni					2	6	6	3	2	0	1	2	No
15	Uni	37	Female	c.6275_6276del	p.Leu2092ProfsX7	11	1	8	2	4	1 (h)	2	2	Yes

BRCA2, breast cancer gene 2; PGD, preimplantation genetic diagnosis; n, number of; uni, universal protocol; h, haploid; t, triploid; nc, non-conclusive; r, recombination; FET, frozen/thawed embryo transfer cycle

*** Mutation specific protocols (see Table 4.2 for details)

^a Cancel due to hyperstimulation

^b Cancel due to poor response

For a detailed description of the primers used, the fluorescent labels, and PCR product lengths see Table 4.2.

For non-informative PGD couples or couples for whom the risk haplotype could not be established by at least two meioses, a mutation-specific protocol was designed by detection of the private mutation combined with at least one informative marker. In these tests, single substitution mutations were detected using the difference in fragment length in case of base pair deletions/insertions or using the double allele amplification refractory mutation systems technique.¹⁵

Figure 4.1 PGD protocols: Time-line overview of the developed single-cell PGD-PCR protocols for *BRCA1* and *BRCA2* mutation carriers
(A) Developed protocols for *BRCA1*
(B) Developed protocols for *BRCA2*
BRCA1, breast cancer gene 1; *BRCA2*, breast cancer gene 2

Validation of PGD protocols

The primers for the described microsatellite markers were developed using the free web program 'primer3 Input' (<http://frodo.wi.mit.edu/primer3/>). Criteria for primer design were primer size (20–30 nucleotides), guanine-cytosine content (40–60%), melting temperature of the primers (60–70°C, with maximum difference of 4°C), primers preferably ending 3' with a guanine or cytosine, and PCR product lengths <300 base pairs. Fluorescent labels were designed in such a way that PCR products with the same label did not overlap. All primers were mixed in one multiplex PCR following the European Society of Human Reproduction and Embryology (ESHRE)

guidelines.¹⁶ After optimization of the single-cell multiplex PCR, all protocols were validated by testing at least fifty single leucocytes heterozygous for each marker in at least three separate experiments to assess amplification efficiency and ADO rate. Additionally 15–20 PCR blanks were analyzed to determine contamination. Only amplification efficiency rates >90% and ADO rates <10% were acceptable for implementation in the single-cell protocol.

Figure 4.2 *BRCA1* and *BRCA2* loci and markers. Schematic overview of the *BRCA1* and *BRCA2* locus on chromosomes 17 and 13, respectively. The large gene arrows indicate the direction of the loci with respect to chromosome orientation. Marker names and their genetic (cM) and physical distances (Mb) to the *BRCA* loci are indicated in the figure. Genetic distance is based on the Genethon genetic map. Heterozygosity of marker alleles (if known) are indicated between brackets. Underlined markers are the markers used in the described universal tests. *BRCA1*, breast cancer gene 1; *BRCA2*, breast cancer gene 2; Mb, megabases; kb, kilobases; het, heterozygosity; cM, centimorgan

Table 4.2 *BRCA1* and *BRCA2* markers, primers, and labels for universal PGD tests

Protocol	Marker	Primer sequence (5' → 3')	Tag	Size (bp)
<i>BRCA1</i>				
(6 markers)	D17S932	Fw - ACACGGATGGCCTTTAGAAAAGTGGTC Rev - AACACACAGACTTGCTCTACTGCCAT	VIC	145-157
	D17S1814	Fw - ATGCTCCCCAATGACGGTGATG Rev - AGCTGGAGGTTGGCTTGTGGAT	NED	150-166
	D17S950	Fw - CATAACAGCACTTGCCCCCATGT Rev - ACAACAGCACAAACGCCCTGCAC	FAM	169-187
	D17S1787	Fw - TGCAAGACCCTTCACGCTTTGTC Rev - CTTGGTGGTTCCTTCGTCCTTG	NED	186-198
	BRCA1_dist24AC	Fw - TGCAGAACAATTGTAGCAGCACACAG Rev - GTGGTCAGAACAATGCAAATTGAAGC	VIC	205-235
	D17S800	Fw - ACATCACCCAGGGAGGTGAGTTC Rev - AAGTGGGAGGAGCCATGAATGA	NED	266-276
<i>BRCA2</i>				
(8 markers)	D13S1695	Fw - TGTCTAATGCCTGGGTATCATCC Rev - CAGGTGATCTGAGACTCAATAGCTTAACA	VIC	98-122
	D13S267	Fw - TCCTCCCCATCCACCTTTCTCC Rev - CAGGTCCCACCATAAGCACAAGC	FAM	133-147
	D13S171	Fw - AAGGGAAGGAGAAAGGGGAGGTG Rev - GCATTGACCTTAGGGCCATCCA	NED	150-166
	D13S289	Fw - GGTGAGCGGCATTGAAAACAG Rev - CACCTTCATCACACCTTGATATGG	FAM	163-177
	BRCA2_dist18AC	Fw - GCCGCCTTTCACGTAAGCACAG Rev - AATGGGAACCCAATTCAGCAAGG	PET	180-210
	D13S260	Fw - GGATCTGCTTGCAATGCCCAA Rev - TCTCCAGATATAAGGACCTGGCTATG	VIC	210-224
	D13S1698	Fw - TGGGATTACAGGCTTGAGCCACA Rev - TCTGACACAGCTGGTTGTCTATTCACC	FAM	215-235
	BRCA2STR19	Fw - GAATTTGTGTTCCAGGTGAGAATTGC Rev - ATGGGGTGCCTATGCCTGAA	NED	235-259

BRCA1, breast cancer gene 1; *BRCA2*, breast cancer gene 2; PGD, preimplantation genetic diagnosis; Fw, forward; Rev, reverse; Tag, fluorescent dyes tagged to 5' end of forward primers; bp, base pairs

IVF/ICSI/PGD procedure

Controlled ovarian hyperstimulation was performed as described earlier.¹⁷ Oocytes were retrieved under ultrasound guidance. After five hours of maturation, MII oocytes were fertilized by means of ICSI¹⁸ followed by embryo culturing.¹⁹ Embryo morphology grade was used as one of the parameters to assess the embryo quality. On the morning of day three post-fertilization, blastomeres were biopsied from cleavage-stage embryos. From each cleavage-stage embryo, one (4–7 cells) or two blastomeres (>8 cell stage) were biopsied according to the ESHRE PGD guidelines.²⁰ Of 4–7 cell cleavage-stage embryos, only one cell was biopsied as this has been a general policy in our IVF center for more than fifteen years. Rationale for biopsying two cells is to increase the number of conclusive genetic results, in case of (partly) PCR failure.

Biopsied blastomeres were rinsed three times in washing buffer (Ca^{2+} - and Mg^{2+} -free phosphate-buffered saline with 1% polyvinylpyrrolidone (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) and 0.1 mg/ml Phenol Red (Sigma-Aldrich Chemie BV)) and 2 μl washing buffer was transferred into 0.2 ml PCR tubes. Blank samples, containing 2 μl of the last washing droplet, were collected for each blastomere to monitor contamination. Single blastomere and blank samples were stored at least for 20 minutes at -20°C , until the PCR was performed.

PCR and genescan analysis

Single cells (blastomeres and leukocytes) were lysed by incubation at 65°C for ten minutes in lysisbuffer (50 mM DTT and 200 mM NaOH) before amplification. Multiplex PCR for the polymorphic markers and allele-specific PCR for a private mutation contained 1 x QIAGEN Multiplex PCR Master Mix (QIAGEN, Venlo, the Netherlands), 20 mmol/l Tricine (Sigma-Aldrich Chemie BV), and primers (Applied Biosystems, Warrington, United Kingdom) in a final volume of 25 μl . The primer concentrations of the individual sets in the multiplexed PCRs varied between 100 nM and 1 mM per primer pair. All PCRs were performed with an initial activation step of 15 minutes denaturation at 95°C . The denaturation–annealing–elongation cycles for the *BRCA1* multiplex PCR were 42 cycles of ten seconds at 95°C , 90 seconds at 62°C , 60 seconds at 72°C , and for the *BRCA2* multiplex ten initial cycles of ten seconds at 95°C , 60 seconds at 63°C , 60 seconds at 72°C , followed by 32 cycles of ten seconds at 95°C , 45 seconds at 63°C , and 60 seconds at 72°C . The PCR products were diluted (ten times *BRCA1* PCR, twenty times *BRCA2* PCR) and separated based on fragment length using capillary electrophoresis on an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, Warrington, United Kingdom). Fragment lengths were analyzed using the GeneMapper software provided by the manufacturer. For an example of genescan analysis and the corresponding haplotypes, see Figure 4.3.

Quality assessment

After genetic diagnosis of embryos and embryo transfer of one or two healthy embryo(s) into the uterus and cryopreservation of the remaining unaffected, good quality embryos, the remaining embryos were collected for quality assessment purposes after informed consent of the couple involved. Usually, the total embryo is selected as a whole to confirm the genetic diagnosis made during the PGD cycle. In a minority of cases, embryos were split into single blastomeres and analyzed separately. Of the 30 PGD couples, the untransferred embryos of sixteen couples (8 *BRCA1*, 8 *BRCA2*) were collected to determine accuracy of genetic diagnosis/misdiagnosis rate.

Results

PGD protocols

Five protocols for *BRCA1* and four protocols for *BRCA2* have been developed and validated at the single-cell level (Figures 4.1a and b). For *BRCA1*, we started with the first protocol of three markers of which two were intragenic and one distal (protocol 1, Figure 4.1a). The next couple had too few family members and the risk haplotype could not be determined with certainty. Therefore the mutation was included (protocol 2, Figure 4.1a). Thereafter many more couples applied with mutations beyond exon 19, leading to non-flanking markers. To avoid repetitive redesign of the protocol, it was adapted to the universal six marker protocol (protocol 3, universal, Figure 4.1a). For two couples thereafter the distal markers of the universal protocol were not informative and mutation-specific protocols were developed (protocols 4 and 5, Figure 4.1a).

For *BRCA2*, a first protocol with a mutation and three markers, two proximal, one distal, was set up (protocol 1, Figure 4.1b). Thereafter couples with different mutations applied and in many couples the one distal marker was not informative. The protocol was changed to four markers (protocol 2, Figure 4.1b). As for some additional couples the two distal markers were not informative, the specific mutation was built in the existing protocol (protocol 3, Figure 4.1b). In the last phase, four additional markers were added to increase informativity (protocol 4, universal, Figure 4.1b).

The universal PGD protocols were developed approximately two years after continuously changing protocols and were thereafter applied to all new PGD couples. A detailed description of the validation of these protocols is depicted in Table 4.3. Also for couples who had a first PGD cycle performed with an old protocol, the next cycles were adapted to the novel universal protocols (see Table 4.1 for details). Since their introduction, 12 of the 14 *BRCA1* couple haplotypes (86%) were informative and all (100%) *BRCA2* family haplotypes were informative. These couples were ready to be scheduled for the PGD procedure within one to two months. For two PGD couples, the *BRCA1* universal protocol was not applicable, because the markers at the distal side were not informative.

Table 4.3 Validation of universal protocols

Gene	Marker	n single cells tested	n hetero-zygotes	ADO (%) per locus	PCR efficiency (%) per locus	Positive blanks per locus
<i>BRCA1</i>	D17S1814	57	54	1,8	96,5	0/13
	D17S800	57	55	1,8	96,5	0/13
	D17S1787	57	55	1,8	96,5	0/13
	D17S932	57	55	1,8	96,5	0/13
	BRCA1_dis24AC	57	55	0,0	96,5	1/13
	D17S950	57	54	0,0	96,5	0/13
<i>BRCA2</i>	D13S289	106	104	1,9	98,1	0/24
	D13S260	106	69	5,8	98,1	0/24
	D13S1698	106	104	5,8	98,1	0/24
	BRCA2STR19	106	103	1,9	97,2	0/24
	D13S171	106	35	2,9	98,1	0/24
	D13S1695	106	34	0,0	97,2	1/24
	BRCA2_dist18AC	106	70	0,0	97,2	0/24
	D13S267	106	69	0,0	98,1	0/24

n, number of; ADO, allelic drop-out; PCR, polymerase chain reaction; *BRCA1*, breast cancer gene 1; *BRCA2*, breast cancer gene 2

Clinical cycles

The results of the individual clinical cycles are summarized in Table 4.1. Thirty couples underwent a total of 47 cycles. Two of these 47 cycles concerned PGD analyses of embryos cryopreserved before biopsy in another IVF center. Of the remaining 45 cycles, eight cycles were cancelled before oocyte pick-up due to hyperstimulation ($n = 2$) or hypostimulation ($n = 6$). In the eighteen *BRCA1* cycles, a total of 134 embryos were biopsied and a genetic diagnosis was obtained in 93% (125/134); in the 21 *BRCA2* cycles, a total of 127 embryos were biopsied and a genetic diagnosis was obtained in 93% (118/127). Of the embryos with a genetic diagnosis for *BRCA1*, 44.4% carried the mutation and 35.7% was unaffected; for *BRCA2*, 46.6% carried the mutation and 42.4% was unaffected. All remaining embryos were genetically abnormal or inconclusive.

In 34 of the 39 cycles (15 *BRCA1*, 19 *BRCA2*, 87.2%) an embryo transfer was possible and resulted in ten pregnancies of which eight were with a fetal heart beat (four *BRCA1*, four *BRCA2*), leading to a fetal heart beat per oocyte pick-up of 20.5% and a fetal heart beat per embryo transfer of 23.5%. For the *BRCA1* cycles, four pregnancies were obtained (see Table 4.1): couple 3 delivered a healthy girl at 41⁺¹ weeks with a birth weight of 3625 grams, couple 6 delivered a healthy twin, a boy and a girl (two embryos transferred) with a birth weight of 3220 and 2660 grams respectively. Couple 10 had an early miscarriage at five weeks. Couple 11 has an ongoing (singleton) pregnancy. For *BRCA2* PGD couples, four pregnancies were obtained: couple 3 delivered a healthy girl with a birth weight of 3120 grams in August 2010 and is currently pregnant with a second child after a cryo-embryo transfer from the first

cycle (at term 14 October 2012). Couple 4 delivered a healthy girl at 40⁺⁴ weeks with a birth weight of 2620 grams. The pregnancy of couple 15 is still ongoing. So far, none of the couples opted for prenatal diagnosis to confirm the determined unaffected genetic status in the PGD cycle.

Quality assessment

A total of 73 embryos were collected after PGD for re-analysis. In all, 13, 45, and 15 embryos were genotyped as unaffected, affected, or aberrant during the PGD cycles. As the unaffected morphological good embryos are used for embryo transfer, the number of affected embryos succeeds the number of unaffected embryos in the re-analysis procedure. Of the 13 unaffected embryos, 11 showed conclusive genotype results after re-analysis. Two of 11 embryos gave one parental haplotype (monosomy), the non-risk marker haplotype of the affected parent. The two remaining embryos were not conclusive and could not be genotyped due to contamination after re-analysis. In 42/43 (98%) of the affected embryos, the PGD results were confirmed, one embryo gave no result; 5/15 aberrant embryos were confirmed (33%), one monosomy gave no result, seven monosomies were disomies, and two trisomies were disomies in the re-analyses. Two recombinations were detected during the PGD cycles (Table 4.1), one around the *BRCA1* locus and another around the *BRCA2* locus. Both recombinations were confirmed in the re-analyses. The recombination around the *BRCA1* locus crossed the two markers between which the mutation is located, and therefore the presence/absence of the mutation remained unknown in this embryo.

Discussion

Since its introduction in the early nineties, PGD is considered a well-established clinical service in many countries for many human genetic diseases.²¹ The range of indications has expanded from sex determination to prevention of chromosomal rearrangements to monogenic disorders. During the past few years, diseases of late age of onset and high (but not complete) penetrance with some therapeutic or preventive options such as HBOC have also been added to this list. PGD for this indication has therefore been a vigorous topic of debate in several countries in the past couple of years.²² Since 2008, several *BRCA1/2* mutation carrier couples applied for PGD. We started with a mutation-specific protocol with few markers and protocols with some informative markers for first couples. These protocols were not applicable to all couples and thus were adapted by either replacing the mutation-specific primers or adding/replacing markers. Also in the literature the same laborious strategy is visible; Spits *et al.*¹³ have described a protocol for *BRCA1* using two markers, flanking

the *BRCA1* locus. These markers are both intragenic, making the protocol suitable only if both markers are informative and the mutation itself is located between the two intragenic markers. Jasper *et al.*²³ have included one specific mutation in *BRCA1* combined with two proximal markers but used two rounds of PCR. Sagi *et al.*¹² have described one *BRCA1* and two *BRCA2* founder-mutation-specific protocols, including three or four markers flanking the *BRCA* loci, applied to ten PGD couples from Israel, and finally Ramón *et al.*²⁴ have described one mutation-specific *BRCA1* protocol with two markers. These protocols are not universal as they are mutation specific and/or include only a very small number of markers limiting informativity and using intragenic markers creates the limitation that the mutation must be located between the markers. Additionally, it takes a lot of time to design and optimize multiplex single-cell PCRs to maximize PCR efficiency and minimize ADO percentages for each specific mutation. To circumvent this costly and laborious procedure, other strategies have already been illustrated. Preimplantation genetic ‘haplotyping’ has been described as a more universal strategy for several inherited monogenic disorders.²⁵ However, this strategy uses an extra step of multiple displacement amplification (MDA) of the single cell. This additional step is needed to allow a combination of several multiplexed PCRs in the following step, which would otherwise not be feasible on a single blastomere. This principle is excellent in the sense that it is applicable to all familiar monogenic diseases and using several multiplexed PCRs will optimize informativity of markers and detection of recombinations. One major drawback of MDA is, however, the high percentage of ADO which needs to be compensated with a higher number of markers tested. A second drawback can be the extra time needed (sixteen hours) to perform the MDA, which may put pressure on the timing of embryonic transfer if transfer is performed on day four post-fertilization (which is the case in our center). Therefore, for centers that transfer at day five, the time needed to do MDA is not an issue. We developed an ‘off-the-shelf’ and fast test for *BRCA1* and *BRCA2* mutation carriers applicable to nearly all couples; we designed one multiplexed PCR test, including as much as possible flanking informative markers, working on a single biopsied blastomere.

For the *BRCA1* locus, we were able to multiplex six highly polymorphic markers in one PCR. The maximal distance of the two outer markers is 2.1 cm. Three markers are located in the proximal region, two in the distal region and one is located intragenic (intron 20). For the *BRCA2* locus, we were able to multiplex eight highly polymorphic markers in one PCR, with a maximal distance between the outer markers of 4.65 cm. All eight markers are flanking the *BRCA2* locus, four proximally and four distally. As mentioned earlier, we started out with approximately 20–30 markers per locus in the early stages of development. Taking several criteria (heterozygosity, ADO percentage, competition, etc.) as well as single-cell PCR limitations into account, the final number as well as the specific location of markers chosen in the universal protocols are not

unique; other numbers or combinations of markers would be possible as long as they meet the same validation criteria and are informative in the majority of new couples. Eighty-six percent of the *BRCA1* and all *BRCA2* mutation carriers could so far be analyzed with the universal tests. If the pedigrees of the 'old protocol' cycles are reassessed and the universal protocols would have been used, 13/15 *BRCA1* couples (87%) and 14/15 (93%) were informative. Only two *BRCA1* PGD couples were not informative at the distal side and one *BRCA2* couple would not have been informative at the distal side. Preparation time is limited to one to two months, let alone the cost reduction saved by using the 'off-the-shelf' tests.

The robustness of the multiplex *BRCA* PCR tests are in accordance with the latest edition of the ESHRE PGD consortium guidelines,¹⁶ with a PCR efficiency >90% and ADO rates <10%.

Conclusions

In summary, we present universal 'off-the-shelf' PGD tests for *BRCA1* and *BRCA2* mutation carriers that are robust, easy, and quick to implement for a wide range of families choosing PGD to avoid transmission of a *BRCA1/2* mutation to future offspring.

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Part II

Oncological safety of IVF in female
BRCA1/2 mutation carriers

Chapter 5

Ovarian stimulation for IVF and risk of primary breast cancer in *BRCA1/2* mutation carriers

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Abstract

Introduction

The effect of *in vitro* fertilization (IVF) on breast cancer for *BRCA1/2* mutation carriers is rarely examined. As carriers may increasingly undergo IVF as part of preimplantation genetic diagnosis, we examined the impact of ovarian stimulation for IVF on breast cancer risk in *BRCA1/2* mutation carriers.

Methods

The study population consisted of 1,550 *BRCA1* and 964 *BRCA2* mutation carriers, derived from the nationwide HEBON-study and the nationwide preimplantation genetic diagnosis registry. Questionnaires, clinical records and linkages with the Netherlands Cancer Registry were used to collect data on IVF exposure, risk-reducing surgeries and cancer diagnosis, respectively. Time-dependent Cox regression analyses were conducted, stratified for birth cohort and adjusted for infertility.

Results

IVF exposure was not associated with risk of breast cancer for *BRCA1/2* mutation carriers (HR 0.79, 95% CI 0.46-1.36). Similar results were found for the subgroups of infertile women ($n = 232$; HR 0.73, 95% CI 0.39-1.37) and *BRCA1* mutation carriers (HR 1.12, 95% CI 0.60-2.09). In addition, age at first IVF treatment and recency of IVF treatment were not associated with breast cancer risk.

Conclusion

No evidence was found for an association between ovarian stimulation for IVF and breast cancer risk in *BRCA1/2* mutation carriers.

Introduction

Previously, we reported a 28-year old *BRCA1* mutation carrier diagnosed with triple negative invasive ductal carcinoma, stage T1bN1aM0, three months after ovarian stimulation for *in vitro* fertilization (IVF) as part of preimplantation genetic diagnosis (PGD).¹ The tumor was detected by magnetic resonance imaging (MRI) of the breast whereas two months before the IVF/PGD treatment the breast MRI was normal. The question was raised whether exposure to ovarian stimulation for IVF has an influence on breast cancer risk in *BRCA1/2* mutation carriers.

In the general population, genetic, lifestyle, hormonal, and histopathologic characteristics (e.g., breast density) are associated with the risk of breast cancer.²⁻⁴ Mature breast tissue arises from mammary stem and progenitor cells which differentiate during pregnancy.⁵ Prolonged exposure to cyclic levels of estrogen and progesterone has been reported to increase the risk of breast cancer in the general population, especially in nulliparous women.^{4,6-10} The biological mechanisms underlying this observation are not clear. It has been suggested that estrogens act upon carcinogenesis via their role in breast tissue proliferation, whereas differentiation of mammary tissue by pregnancy at younger age protects against breast cancer due to the expression of genes involved in apoptosis, cell cycle control, and cell signalling.^{11,12}

The use of a long agonist protocol for ovarian stimulation for IVF results in a period of decreased levels of estrogen and progesterone due to down regulation of the natural hormonal cycle,¹³ followed by a temporary hyperestrogenic state.¹⁴ Recent meta-analyses did not report an association between exposure to ovarian stimulation for IVF and breast cancer risk in the general population.^{15,16} A large recent cohort study in over 19,000 Dutch women did also not show an adverse effect of IVF on breast cancer risk.¹⁷ Little is known about the influence of ovarian stimulation for IVF on the risk of breast cancer in carriers of a pathogenic mutation in the *BRCA1* or *BRCA2* gene. Women carrying such a mutation have a high *a priori* risk of breast cancer¹⁸ and other cancers.¹⁹ Prospective studies have estimated the risk of breast cancer to be 72% (95% CI 65-79%) for *BRCA1* and 69% (95% CI 61-77%) for *BRCA2* mutation carriers by the age of 80. The risk of breast cancer in female *BRCA1/2* mutation carriers starts to rise from age 25 onwards, reaching a 20% cumulative risk already at ages 38 and 43 for *BRCA1* and *BRCA2*, respectively, i.e., ages at which women may be exposed to IVF.¹⁸ However, the association between exposure to ovarian stimulation for IVF and breast cancer risk for women with a *BRCA1/2* mutation has been hardly studied. The only case-control study conducted did not find an elevated risk of breast cancer associated with IVF exposure.²⁰

BRCA1/2 mutations are relatively frequent, with heterozygote germline mutations in 1:400 up to 1:200 persons in general populations²¹ and 1:40 in certain ethnic groups.²² As a consequence, a substantial number of female *BRCA1/2* mutation carriers will be

exposed to IVF for (1) infertility, (2) fertility preservation (i.e., banking of embryos or oocytes prior to oncologic treatment, risk-reducing salpingo-oophorectomy (RRSO), or to postpone motherhood on social grounds),^{23,24} or (3) PGD.^{1,25} In PGD, embryos obtained by IVF are genotyped for the presence of a known familial mutation in order to establish a pregnancy of a fetus without the genetic condition.²⁶

The aim of the current study is to evaluate whether exposure to ovarian stimulation for IVF increases the risk of primary breast cancer in *BRCA1* and *BRCA2* mutation carriers.

Patients and methods

Women were eligible if they were 18 years or older and had been tested positive for a pathogenic mutation in either the *BRCA1* or *BRCA2* gene. Women born before 1940 were excluded because they had their reproductive years before IVF was available.

Our study population was derived from two sources: the Dutch HEBON study (Hereditary Breast and Ovarian cancer study, the Netherlands) and the national PGD registry.

The HEBON study (initiated in 1999) is an ongoing nationwide retrospective cohort study among members of *BRCA1/2* mutation families with prospective follow-up.²⁷ Current analyses were restricted to participants that entered the HEBON study between 2010-2013 and subsequently completed the most recent HEBON baseline questionnaire (response, 57%), which included questions concerning exposure to fertility treatments. In the Netherlands, PGD for *BRCA1/2* mutations has been offered since 2008 in Maastricht University Medical Center, in collaboration with IVF transport clinics in the University Medical Centers of Utrecht and Groningen, and the Academic Medical Center Amsterdam. All women who undergo PGD are registered in the PGD registry. Some women were eligible through the HEBON study and had also been registered in the PGD-registry.

The HEBON study was approved by the medical ethics committees of all participating Dutch Clinical Genetic Centers or Family Cancer Clinics. All HEBON participants gave their informed consent for linkage with the nationwide cancer and pathology registries. If women completed and returned the study questionnaire this was taken as approval for use for research. The PGD program has been approved by the Institutional Review Board of Maastricht University Medical Center. All patients undergoing PGD gave their written informed consent for this treatment and use of their medical data for scientific research .

Data collection

For *BRCA1/2* mutation carriers included through the HEBON study, data on exposure to IVF and other fertility treatments (i.e., ovulation induction and intrauterine

insemination), prophylactic surgeries, and confounders were collected with the HEBON baseline questionnaire. In case of female infertility, women reported the type(s) of fertility treatment(s) they underwent and their age at first and last treatment. Information on cancer history was self-reported but also obtained by linkage with both the Dutch national Pathology Database (PALGA) and the Netherlands Cancer Registry (NCR) for the period after 1988. For the period when the registries were not yet available (≤ 1988) cancer diagnoses were solely self-reported. For the women included through the PGD registry, data on exposure (type of fertility treatment(s) and age at treatment(s)), potential confounders, prophylactic surgeries, and cancer history were retrieved from medical files.

For women eligible through the HEBON study and also registered in the PGD-registry data from medical files and the HEBON questionnaire were combined. No conflicting data were found.

Statistical analysis

The association between IVF exposure and risk of breast cancer was analyzed using a time-dependent Cox proportional hazards regression model with age as the timescale. Main analyses were stratified for birth cohort (based on tertiles: 1940-1957, 1958-1968, 1969-1993) and adjusted for infertility (no, yes, or missing). Observation time started at birth and ended at age at diagnosis of first invasive breast cancer (event of interest), other invasive cancer diagnosis (excl. basal cell carcinoma), or bilateral prophylactic mastectomy (BPM), whichever occurred first. In case these events did not take place before baseline questionnaire or last PGD contact, observation time ended at the age of baseline questionnaire completion or last PGD contact, whichever was last.

The exposed group, in terms of person-time, consisted of observation time of *BRCA1/2* mutation carriers who had undergone ovarian stimulation for IVF before censoring. Women were considered as exposed to IVF from the first IVF treatment onwards. The unexposed (comparison) person-time consisted of observation time of *BRCA1/2* mutation carriers who had not (yet) been exposed to ovarian stimulation for IVF before censoring. Cut-offs of categories in variables related to IVF characteristics are based on numbers of cases available in the exposed group.

Potential confounders, besides infertility and birth cohort, were exposure to other fertility treatments (clomid and/or intra-uterine insemination (in which hormonal treatment may be included); time-dependent: ever/never), use of oral contraceptives (time dependent: ever/never), parity (time-dependent: nulliparous/parous), and age at first childbirth (time-dependent: nulliparous / <25 years / 25-29 years / 30+ years). None of these changed the hazard ratio (HR) for IVF treatment by more than 10% and therefore they were not included. The uptake of a RRSO could only be tested as a potential confounder in an unbiased manner by comparing univariate and

multivariate analyses between IVF uptake and breast cancer risk with restricted follow-up time until age at DNA test, since the uptake of RRSO mainly depends on the outcome of the DNA test.²⁸ As univariate and multivariate HRs were similar, RRSO uptake was not considered to be a confounder.

BRCA mutation-specific analyses were only conducted in the subgroup of *BRCA1* mutation carriers since power in *BRCA2* mutation carriers was too limited. An additional sensitivity analysis restricted to infertile women was conducted to further assess any association in this stratum.

All statistical analyses were performed using STATA version 13 (StataCorp, College Station, TX).

Results

Patient characteristics

The final group eligible for analyses comprised 2,514 *BRCA1/2* mutation carriers (1,550 *BRCA1*, 964 *BRCA2*). Of these, 2,502 (99.5%) women participated in the HEBON study. The PGD group included a total of 25 eligible women, including 13 responders to the HEBON-study (Figure 5.1).

Figure 5.1 Composition of study groups
BRCA1, breast cancer gene 1; *BRCA2*, breast cancer gene 2; HEBON, Hereditary Breast and Ovarian cancer study, the Netherlands; PGD, preimplantation genetic diagnosis; n, number of women; BC, breast cancer; BPM, bilateral prophylactic mastectomy; IVF, *in vitro* fertilization

In total 938 women (37.3%) were diagnosed with breast cancer, 630 *BRCA1* (40.7%) and 308 *BRCA2* mutation carriers (32.0%, Table 5.1).

The average age at breast cancer diagnosis was 40.1 years for *BRCA1* and 44.4 years for *BRCA2* mutation carriers. Of the remaining participants 163 (10.3%) were censored at another cancer diagnosis, 423 (26.8%) at BPM, and 990 women (62.8%) at the age of the questionnaire or last PGD contact. Their average age at censoring was 43.7 years (*BRCA1* 43.1 years, *BRCA2* 44.6 years). Those women only identified through the PGD registry were on average younger at censoring compared to the women included by the HEBON study (33.0 years versus 43.0 years). Of all mutation carriers women unaffected with breast cancer were slightly more often born in more recent birth years (1969-1993: 43.4% mutation carriers unaffected with breast cancer versus 22.3% affected with breast cancer).

In total, 3% (n = 76) of all *BRCA1/2* mutation carriers included were exposed to ovarian stimulation for IVF; 41 *BRCA1* and 35 *BRCA2* mutation carriers. For 51 mutation carriers IVF exposure was identified through the HEBON study and for 25 through the PGD registry. Due to the nature of the IVF exposure question in the HEBON study (infertile women only), data on IVF exposure of eight mutation carriers was collected through the PGD registry while they were exposed before date of HEBON questionnaire. Mutation carriers were on average 31.7 years at first IVF treatment. At censoring their first IVF treatment was on average 6.5 years ago (Table 5.2). The year of first treatment varied between 1989 – 2015, while mutation carriers included through the PGD registry received their first IVF treatment in more recent years (HEBON: 1989-2013, median 2003; PGD: 2010-2015, median 2013). Mutation carriers exposed to IVF were more often infertile (64.5% versus 7.4%) and nulliparous (36.8% versus 19.3%) compared to unexposed mutation carriers.

Breast cancer risk related to IVF

At the end of follow-up 15 mutation carriers (12 *BRCA1* and three *BRCA2*) exposed to ovarian stimulation for IVF had developed breast cancer. Exposure to ovarian stimulation for IVF was not associated with the risk of breast cancer (HR 0.79, 95% CI 0.46-1.36, Table 5.2). Also, specific IVF characteristics, e.g., age at first IVF treatment were not associated with breast cancer risk (no IVF, HR = 1.0 (reference), ≤32 years HR 1.13 95% CI 0.57-2.20, >32 years HR 0.54 95% CI 0.24-1.24, Table 5.2).

Table 5.1 Cohort characteristics of *BRCA1/2* mutation carriers

	<i>BRCA1</i> n = 1550		<i>BRCA2</i> n = 964		<i>BRCA1/2</i> combined n = 2514	
	BC+*	BC-*	BC+*	BC-*	BC+*	BC-*
n (%)	630 (40.7)	920 (59.3)	308 (31.9)	656 (68.1)	938 (37.3)	1576 (62.7)
Data source, n (%)						
HEBON	629 (99.8)	913 (99.2)	308 (100)	639 (97.4)	937 (99.9)	1552 (98.5)
HEBON + PGD						
PGD<HEBON	1 (0.2)	2 (0.2)	0 (0.0)	5 (0.8)	1 (0.1)	7 (0.4)
PGD>HEBON	0 (0.0)	1 (0.1)	0 (0.0)	4 (0.6)	0 (0.0)	5 (0.3)
PGD only	0 (0.0)	4 (0.4)	0 (0.0)	8 (1.2)	0 (0.0)	12 (0.8)
Mean age at censoring (SD)	40.1 (8.5)	43.1 (11.6)	44.4 (8.9)	44.6 (12.0)	41.5 (8.9)	43.7 (11.8)
Age at censoring (years) (%)						
<35	176 (27.9)	247 (26.9)	36 (11.7)	149 (22.7)	212 (22.6)	396 (25.1)
35-44	276 (43.8)	259 (28.2)	132 (42.9)	190 (29.0)	408 (43.5)	449 (28.5)
45-64	175 (27.8)	384 (41.7)	139 (45.1)	276 (42.1)	314 (33.5)	660 (41.9)
65+	3 (0.5)	30 (3.3)	1 (0.3)	41 (6.3)	4 (0.4)	71 (4.5)
Censoring reason						
Baseline Q/PGD contact	0 (0.0)	561 (61.0)	0 (0.0)	429 (65.4)	0 (0.0)	990 (62.8)
Breast cancer	630 (100)	0 (0.0)	308 (100)	0 (0.0)	938 (100)	0 (0.0)
Other Cancer	0 (0.0)	90 (9.8)	0 (0.0)	73 (11.1)	0 (0.0)	163 (10.3)
BPM	0 (0.0)	269 (29.2)	0 (0.0)	154 (23.5)	0 (0.0)	423 (26.8)
Birth year (%)						
1940-1957	244 (38.7)	216 (23.5)	153 (49.7)	191 (29.1)	397 (42.3)	407 (25.8)
1958-1968	231 (36.7)	296 (32.2)	101 (32.8)	189 (28.8)	332 (35.4)	485 (30.8)
1969-1993	155 (24.6)	408 (44.4)	54 (17.5)	276 (42.1)	209 (22.3)	684 (43.4)
IVF (%)						
No	616 (97.8)	889 (96.6)	305 (99.0)	624 (95.1)	921 (98.2)	1513 (96.0)
Yes	12 (1.9)	29 (3.2)	3 (1.0)	32 (4.9)	15 (1.6)	61 (3.9)
Missing	2 (0.3)	2 (0.2)	0 (0.0)	0 (0.0)	2 (0.2)	2 (0.1)

BRCA1, breast cancer gene 1; *BRCA2*, breast cancer gene 2; n, number of women; BC, breast cancer; HEBON, Hereditary Breast and Ovarian cancer study, the Netherlands; PGD, preimplantation genetic diagnosis; SD, standard deviation; Q, questionnaire; BPM, bilateral prophylactic mastectomy; IVF, *in vitro* fertilization

* Distribution of variable at time of censoring

Table 5.2 IVF exposure and breast cancer risk in *BRCA1/2* mutation carriers

n = 2,514	BC+*	BC-*	HR (95% CI)†
n (%)	938 (37.3)	1576 (62.7)	
IVF treatment (%)			
No	921 (98.2)	1513 (96.0)	1.00
Yes	15 (1.6)	61 (3.9)	0.79 (0.46-1.36)
Missing	2 (0.2)	2 (0.1)	
Year at first IVF (years)			
Median (min-max)	2000 (1988-2010)	2005 (1985-2015)	
Age at first IVF (%)			
Median (min – max; years)	30.9 (23-37)	31.9 (24-42)	
No IVF	921 (98.2)	1513 (96.0)	1.00
≤32 years	9 (1.0)	36 (2.3)	1.13 (0.57-2.20)
>32 years	6 (0.6)	25 (1.6)	0.54 (0.24-1.24)
Missing	2 (0.2)	2 (0.1)	
Time since first IVF treatment (%)			
No IVF	921 (98.2)	1513 (96.0)	1.00
<5 years ago started	7 (0.8)	34 (2.2)	0.83 (0.39-1.78)
≥5 years ago started	8 (0.9)	27 (1.7)	0.75 (0.36-1.56)
Missing	2 (0.2)	2 (0.1)	
Time since last IVF treatment (%)			
No IVF	921 (98.2)	1513 (96.0)	1.00
<2 years ago stopped	6 (0.6)	20 (1.3)	0.99 (0.43-2.25)
≥2 years ago stopped	7 (0.8)	40 (2.5)	0.60 (0.28-1.30)
Age of last treatment before censoring unknown	2 (0.2)	1 (0.1)	
Missing	2 (0.2)	2 (0.1)	

IVF, *in vitro* fertilization; *BRCA1*, breast cancer gene 1; *BRCA2*, breast cancer gene 2; n, number of women; BC, breast cancer; HR, hazard ratio; CI, confidence interval

* Distribution of variable at time of censoring

† Intrinsically stratified on birth year (1940-1957, 1958-1968, 1969-1993); adjusted for infertility (no, yes, missing)

Overall, additional analyses in the subgroup of *BRCA1* mutation carriers and in infertile women gave similar results. Both in *BRCA1* mutation carriers (HR 1.12, 95% CI 0.60-2.09; Table 5.3) and in infertile women (HR 0.73, 95% CI 0.39-1.37; Table 5.4) IVF exposure was not associated with breast cancer risk. However, in *BRCA1* mutation carriers a younger age at first IVF treatment seemed to be more strongly related with breast cancer risk than first IVF treatment at older ages (≤32 years HR 1.91 95% CI 0.88-4.16, >32 years HR 0.70 95% CI 0.28-1.76), but numbers were small.

Table 5.3 IVF exposure and breast cancer risk in *BRCA1* mutation carriers

n = 1,550	BC+*	BC-*	HR (95% CI)†
n (%)	630 (40.7)	920 (59.4)	
IVF treatment			
No	616 (97.8)	889 (96.6)	1.00
Yes	12 (1.9)	29 (3.2)	1.12 (0.60-2.09)
Missing	2 (0.3)	2 (0.2)	
Age at first IVF (%)			
Median (min – max; years)	31.3 (23-36)	32.3 (24-42)	
No IVF	616 (97.8)	889 (96.6)	1.00
≤32 years	7 (1.1)	14 (1.5)	1.91 (0.88-4.16)
>32 years	5 (0.8)	15 (1.6)	0.70 (0.28-1.76)
Missing	2 (0.3)	2 (0.2)	
Time since first IVF treatment (%)			
No IVF	616 (97.8)	889 (96.6)	1.00
<5 years ago started	6 (1.0)	13 (1.4)	1.15 (0.49-2.66)
≥5 years ago started	6 (1.0)	16 (1.7)	1.10 (0.47-2.56)
Missing	2 (0.3)	2 (0.2)	
Time since last IVF treatment (%)			
No IVF	616 (97.8)	889 (96.6)	1.00
<2 years ago stopped	5 (0.8)	7 (0.8)	1.49 (0.60-3.73)
≥2 years ago stopped	5 (0.8)	21 (2.3)	0.84 (0.33-2.10)
Age of last treatment before censoring unknown	2 (0.3)	1 (0.1)	
Missing	2 (0.3)	2 (0.2)	

IVF, *in vitro* fertilization; *BRCA1*, breast cancer gene 1; n, number of women; BC, breast cancer; HR, hazard ratio; CI, confidence interval

* Distribution of variable at time of censoring

† Intrinsically stratified on birth year (1940-1957, 1958-1968, 1969-1993); adjusted for infertility (no, yes, missing)

Discussion

We show that for *BRCA1* and *BRCA2* mutation carriers breast cancer risk was not increased after exposure to ovarian stimulation for IVF. This was also the case when assessing this risk in subgroups of infertile women and *BRCA1* mutation carriers alone. The association between exposure to ovarian stimulation for IVF and the incidence of breast cancer in *BRCA1/2* mutation carriers was examined in only one previous study. Kotsopoulos et al.²⁰ studied the risk of breast cancer associated with infertility, fertility treatment, and IVF treatment in a case-control design. They included 1,380 women with a *BRCA1* or *BRCA2* mutation with a history of breast cancer (cases) and matched them to 1,380 female *BRCA1* or *BRCA2* mutation carriers without a history of breast cancer (controls). No association between exposure to IVF and breast cancer was observed (multivariable odds ratio 0.98, 95% CI 0.39-2.45, based on nine cases who received IVF), while a non-significantly increased risk was found between exposure to

gonadotropin-containing fertility medication and breast cancer risk (multivariable odds ratio 2.32, 95% CI 0.91-5.95). Data regarding types of fertility treatment and medication used were self-reported in Kotsopoulos *et al.* and information concerning the latter was missing in 27% of the study subjects.

Table 5.4 IVF exposure and breast cancer risk in *infertile BRCA1/2* mutation carriers

n = 232	BC+*	BC-*	HR (95% CI)†
n (%)	93 (40.1)	139 (59.9)	
IVF treatment			
No	79 (85.0)	100 (71.9)	1.00
Yes	12 (12.9)	37 (26.6)	0.73 (0.39-1.37)
Missing	2 (2.2)	2 (1.4)	
Age at first IVF (%)			
Median (min – max; years)	31.9 (24-37)	32.6 (24-41)	
No IVF	79 (85.0)	100 (71.9)	1.00
≤32 years	6 (6.5)	17 (12.2)	0.93 (0.40-2.17)
>32 years	6 (6.5)	20 (14.4)	0.60 (0.25-1.41)
Missing	2 (2.2)	2 (1.4)	
Time since first IVF treatment (%)			
No IVF	79 (85.0)	100 (71.9)	1.00
<5 years ago started	6 (6.5)	11 (7.9)	0.94 (0.40-2.22)
≥5 years ago started	6 (6.5)	26 (18.7)	0.59 (0.25-1.40)
Missing	2 (2.2)	2 (1.4)	
Time since last IVF treatment (%)			
No IVF	79 (85.0)	100 (71.9)	1.00
<2 years ago stopped	5 (5.4)	7 (5.0)	1.05 (0.41-2.65)
≥2 years ago stopped	6 (6.5)	29 (20.9)	0.55 (0.23-1.31)
Age of last treatment before censoring unknown	1 (1.1)	1 (0.7)	
Missing	2 (2.2)	2 (1.4)	

IVF, *in vitro* fertilization; *BRCA1*, breast cancer gene 1; *BRCA2*, breast cancer gene 2; n, number of women; BC, breast cancer; HR, hazard ratio; CI, confidence interval

* Distribution of variable at time of censoring

† Intrinsically stratified on birth year (1940-1957, 1958-1968, 1969-1993)

Our data regarding type of fertility treatment was self-reported as well for the greater part of exposed women, and we did not collect information regarding the type of medication used. In the Netherlands, IVF is in the vast majority of cases performed using gonadotropins for ovarian stimulation. Gonadotropins are also used but to a lesser extent for ovulation induction, as a second choice for clomiphene citrate-containing drugs (e.g., clomid). In the study by Kotsopoulos *et al.* it was not described for which fertility treatments gonadotropin-containing medications were used, nor which types of medication were used for ovarian stimulation for IVF. Additionally, no information regarding the indication for fertility treatment was provided. As a consequence, it is difficult to interpret their apparent different findings between

exposure to IVF treatment and exposure to gonadotropin-containing drugs. Only nine and 15 cases and 11 and 61 controls were exposed to IVF in the study of Kotsopoulos *et al.* and our study, respectively. Thus, the power in the present study is larger but still too limited to exclude a true weak association.

The association between ovarian stimulation for IVF and the risk of breast cancer has been extensively studied in the non-*BRCA* population. In a recently published cohort study in over 19,000 Dutch women, the risk of breast cancer after exposure to ovarian stimulation for IVF was neither different from the risk of breast cancer in the general population (standardized incidence ratio 1.01, 95% CI 0.93-1.09) nor from the risk of breast cancer in a non-IVF infertile comparison group (HR 1.01, 95% CI 0.86-1.19).¹⁷ A recent meta-analysis¹⁶ drew the same conclusion: in a cumulative cohort of over 1.5 million women, no association was found between ovarian stimulation for IVF and breast cancer risk in the studies conducted in the entire general population (RR 0.91, 95% CI 0.74-1.11), nor in studies restricted to infertile women (RR 1.02, 95% CI 0.88-1.18).¹⁶

The role of estrogens in the pathophysiology of breast cancer in the general population has only been partly elucidated. The dissimilarities in risks observed associated with exposure to long-lasting low levels of estrogens, as is the case in oral contraceptives use and hormone replacement therapy,^{29,30} and with exposure to peak levels of estrogens during a short period of time as in IVF are not yet understood. The existence of an estrogen-receptor alpha (ER α) dependent and independent pathway has been suggested in breast carcinogenesis.¹² The estrogen-receptor independent route consists of the enzymatic conversion of estrogens into metabolites that damage the DNA.³¹ Since the *BRCA1* and *BRCA2* gene are involved in DNA repair, it is possible that the potential detrimental effect of estrogens on mammary tissue is aggravated in *BRCA1/2* mutation carriers.

We speculated that the effect of exposure to ovarian stimulation for IVF on breast cancer risk might be different in *BRCA1* and/or *BRCA2* mutation carriers from the effect in the general population. Our previous observation of a breast cancer diagnosis in a young *BRCA1* mutation carrier shortly after ovarian stimulation for IVF/PGD despite normal MRI screening just before treatment,¹ could either be the result of a shorter time interval between MRIs or of a stimulating effect of elevated estrogen levels on growth of pre-existing cancerous cell foci. *BRCA1/2* mutation-associated breast tumors exhibit histopathologic features different from non-*BRCA1/2* mutation-associated breast cancers and tend to be fast-growing. Although a surplus of *BRCA1* mutation-associated tumors are triple negative (i.e., with a negative estrogen and progesterone receptor and Her2Neu status),³²⁻³⁶ an effect of hormonal factors on cancer risk was observed in these genetically predisposed women as well. In *BRCA1* mutation carriers a late age at menarche, late age at first birth, and breastfeeding appear to reduce the risk of breast cancer, while in *BRCA2* mutation carriers no protective factors have been identified up to now.³⁷ However, not all observations

support a (substantial) role of estrogens in the etiology of *BRCA*-associated breast cancer. For instance, whereas retrospective studies suggested that a RRSO reduced the risk for breast cancer with approximately 50%, there was no evidence for a short- or medium-term protective effect in a more recent study using a different design, maximally eliminating bias.²⁸ Prospective studies are needed to provide more insight into the etiology of breast cancer in *BRCA1/2* mutation carriers and to further assess the involvement of hormonal factors in the pathophysiology.

There are several potential explanations for the lack of an effect of exposure to ovarian stimulation for IVF and the risk of breast cancer in our study: (1) there is an effect, but the effect size was too small to be detected due to limited power or observation time in the current study, (2) there is no true effect, as (a) ovarian stimulation for IVF has no influence on breast tissue carcinogenesis in *BRCA1/2* mutation carriers, or (b) a protective influence of temporary low levels of estrogens and progesterone due to down regulation of the natural cycle in IVF practice which directs the effect size to the null, as suggested by van den Belt-Dusebout.¹⁷

In total, three percent of *BRCA1/2* mutation carriers included in the current study were exposed to IVF. The exposed group consisted of both women undergoing IVF because of infertility as well as women opting for IVF because of PGD. After correction for oversampling by excluding the women ascertained via the national PGD registry, 2% of the women were exposed to IVF. In the general population approximately 1% of all women are exposed to IVF.³⁸ Several studies have suggested a reduced ovarian reserve in female carriers of a *BRCA1* mutation,³⁹⁻⁴³ but there is no convincing evidence for a clinically relevant adverse effect on fecundity.

Our study has several limitations. Firstly, despite the availability of a nation-wide *BRCA1* and *BRCA2* mutation cohort, questions regarding fertility treatments were only recently added to the questionnaire and power was still limited due to the low proportion of women exposed to IVF. To increase power we included exposed carriers that were so far not participating in HEBON or were exposed to IVF after completion of the questionnaire, through a merge of the HEBON cohort with *BRCA1/2* mutation carriers included in the PGD registry. Although there was a difference in data collection between the two sources, self-reported in a questionnaire versus medical records, it is unlikely that IVF exposure (yes/no) was confounded by recall bias. The difference in age at censoring between the included groups HEBON and PGD is accounted for by the age-dependent analysis. Secondly, the subgroup of *BRCA2* mutation carriers was too small for a separate analysis (only three exposed *BRCA2* mutation carriers were diagnosed with breast cancer). Thirdly, the retrospective study design may have resulted in survival bias if IVF exposed women developed tumors with worse prognosis. Although the interval between breast cancer diagnosis and age at questionnaire was somewhat lower in IVF-exposed women compared to IVF-unexposed women (7.1 years $\pm$ 5.5 years versus 10.5 $\pm$ 8.0, respectively), this difference was not statistically significant ($p = 0.052$). Fourthly, since data were self-

reported specific information on IVF protocols used was missing. In addition, in the questionnaire women were only asked to fill in their IVF history if they were infertile. However, since fertile women with a PGD indication for IVF treatment were included by the PGD registry, only women opting for IVF treatment because of male infertility have possibly been missed, which was the case in 22.3% of the population of IVF-exposed women.¹⁷ Lastly, in our retrospective cohort approach breast cancer patients were oversampled due to the non-random uptake of DNA test. To account for this a weighted cohort approach is suggested by Antoniou *et al.*,⁴⁴ in which women with breast cancer and unaffected women are differentially weighted such that the breast cancer incidence rates in the study cohort are consistent with age- and birth cohort-specific breast cancer risk estimates for *BRCA1* and *BRCA2* mutation carriers. Unfortunately, power per age group was too limited to calculate reliable weights.

Conclusion and recommendations

We did not find an increased risk of breast cancer after ovarian stimulation for IVF in *BRCA1/2* mutation carriers. Obviously, ruling out pre-existing lesions before an IVF treatment is started remains important, but based on the present knowledge there is no reason to exclude women with a *BRCA1/2* mutation from IVF for fertility treatment, fertility preservation, or PGD.

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Part III

Ovarian reserve in female
BRCA1/2 mutation carriers

Chapter 6

***BRCA1* mutation carriers have a lower number of mature oocytes after ovarian stimulation for IVF/PGD**

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Abstract

Purpose

The aim of this study was to determine whether *BRCA1/2* mutation carriers produce fewer mature oocytes after ovarian stimulation for *in vitro* fertilization (IVF) with preimplantation genetic diagnosis (PGD), in comparison to a PGD control group.

Methods

A retrospective, international, multicenter cohort study was performed on data of first PGD cycles performed between January 2006 and September 2015. Data were extracted from medical files. The study was performed in one PGD center and three affiliated IVF centers in the Netherlands and one PGD center in Belgium.

Exposed couples underwent PGD because of a pathogenic *BRCA1/2* mutation, controls for other monogenic conditions. Only couples treated in a long Gonadotropin-Releasing Hormone (GnRH) agonist-suppressive protocol, stimulated with at least 150 IU follicle stimulating hormone (FSH), were included. Women suspected to have a diminished ovarian reserve status due to chemotherapy, auto-immune disorders, or genetic conditions (other than *BRCA1/2* mutations) were excluded. A total of 106 *BRCA1/2* mutation carriers underwent PGD in this period, of which 43 (20 *BRCA1* and 23 *BRCA2* mutation carriers) met the inclusion criteria. They were compared to 174 controls selected by frequency matching.

Results

Thirty-eight *BRCA1/2* mutation carriers (18 *BRCA1* and 20 *BRCA2* mutation carriers) and 154 controls proceeded to oocyte pick-up. The median number of mature oocytes was 7.0 (interquartile range (IQR) 4.0-9.0) in the BRCA group as a whole, 6.5 (IQR 4.0-8.0) in *BRCA1* mutation carriers, 7.5 (IQR 5.5-9.0) in *BRCA2* mutation carriers, and 8.0 (IQR 6.0-11.0) in controls. Multiple linear regression analysis with the number of mature oocytes as dependent variable and adjustment for treatment center, female age, female body mass index (BMI), type of gonadotropin used, and the total dose of gonadotropins administered revealed a significantly lower yield of mature oocytes in the BRCA group as compared to controls ($p = 0.04$). This finding could be fully accounted for by the *BRCA1* subgroup (*BRCA1* mutation carriers versus controls $p = 0.02$, *BRCA2* mutation carriers versus controls $p = 0.50$).

Conclusions

Ovarian response to stimulation, expressed as the number of mature oocytes, was reduced in *BRCA1* but not in *BRCA2* mutation carriers. Although oocyte yield was in correspondence to a normal response in all subgroups, this finding points to a possible negative influence of the *BRCA1* gene on ovarian reserve.

Introduction

Contradicting results have been published on a potential influence of mutations in the *BRCA1* and *BRCA2* gene on ovarian reserve. Mutations in the *BRCA* genes are primarily known for their predisposition to breast and ovarian cancer.¹ The *BRCA* genes act as tumor suppressor genes and are involved in DNA double-strand break repair.² An impaired function leads to an accumulation of intracellular DNA damage. This may affect cellular growth mechanisms, leading to carcinogenic transformation.³ Alternatively, accumulating DNA damage may induce growth arrest, leading to apoptosis.⁴ Hypothetically, this may be illustrated in non-dividing cell populations, e.g., the ovarian follicle pool.

Oktay et al.⁵ were the first to observe a reduced ovarian response to ovarian stimulation for *in vitro* fertilization (IVF) in *BRCA1* mutation-positive cancer patients undergoing fertility preservation. This was not confirmed by another report on the ovarian response to IVF stimulation in a combined group of *BRCA1/2* mutation carriers undergoing fertility preservation because of breast cancer and asymptomatic *BRCA1/2* mutation carriers undergoing IVF with preimplantation genetic diagnosis (PGD).⁶ Contradicting results have also been published when assessing ovarian reserve in *BRCA1/2* mutation carriers using other endpoints. Several studies on age of natural menopause reported an earlier menopause in both *BRCA1* and *BRCA2* mutation carriers.⁷⁻⁹ The majority of studies using anti-Müllerian hormone (AMH) as an indicator for the number of (pre-)antral follicles in the ovaries detected lower levels of AMH in *BRCA1* mutation carriers, not in *BRCA2* mutation carriers.¹⁰⁻¹³ Studies using several other reproductive outcome parameters (e.g., parity) did not point to a reduced fecundity in *BRCA1/2* mutation carriers.¹⁴⁻¹⁸

Ovarian response to stimulation for IVF is a strong indicator for ovarian reserve status.¹⁹ Sufficient ovarian response is particularly important in PGD, where transfer criteria primarily involve genetic results. After a second selection on embryo quality, only a minority of the obtained embryos will be available for transfer. If a mutation in the *BRCA1* and/or *BRCA2* gene is associated with a lower ovarian reserve, this may have a negative effect on success chances of mutation carriers undergoing IVF for infertility reasons, for fertility preservation, as well as for PGD. PGD for *BRCA1/2* mutations has been performed for a decade now and the number of couples treated each year has been growing steadily.^{20,21}

The objective of the current study is to clarify whether *BRCA1/2* mutation carriers produce less mature oocytes after ovarian stimulation for IVF/PGD.

Material and methods

A retrospective, observational cohort study was carried out in five centers: Maastricht University Medical Center (center 1) and affiliated IVF-centers University Medical Center Utrecht (center 2), University Medical Center Groningen (center 3), and Academic Medical Center Amsterdam (center 4), united in the Dutch consortium for PGD, and Universitair Ziekenhuis Brussel, Brussels, Belgium (center 5). The study period lasted from the introduction of PGD for hereditary cancer syndromes (i.e., 2006 for Brussels and 2008 for the Netherlands) until September 2015.

The exposed group consisted of couples who underwent IVF/PGD because of a pathogenic mutation in the *BRCA1* or *BRCA2* gene in the female (the 'BRCA group'). All mutations were proven pathogenic by means that they had a verified significant disturbing effect on protein translation. The control group consisted of couples who underwent PGD because of an autosomal dominant or recessive disorder not known to be associated with a reduced ovarian reserve. For the selection of controls, frequency matching was used: control couples were selected blinded for outcome, based on treatment center and treatment period in order to obtain an equal distribution in both groups.²² For this purpose a chronological overview of PGD treatments performed per PGD center for autosomal dominant and recessive disorders (excluding conditions known for a (potential) effect on ovarian reserve (e.g., fragile X syndrome, myotonic dystrophy type 1) and male *BRCA1/2* mutation carriership) was created. Matching was done per PGD center: PGD treatments for female *BRCA1/2* mutations were identified and (if available) four PGD treatments for autosomal dominant or recessive disorders chronologically performed closely before or after the PGD treatment for *BRCA1/2* were included as controls. In order to rule out bias from repetitive cycles, only first treatment cycles were included. First cycles with and without oocyte pick-up were included in order to assess the cancellation rate because of poor ovarian response in both groups.

Only treatments in a long Gonadotropin-Releasing Hormone (GnRH) agonist-suppressive protocol, with stimulation with at least 150 IU follicle stimulating hormone (FSH) or human Menopausal Gonadotropin (hMG) per day, were included in order to obtain a homogenous study population with optimal ovarian stimulation.²³ Other inclusion criteria for both groups were: female age <43 years, female body mass index (BMI) <35 kg/m², and female endogenous FSH <15 IU/l. Exclusion criteria were a history of invasive (breast) cancer up to two years prior to IVF/PGD treatment, ovarian surgery, chemotherapy, pelvic radiation, polycystic ovary syndrome that conforms the Rotterdam criteria,²⁴ and known endocrine, autoimmune, or genetic abnormalities (potentially) associated with a reduced ovarian reserve (e.g., Fragile X premutation carriers, myotonic dystrophy type 1). Final oocyte maturation was induced when sufficient dominant follicles were seen at ultrasound (i.e., at least four follicles >14 millimeters in the Netherlands and at least three follicles >17 millimeters in

Brussels). The number of mature oocytes was assessed at the moment of intracytoplasmic sperm injection (ICSI). ICSI was used for fertilization in order to avoid contamination of the zona pellicuda with residual spermatozoa. Embryo biopsy was performed on day three after fertilization. Single cell analysis of the removed blastomeres was performed using multiplex polymerase chain reaction (PCR), as described elsewhere.^{20,25,26} Data were extracted from medical files.

Ethical approval

The study was approved by the Institutional Review Boards of Maastricht University Medical Center (METC 14-4-163) and Universitair Ziekenhuis Brussel (2014/383). All couples gave their written informed consent for IVF/PGD treatment and the usage of their PGD data for scientific research before the treatment was started.

Statistical analysis

Patient characteristics and outcome data are presented as mean and standard deviation, median and interquartile range (IQR), or frequency and percentage, depending on the distribution of the variable. Where outcome data were not normally distributed, bivariate analyses were performed using non-parametric tests (Mann-Whitney U test). A linear regression model was used to assess an association between *BRCA1/2* mutation status and ovarian response in terms of number of obtained mature oocytes. The number of mature oocytes was transformed using the square root, in order to obtain an approximately normal distribution of the residuals. Adjustments were made for potential confounding factors, i.e., treatment center, female age, female BMI, type of gonadotropin administered (FSH or hMG), and total dose of gonadotropin administered. These factors were incorporated because of a potential negative influence of an advanced age, higher BMI, and the use of hMG on the number of mature oocytes yielded and because an effect of the treatment center and the cumulative dose of gonadotropins applied could not be ruled out. Age and BMI were both assessed as continuous and categorical variables (age ≤ 30 versus >30 years, age ≤ 35 versus >35 years, BMI ≤ 25 versus BMI >25). Subgroup analyses were conducted to determine potential differences in primary outcome between *BRCA1* mutation carriers and the control group and *BRCA2* mutation carriers and the control group. A sensitivity analysis was performed excluding center 5, since this center used the long agonist protocol particularly for expected poor responders. Statistical analyses were performed using SAS statistical analysis software for Windows, version 9.3.

The study was powered on a previously reported difference in obtained oocytes following IVF in *BRCA* carriers (7.9 (95% CI 4.6-13.8) oocytes in *BRCA* mutation carriers compared to 11.3 (95% CI 9.1-14.1) oocytes in women without a *BRCA* mutation).⁵ The

inclusion of 50 *BRCA* mutation carriers and 200 controls would be sufficient to detect a difference of the aforementioned magnitude, with alpha set at 0.05 and beta at 0.8.

Results

Patient characteristics

In total, 106 female *BRCA1/2* mutation carriers underwent PGD in the study period, of whom 66 (62.3%) had a *BRCA1* mutation and 40 (37.7%) a *BRCA2* mutation. Twelve mutation carriers had a history of invasive breast cancer and chemotherapy (nine *BRCA1* and three *BRCA2* mutation carriers), 51 mutation carriers were excluded for other reasons (Table 6.1). Of the 43 included mutation carriers, twenty (46.5%) had a *BRCA1* mutation and 23 (53.5%) a *BRCA2* mutation. Of the 174 controls, 119 (68.4%) underwent PGD because of an autosomal dominant condition and 55 (31.6%) because of an autosomal recessive condition (Table 6.2). An overview of the distribution of the couples over the five centers is provided in Supplemental Table S6.1.

Table 6.1 The number of eligible women and the reasons for exclusion

	BRCA group n = 106	Control group n = 174
Reason for exclusion (n)		
Breast cancer + chemotherapy	12 ^a	
Endocrine / auto-immune disorder	5 ^b	
Polycystic ovary syndrome	3	
Ovarian surgery	1	
Other genetic condition	1 ^c	
Regular IVF prior to PGD	0	
Different IVF protocols ^d	36	
Only cycles with <150 IU FSH per day	5	
First cycles included (n)	43	174
Cancel in the first cycle (n, %)	5/43 (11.6%)	20/174 (11.5%)
First cycles with oocyte pick-up	38/43 (88.4%)	154/174 (88.5%)

BRCA, breast cancer gene; n, number of women; IVF, *in vitro* fertilization; PGD, preimplantation genetic diagnosis; IU, international units; FSH, follicle stimulating hormone

^a Five of these women were also treated in an IVF protocol other than a long GnRH agonist-suppressive protocol

^b Two of these women were also treated in an IVF protocol other than a long GnRH agonist-suppressive protocol

^c Female *CHEK2* mutation

^d Treatment in an IVF protocol other than a long GnRH agonist-suppressive protocol

Table 6.2 Patient characteristics

	BRCA group n = 43	Control group n = 174
Female age (mean, SD)	31.4 ± 3.7	32.1 ± 4.1
Female BMI (mean, SD)	23.8 ± 3.0	23.9 ± 3.5
AD disorders	43 (100.0%)	119 (68.4%)
Female carriers	42	59 ^a
Male carriers	n/a	57
Both partners	1	3
BRCA1	20 (46.5%)	n/a
BRCA2	22 (51.2%)	n/a
BRCA2 female + retinoblastoma male	1 (2.3%)	n/a
Huntington	n/a	25 ^b
Neurofibromatosis type 1	n/a	12 ^c
Myotonic dystrophy ^d	n/a	10
Familial adenomatous polyposis	n/a	10
Spinocerebellar ataxia type 3	n/a	8
Marfan syndrome	n/a	7
Other	n/a	47 ^e
AR disorders	n/a	55 (31.6%)
Cystic fibrosis	n/a	16
Spinal muscular atrophy	n/a	13 ^e
Other	n/a	26

n, number of women; SD, standard deviation; BMI, body mass index; AD, autosomal dominant; *BRCA1*, breast cancer gene 1; *BRCA2*, breast cancer gene 2; AR, autosomal recessive; n/a, not applicable

^a One woman had both Peutz Jeghers syndrome and porencephalia

^b Of which five couples opted for exclusion PGD

^c Of which two couples had two indications for PGD

^d Only males with myotonic dystrophy type 1 were included, since myotonic dystrophy type 1 is potentially associated with a reduced ovarian reserve

^e Of which one couple had two indications for PGD

Bivariate analyses

Thirty-eight out of 43 BRCA cycles and 154 out of 174 control cycles proceeded to oocyte pick-up. The cancellation rate due to a poor response was 3/43 (7.0%) in the BRCA group and 16/174 (9.3%) in the control group ($p = 0.35$). The median number of cumulus oocyte complexes was 9.0 (IQR 5.8-11.0) and 10.0 (IQR 7.0-14.0) in the BRCA and control group, respectively ($p = 0.05$, Table 6.3). The median number of mature oocytes was 7.0 (IQR 4.0-9.0) and 8.0 (IQR 6.0-11.0, $p = 0.02$), respectively. The observed difference in mature oocytes could be fully accounted to women with a *BRCA1* mutation: *BRCA1* mutation carriers ($n = 18$) produced a median of 6.5 (IQR 4.0-8.0) mature oocytes, compared to 8.0 (IQR 6.0-11.0) in the control group ($p = 0.01$). This difference was not observed in the *BRCA2* subgroup ($n = 20$, median 7.5 (IQR 5.5-9.0) in the *BRCA2* subgroup, $p = 0.20$).

There was no difference in the cumulative dose of exogenous FSH administered between groups (1987.5 IU (IQR 1762.5-2812.5 IU) in the BRCA group as a whole,

1950.0 IU (IQR 1650.0-2550.0 IU) in the BRCA1 subgroup, 2137.5 IU (IQR 1800.0-3356.3 IU) in the BRCA2 subgroup, and 1950.0 IU (IQR 1650.0-2575.0 IU) in controls (all $p > 0.05$, Table 6.3). As the number of mature oocytes was lower in the BRCA group, we explored whether the ratio of administered FSH per obtained mature oocyte obtained was higher in this group (i.e., whether *BRCA* mutation carriers needed more FSH to obtain the same amount of oocytes and/or produced less oocytes when the same dose of FSH was applied). In the BRCA group as a whole, more FSH was administered per obtained mature oocyte when compared to the control group (median ratio FSH/mature oocyte 353.0 (IQR 210.7-521.9) and 250.0 (IQR 168.6-375.0) respectively, $p = 0.03$). FSH/mature oocyte ratio was highest in the BRCA1 subgroup (median ratio FSH/mature oocyte 383.0 (IQR 208.3-521.9) in the BRCA1 subgroup and 326.3 (IQR 203.6-600.0) in the BRCA2 subgroup). The fraction of normally fertilized oocytes (2PN oocytes) was comparable between groups (Table 6.3). The pregnancy rate was lower in women with a *BRCA1* mutation but this did not reach significance.

Multivariable analyses

Linear regression analyses with the square root transformed number of mature oocytes as the dependent variable showed that the difference in the number of mature oocytes between the BRCA group and control group remained statistically significant after adjustment for treatment center, female age, female BMI, type of gonadotropin (FSH or hMG), and cumulative dose of FSH administered ($p = 0.04$, Table 6.4). Again, this difference was only present in *BRCA1* mutation carriers as compared to controls ($p = 0.02$), not in *BRCA2* mutation carriers ($p = 0.50$).

Additional analyses were performed to allow for a possible non-linear effect of female age and female BMI on the number of mature oocytes, by introducing these variables as dichotomous (instead of linear) variable in the multivariable model (age ≤ 30 versus > 30 years, age ≤ 35 versus > 35 years, and BMI ≤ 25 versus BMI > 25). This did not change the outcome. A sensitivity analyses excluding center 5 (as stated above, the fact that in this center the long agonist protocol was primarily used for expected poor responders could have introduced bias) did neither change the outcome.

Table 6.3 First IVF/PGD cycles

	BRCA group (n = 38)	Control group (n = 154)	p-value	BRCA1 subgroup (n = 18)	p-value ^a	BRCA2 subgroup (n = 20)	p-value ^a
Cumulative dose of exogenous FSH administered (median, IQR) ^b	1987.5 (1762.5-2812.5)	1950.0 (1650.0-2575.0)	0.65	1950.0 (1650.0-2550.0)	0.98	2137.5 (1800.0-3356.3)	0.49
Cumulus oocyte complexes (median, IQR) ^b	9.0 (5.8-11.0)	10.0 (7.0-14.0)	0.05	8.5 (5.0-11.3)	0.13	9.0 (6.0-10.8)	0.14
Mature oocytes (median, IQR) ^b	7.0 (4.0-9.0)	8.0 (6.0-11.0)	0.02	6.5 (4.0-8.0)	0.02	7.5 (5.5-9.0)	0.20
FSH/mature oocyte (median, IQR) ^b	353.0 (210.7-521.9)	250.0 (168.6-375.0)	0.03	383.0 (208.3-521.9)	0.06	326.3 (203.6-600.0)	0.14
Fraction of normally fertilized oocytes (2 PN) per injected oocyte (median, IQR) ^b	0.7 (0.7-0.8)	0.7 (0.6-0.9)	0.89	0.8 (0.7-0.8)	0.46	0.7 (0.6-0.8)	0.62
Fraction of embryos biopsied for PGD per injected oocyte (median, IQR) ^b	0.7 (0.6-0.8)	0.7 (0.6-0.8)	0.63	0.8 (0.7-0.8)	0.21	0.7 (0.5-0.8)	0.63
Fraction of aneuploid embryos per injected oocyte (median, IQR) ^{b,c}	0.0 (0.0-0.1)	0.1 (0.0-0.2)	0.04	0.1 (0.0-0.2)	0.95	0.0 (0.0-0.0)	0.00
Cycles with embryonic transfer ^d	34/38 (89.5%)	129/154 (83.8%)	0.38	17/18 (94.4%)	0.23	17/20 (85.0%)	0.89
Pregnancy with fetal heart beat at 7 weeks of gestation (n, %) ^{d,e}	10/34 (29.4%)	39/129 (30.2%)	0.93	3/17 (17.6%)	0.28	7/17 (41.2%)	0.36

IVF, *in vitro* fertilization; PGD, preimplantation genetic diagnosis; BRCA, breast cancer gene; BRCA1, breast cancer gene 1; BRCA2, breast cancer gene 2; n, number of women; FSH, follicle stimulating hormone; IQR, interquartile range; PN, pronuclei

^a Compared to the control group

^b Analyzed using Mann-Whitney U tests

^c Aneuploid for the chromosome analyzed during PGD

^d Analyzed using Chi-square tests

^e Only cycles included which resulted in embryonic transfer

Table 6.4 Multivariable analyses

	Number of mature oocytes (linear regression analysis)		
	B	SE	p
<i>BRCA1/2</i> vs. controls	-0.28	0.13	0.04
<i>BRCA1</i> vs. controls	-0.45	0.18	0.02
<i>BRCA2</i> vs. controls	-0.12	0.17	0.50

BRCA1, breast cancer gene 1; *BRCA2*, breast cancer gene 2

Adjusted for treatment center, female age, female body mass index, type of gonadotropin used, and total dosage of gonadotropins administered

Discussion

In this study, a lower number of mature oocytes was found in women with a *BRCA1* mutation in response to ovarian stimulation for IVF/PGD.

Diverse studies have been reported on a possible diminished ovarian reserve in *BRCA* mutation carriers, using different primary outcomes and study designs. Oktay et al.⁵ were the first to report a lower yield of oocytes in eight *BRCA1*, but not in four *BRCA2* mutated breast cancer patients. A case-control study by Shapira et al.⁶ found no difference in oocyte yield according to *BRCA* mutation status in 62 *BRCA* mutation-positive women. However, the inclusion of cancer patients and patients stimulated in different IVF protocols, and the lack of clarity regarding minimal stimulation doses applied may have obscured an existing difference.

Previous studies on ovarian reserve in *BRCA1/2* mutation carriers using non-IVF related parameters did not show consistent results. It is challenging however to study ovarian reserve in *BRCA1/2* mutation carriers because of the presence of several confounding factors in this particular population. Firstly, (breast) cancer itself^{27,28} as well as its potential gonadotoxic treatment²⁹ has a negative effect on ovarian reserve. Secondly, many *BRCA1/2* mutation carriers opt for a risk-reducing salpingo-oophorectomy. The timing of this event may be influenced by personal cancer history and related to the menopausal transition. As a consequence, studies on age at natural menopause in *BRCA1/2* mutation carriers have important limitations, as set out by van Tilborg et al.³⁰ Two studies reported a younger age of natural menopause in both *BRCA1* and *BRCA2* mutation carriers.^{8,9} A third study found a younger age of menopause in *BRCA1* mutation carriers with and without breast cancer.⁷ Two other studies did not detect a difference in age of natural menopause between carriers and non-carriers.^{30,31} Studies were troubled by both the inclusion⁷ and exclusion⁸ of breast cancer patients, the exclusion of women who experienced menopause due to other reasons than natural menopause,⁸ bias resulting from informative censoring due to risk-reducing salpingo-oophorectomy uptake,⁹ the inclusion of only few women who had actually reach natural menopause,³¹ and/or other forms of bias.³⁰ Three studies

have found a lower AMH in *BRCA1* mutation carriers and not in *BRCA2* mutation carriers,^{10,12,13} while two other studies did not detect a difference between *BRCA1* and/or *BRCA2* mutation carriers and controls.^{11,32} Differences in outcome may be the result of variances in study design, in particular the inclusion of breast cancer patients¹⁰ and women with irregular menstrual cycles and/or polycystic ovary syndrome,¹¹⁻¹³ the lack of appropriate adjustment for potential confounding factors in the analysis,^{10,11} and/or power issues.³² Pregnancy rate and parity in *BRCA1/2* mutation carriers was not different from controls.¹⁴⁻¹⁶ Some studies even report more pregnancies and children born per mother among *BRCA1/2* mutation carriers.^{17,18}

Our study provides additional evidence for a reduced ovarian reserve in *BRCA1* mutation carriers, although the effect size was rather small and the oocyte yield was in the range of a normal response for all subgroups. Consequently, our finding may be more interesting from a biological point of view than relevant for clinical practice. The strengths of our study are (1) the large homogeneous cohort of *BRCA1/2* mutation carriers without recent malignant disease,^{27,28} (2) the use of the same IVF protocol including only first cycles, and (3) the application of frequency matching, resulting in a representative control group. Our study also has limitations, mainly associated with the retrospective study design although the most important outcome data were complete for all inclusions (Supplemental Table S6.2). Firstly, during the study period different IVF protocols were used in the participating centers. In order to obtain a homogeneous stimulated cohort, we only included couples treated in a long GnRH agonist-suppressive protocol with at least 150 IU gonadotropins per day. This selection led to a smaller cohort than initially powered. Nevertheless, the effect size in the *BRCA1* subgroup was large enough to be detectable. Additionally, this strategy may have introduced bias due to the exclusion of expected hyperresponders (treated with lower doses of FSH per day) and the inclusion of an excess of suspected poor responders, since in center five this IVF protocol was only the first choice in this subgroup of patients. However, this may have had an effect on both the *BRCA* and control groups and a sensitivity analysis excluding center five did not change the primary outcome. Secondly, since the poor response rate was (non-significantly) higher in the control group, this could have biased our primary outcome. Thirdly, we did not have the opportunity to correct for lifestyle factors (e.g., smoking). Finally, the *BRCA1* and *BRCA2* subgroups were still relatively small. Consequentially, the absence of an effect of *BRCA2* dysfunction on ovarian response may have been the result of insufficient power.

Despite these limitations, our finding of an impaired response to ovarian stimulation in *BRCA1* mutation carriers and not in *BRCA2* mutation carriers is interesting and confirms several previous studies. The absence of a(n) (detectable) effect of *BRCA2* dysfunction on ovarian reserve in most studies may be the result of a true lack of a difference, of insufficient power, and/or of either a later in life occurring or more subtle decline in ovarian reserve, corresponding to the lower risk and higher age at

diagnosis of breast and ovarian cancer associated with *BRCA2* mutations.³³ Both *BRCA* genes are involved in DNA double-strand break repair, but their biological functions differ. The association between *BRCA1* and *BRCA2* and (reproductive) ageing is demonstrated by the involvement of the *BRCA* genes in telomere maintenance: telomeres shorten with age.^{34,35} In human oocytes, DNA double-strand breaks are more prevalent with increasing age, while *BRCA1* expression is reduced by then.¹⁰ *BRCA1* plays an important role in meiotic spindle formation in mice and *BRCA1* mutant mice had fewer primordial follicles, produced fewer oocytes in response to ovarian stimulation, had a smaller litter size, and showed more DNA double-strand breaks in their oocytes with increasing age than wild-type mice.^{10,36} *BRCA2* dysfunction in mice has been associated with insufficient spermatogenesis, a depletion of germ cells in female mice, and a higher frequency of nuclear aberrations in mutant oocytes.³⁷ However, the involvement of *BRCA2* in DNA double-strand break repair is probably less comprehensive than *BRCA1* involvement.³⁸ Consequentially, it can be hypothesized that the effect of *BRCA2* dysfunction on ovarian reserve is less powerful than the effect of a *BRCA1* mutation and potentially only becomes visible at increasing age.

If *BRCA1/2* mutation carriers are affected with a reduced ovarian reserve this might have several clinical consequences, such as a higher need for fertility treatment, a worse treatment outcome, an urge for more treatment attempts and/or higher doses of fertility drugs. However, the size of the effect found in our study is probably too small to be of clinical relevance. Future clinical and molecular studies are needed to provide more insight into the role of the *BRCA(1)* gene(s) in the maintenance of the ovarian pool.

Conclusions

A reduced yield of mature oocytes was found in *BRCA1* mutation carriers undergoing IVF/PGD, suggesting a role of the *BRCA1* gene in the maintenance of ovarian reserve.

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Supplemental tables

Table S6.1 Inclusions per PGD center

	Center 1	Center 2	Center 3	Center 4	Center 5
Inclusions	72	60	30	14	41
BRCA	15	13	6	3	6
Control	57	47	24	11	35
Age (mean, SD)	31.6 ± 3.8	32.1 ± 3.5	30.7 ± 4.0	30.7 ± 5.5	33.7 ± 4.1
BMI (mean, SD)	24.5 ± 3.0	24.2 ± 3.6	23.7 ± 3.3	23.7 ± 3.9	22.6 ± 3.2
Couples with cancel in 1 st cycle due to poor response (n, %)	10/72 (13.9%)	6/60 (10.0%)	2/30 (6.7%)	1/14 (7.1%)	0/41 (0.0%)
BRCA (n, %)	1/15 (6.7%)	1/13 (7.7%)	1/6 (16.7%)	0/3 (0.0%)	0/6 (0.0%)
Control (n, %)	9/57 (15.8%)	5/47 (10.6%)	1/24 (4.2%)	1/11 (9.1%)	0/35 (0.0%)
First cycles with oocyte pick-up	59	53	28	13	39
BRCA	13 (22.0%)	11 (20.8%)	5 (17.9%)	3 (23.1%)	6 (15.4%)
Control	46 (78.0%)	42 (79.2%)	23 (82.1%)	10 (76.9%)	33 (84.6%)
Type of gonadotropin					
FSH	44 (74.6%)	52 (98.1%)	16 (57.1%)	0 (0.0%)	5 (12.8%)
hMG	9 (15.2%)	1 (1.9%)	12 (42.9%)	13 (100.0%)	34 (87.2%)
Missing	6 (10.2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Cumulative FSH dose (IU)	2250.0	1600.0	2250.0	1800.0	2250.0
(median, IQR)	(1950.0-3150.0)	(1500.0-1950.0)	(1950.0-2925.0)	(1650.0-2025.0)	(1800.0-2475.0)
Mature oocytes	7.0	8.0	9.0	8.0	9.0
(median, IQR)	(5.0-9.0)	(5.0-10.5)	(6.3-11.5)	(4.0-14.5)	(6.0-11.0)
Pregnancy with fetal heart beat at 7 weeks	14/59 (23.7%)	13/53 (24.5%)	3/28 (10.7%)	4/13 (30.8%)	15/39 (38.5%)
BRCA	3/13 (23.1%)	2/11 (18.2%)	0/5 (0.0%)	1/3 (33.3%)	4/6 (66.7%)
Control	11/46 (23.9%)	11/42 (26.2%)	3/23 (13.0%)	3/10 (30.0%)	11/33 (33.3%)

PGD, preimplantation genetic diagnosis; BRCA, breast cancer gene; SD, standard deviation; BMI, body mass index; n, number of couples; FSH, follicle stimulating hormone; hMG, human menopausal gonadotropin; IU, international units; IQR, interquartile range

Table S6.2 Missing data of included first cycles

	BRCA1 subgroup	BRCA2 subgroup	Control group
<i>BRCA1/2</i> mutation	0	0	n/a
Treatment center	0	0	0
Female age	0	0	0
Female BMI	0	0	0
Type of gonadotropin	1	0	5
Cumulative dose of exogenous FSH administered	0	0	0
Cumulus oocyte complexes	0	0	0
Mature oocytes	0	0	0
FSH/mature oocyte	0	0	0
Normally fertilized oocytes (2 PN)	0	0	7
Embryos biopsied for PGD	0	0	1
Aneuploid embryos	0	0	2
Lost to follow-up	0	0	0

BRCA1, breast cancer gene 1; *BRCA2*, breast cancer gene 2; BMI, body mass index; FSH, follicle stimulating hormone; PN, pronuclei; PGD, preimplantation genetic diagnosis; n/a, not applicable

Chapter 7

Serum AMH levels in healthy women from *BRCA1/2* mutated families: are they reduced?

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Abstract

Study question

Do *BRCA1/2* mutation carriers have a compromised ovarian reserve compared to proven non-carriers, based on serum anti-Müllerian hormone (AMH) levels?

Summary answer

BRCA1/2 mutation carriers do not show a lower serum AMH level in comparison to proven non-carriers, after adjustment for potential confounders.

What is known already

It has been suggested that the *BRCA* genes play a role in the process of ovarian reserve depletion, although previous studies have shown inconsistent results regarding the association between serum AMH levels and *BRCA* mutation status. Hence, it is yet unclear whether *BRCA1/2* mutation carriers may indeed be at risk of a reduced reproductive lifespan.

Study design, size, duration

A multi-center, cross-sectional study was performed between January 2012 and February 2015 in 255 women. We needed to include 120 *BRCA1/2* mutation carriers and 120 proven non-carriers to demonstrate a difference in AMH levels of 0.40 µg/l (SD ± 0.12 µg/l, two-sided alpha-error 0.05, power 80%).

Participants/materials, setting, method

Healthy women aged 18-45 years who were referred to the Clinical Genetics department and applied for predictive *BRCA1/2* testing because of a familial *BRCA1/2* mutation were asked to participate. A cross-sectional assessment was performed by measuring serum AMH levels and filling out a questionnaire. Multivariate linear regression analyses adjusted for age, current smoking, and current hormonal contraceptive use were performed on log-transformed serum AMH levels.

Main results and the role of chance

Out of 823 potentially eligible women, 421 (51.2%) were willing to participate, and of those, 166 (39%) did not meet our inclusion criteria. Two hundred and fifty-five women were available for analyses; 124 *BRCA1/2* mutation carriers and 131 proven non-carriers. The median AMH level in carriers was 1.90 µg/l (range 0.11-19.00 µg/l) compared to 1.80 µg/l (range 0.11-10.00 µg/l) in non-carriers ($p = 0.34$). Adjusted linear regression analysis revealed no reduction in AMH level in the carriers (relative change = 0.98 (95% CI 0.77-1.22, $p = 0.76$).

Limitations, reasons for caution

Participants were relatively young. Besides, power was insufficient to analyze *BRCA1* and *BRCA2* mutation carriers separately. AMH levels may have been influenced by the use of hormonal contraceptives, though similar proportions of carriers and non-carriers were current users and adjustments were made to correct for potential confounding in our analysis.

Wider implications of the findings

Limitations of the current analysis and limitations of the existing literature argue for prospective, well-controlled follow-up studies with recurrent AMH measurements to determine whether *BRCA1/2* mutation carriers might be at risk for low ovarian reserve and to definitively guide care.

Trial registration number

NTR no. 4324.

Introduction

Ovarian aging is the result of the decrease in ovarian reserve, which consists of both the quantity and quality of oocytes, and will eventually lead to menopause. The wide range in age at natural menopause (ANM) in the general population (40-60 years) underlines the substantial variation in this aging process.^{1,2} Timing of menopause is associated with the onset of multiple women's health risks.^{3,4} Therefore, studies on factors that determine decline of ovarian reserve and ANM can help to unravel the underlying biological pathways and the mechanisms of the associated infertility and health risks.

The process of ovarian reserve decline and its variability is mostly explained by a combination of genetic^{5,6} and non-genetic factors such as lifestyle and environment.⁷⁻⁹ Genome wide association studies have identified loci associated with ANM that are mainly involved in DNA repair and immune function.^{5,10} It has been suggested that the *BRCA* genes, which are involved in DNA double-strand break (DSB) repair, are not only of importance in the prevention of cancer but also play a role in the process of ovarian reserve depletion.¹⁰⁻¹² A recent review describes the association between several types of DNA damage and repair on the one hand and aging on the other, and mentions the influence of DNA DSB repair on both cell death and senescence.¹³ An experimental study has demonstrated that *BRCA1* mutant mice have fewer oocytes at birth and that down regulation of *BRCA1* in mice oocytes leads to an increased sensitivity to environmental stress, accumulation of DSBs and cell death.¹² In human studies, contradicting results have been found regarding *BRCA* mutation status and ovarian response to controlled ovarian hyperstimulation.^{14,15}

Anti-Müllerian hormone (AMH) is produced by granulosa cells of ovarian follicles during the later stages of follicle development, and is an accurate biomarker to assess the quantitative ovarian reserve.¹⁶⁻¹⁸ Some studies have reported a significant negative association between *BRCA* mutation status and AMH levels,^{12,19,20} although others could not confirm this finding.²¹ Also, studies have failed to show a reduced fertility defined as number of pregnancies or self-reported fertility problems in *BRCA* mutations carriers.^{20,22-26}

Taken together, it is currently unclear whether there is an association between *BRCA* mutation status and the pacing of the ovarian aging process. If the *BRCA1/2* genes are identified to play a role in the process of declining ovarian reserve, an impact can be assumed on general health and fertility of women carrying a *BRCA* mutation.^{3,4} This study therefore aims to refine previous findings that suggested a reduced quantitative ovarian reserve in *BRCA* mutation carriers, by comparing serum AMH levels between proven *BRCA1/2* mutation carriers and proven non-carriers.

Methods

Design, participants and data collection

We performed a multi-center cross-sectional study to investigate the possible association between *BRCA1/2* mutation status and ovarian reserve, measured by serum AMH level. Subsequently, we assessed the reproductive history by using self-reported data obtained by a questionnaire.

All healthy women aged 18-45 years who were referred to the genetics department and applied for predictive *BRCA1/2* testing because of a familial *BRCA1/2* mutation, were recruited between January 2012 and February 2015. Two approaches were used for participant recruitment: in approach A, participants provided an additional blood sample at the moment of blood collection for the DNA test, while in approach B, women who had had a predictive DNA-test in the previous five years and therefore a known *BRCA1/2* mutation status, were asked to visit the hospital once for taking a blood sample. Both mutation carriers and non-carriers were included by using approach B. Two centers used both recruitment methods (University Medical Center Utrecht, Maastricht University Medical Center), the other centers only used approach A.

When eligible for participation, women applying for *BRCA* screening had to fulfill the criterion of having a regular menstrual cycle (i.e., mean cycle length of 21-35 days, with the next menstrual period predictable within a seven days' time frame), or having a history of a regular menstrual cycle previous to current usage of hormonal contraceptives. This criterion was applied to exclude cases with polycystic ovary syndrome (PCOS). Other reasons for exclusion were a history of breast and/or ovarian cancer, a surgical menopause event (i.e., premenopausal hysterectomy and/or bilateral oophorectomy), a history of ovarian surgery, a history of chemotherapy / radiotherapy, a human immunodeficiency virus infection, known endocrine or autoimmune abnormalities, self-reported PCOS diagnosis, or a known genetic disorder other than a *BRCA1/2* mutation associated with primary ovarian insufficiency.²⁷

Carriers were defined as women with a pathogenic *BRCA1*, *BRCA2*, or a *BRCA1* and *BRCA2* mutation and non-carriers as women who did not carry the pathogenic mutation that was previously identified in their family.

Blood samples were collected irrespective of the cycle day and stored at -80°C. All participants were asked to complete a questionnaire regarding their medical / surgical / menstrual / reproductive history, contraceptive use, lifestyle factors, and fertility treatment.

AMH assay

After blood collection (starting in February 2012), plasma for AMH assessment was separated directly and frozen in aliquots within one to two hours. In March 2015 all AMH levels were measured at the diagnostic endocrine laboratory at the University Medical Center Utrecht (Utrecht, The Netherlands). All measurements were performed in a batch analysis using a DS2 ELISA robot and a single lot reagent analyzer (AMH Gen II ELISA, A79765, Beckman Coulter, Inc., USA). The lower detection limit was 0.16 µg/l. Inter-assay variation was 10% at 0.27 µg/l and 4.7% at 3.9 µg/l (n = 18).

Ethical approval

The study was approved by the Institutional Review Board of all participating centers (n = 5) and registered in The Netherlands Trial Register (www.trialregister.nl; NTR no. 4324). All participants provided written informed consent.

Statistical analyses

Statistical analyses were performed with the SPSS 21.0 for Windows package (SPSS, Chicago, IL). Descriptive parameters were reported as median with range and categorical data as percentages. Comparison of population characteristics was performed by using a Chi-square test or a Wilcoxon-Mann-Whitney U test, depending on the variable. Statistical significance was reached at p-value < 0.05. Linear regression analyses were performed on log-transformed serum AMH levels to determine a possible association between *BRCA* mutation status and ovarian reserve status. Adjustments were made for age at blood sampling, current smoking status (yes/no), and current hormonal contraceptive use (yes/no). Undetectable serum AMH levels were imputed by a single value (0.11 µg/l, calculated by detection limit (0.16 µg/l) divided by square root 2). Secondary analyses were performed to assess a possible interaction of carrier status and age with respect to ovarian reserve. Therefore an interaction variable, based on centered age and centered carrier status in order to deal with multicollinearity, was added to our primary model. Sensitivity analyses were conducted to assess whether there was a difference between *BRCA1* and *BRCA2* mutation carriers with respect to serum AMH levels. Hence, one patient that carried both mutations had to be excluded for analysis. Furthermore, sensitivity analyses were performed excluding participants recruited by approach B.

Power calculation was based on the association between ovarian reserve status and *BRCA* mutation status. As the possible effect of *BRCA* carrier status on age at menopause was reported to be three years²⁸, we considered a difference in AMH levels of 0.40 µg/l (SD ± 0.12 µg/l) as sufficient evidence for a clinically relevant difference in ovarian reserve decline.²⁹ For detection of such a difference the power calculation revealed that with $\alpha < 0.05$, $\beta = 0.80$, and relative effect size (Cohen's d) of

0.35 a number of 120 *BRCA* mutation carriers and 120 proven non-carriers would be sufficient.

Results

Two hundred and fifty-five women were eligible for analyses (Figure 7.1). In total, 191 participants were included by approach A (93 mutation carriers and 98 proven non-carriers) and 64 participants by approach B (31 carriers and 33 proven non-carriers). Characteristics of study participants are shown in Table 7.1. Mutation carriers were significantly younger at study inclusion compared to proven non-carriers (median age: 29 (range 20-45) versus 31 (range 18-44) years respectively, $p = 0.02$). No significant differences were found with respect to body mass index, age at menarche, menstrual cycle length, smoking status, oral contraceptive use, or current hormonal contraceptive use.

Figure 7.1 Study flowchart
PCOS, polycystic ovary syndrome; VUS, variant of unknown significance; *BRCA1/2*, breast cancer gene 1 and 2

Table 7.1 Population characteristics

	BRCA1/2 mutation carriers (n = 124)	Non-carriers (n = 131)	p-value
Carrier status (n, %)			
BRCA1	66 (53)	n/a	n/a
BRCA2	57 (46)		
BRCA1+BRCA2	1 (1)		
Age at blood sample (years; median, range)	29 (20-45)	31 (18-44)	0.02
Missing	0	0	
Body mass index (kg/m ² ; median, range)	23 (18-36)	23 (18-58)	0.56
Missing	2	7	
Ethnicity (n, %)			0.15
Caucasian	120 (98)	125 (100)	
Other	2 (2)	0	
Missing	2	6	
Age at menarche (years; median, range)	13 (9-17)	13 (9-17)	0.32
Missing	9	13	
Menstrual cycle length (days; median, range)	28 (21-32)	28 (21-35)	0.85
Missing	0	0	
Smoking (n, %)			0.80
Current	21 (17)	24 (19)	
Past	29 (24)	33 (26)	
Never	72 (59)	69 (55)	
Missing	2	5	
OCP use			0.06
Ever	120 (98)	119 (92)	
Never	3 (2)	10 (8)	
Missing	1	2	
Current hormonal contraceptive use (n, %)			0.77
Yes	77 (62)	79 (60)	
No	47 (38)	52 (40)	
Missing	0	0	
Types of hormonal contraceptive (n, %)			
Oral contraceptives	50 (65)	48 (61)	
Oral progesterone	2 (3)	0	
Progesterone depot	1 (1)	1 (1)	
Progesterone IUD	19 (25)	28 (35)	
Implants	3 (4)	1 (1)	
Vaginal ring	2 (3)	1 (1)	
Patches	0	0	

BRCA1, breast cancer gene 1; BRCA2, breast cancer gene 2; n, number of women; OCP, oral contraceptive; IUD, intra-uterine device

The median AMH level in mutation carriers was 1.80 µg/l (range 0.11-19.00) compared to 1.90 µg/l (range 0.11-10.00) in proven non-carriers ($p = 0.34$, Figure 7.2 and Table 7.2). Serum AMH levels were below detection limit in ten non-carriers and seven mutation carriers (three *BRCA1* and four *BRCA2* mutation carriers). Linear regression analysis adjusted for age, current smoking, and current hormonal contraceptive use showed no reduction in AMH levels in carriers (relative change 0.98, 95% CI 0.77-1.22, $p = 0.76$, Table 7.3).

Figure 7.2 Scatter plot of serum AMH levels ($\mu\text{g/l}$) in mutation carriers and non-carriers versus age (in years). The Y-axis is represented as a logarithmic scale.
BRCA1/2, breast cancer gene 1 and 2; AMH, anti-Müllerian hormone

Table 7.2 Results of ovarian reserve, fertility, and obstetric history characteristics

	<i>BRCA1/2</i> mutation carriers (n = 124)	Non-carriers (n = 131)	p-value
AMH ($\mu\text{g/l}$; median, range)	1.90 (0.11-19.00)	1.80 (0.11-10.00)	0.34
Infertility (n, %) ^a			0.03
No	37 (70)	64 (85)	
Yes	16 (30)	11 (15)	
Missing	0	1	
Infertility treatment (n, %)	7/16 (44)	4/11 (36)	0.70
Pregnant (n, %)			0.008
Never	71 (58)	52 (41)	
Ever	51 (42)	74 (59)	
Missing	2	5	
Miscarriage < 16 weeks gestational age (n, %) ^b			0.30
0	39 (80)	52 (71)	
≥ 1	10 (20)	21 (29)	
Missing	2	1	
Not applicable; never been pregnant	71	52	
Parity (n, %)			0.007
0	74 (61)	55 (44)	
≥ 1	48 (39)	71 (56)	
Missing	2	5	
Age at first child (median, range)	29 (22-42)	28 (22-40)	0.21
Missing	1	2	
Age at last child (median, range)	31 (22-42)	30 (25-38)	0.34
Missing	1	2	

BRCA1/2, breast cancer gene 1 and 2; n, number of women; AMH, anti-Müllerian hormone

^a Percentage is calculated based on women who reported infertility divided by the sum of women ever pregnant combined with women who reported infertility and never became pregnant

^b Calculated based on women who reported ever being pregnant and supplied miscarriage data

Table 7.3 Association between serum AMH level and *BRCA1/2* mutation status

		AMH (µg/l)	
Determinants	Relative change	95% CI	p-value
<i>BRCA1/2</i> mutation status			
Non-carriers (reference)			
<i>BRCA1/2</i> mutation carriers	1.07	0.89-1.53	0.28
<i>BRCA1/2</i> mutation status, adjusted ^a			
Non-carriers (reference)			
<i>BRCA1/2</i> mutation carriers	0.98	0.77-1.22	0.76

AMH, anti-Müllerian hormone; *BRCA1/2*, breast cancer gene 1 and 2; CI, confidence interval

^a Adjusted for age, current hormonal contraceptive use, and current smoking

Results regarding reproductive history are shown in Table 7.2. Mutation carriers were more often nulliparous (74 (61%) versus 55 (44%) respectively, $p = 0.007$). Besides, mutation carriers reported a significantly higher prevalence of infertility (i.e., more than one year trying to become pregnant) compared to non-carriers (16/53 (30%) versus 11/75 (15%) respectively, $p = 0.03$). Of the women who reported fertility problems, fourteen carriers and nine non-carriers achieved a pregnancy. Unfortunately, no useful data were available regarding infertility diagnosis. No significant differences were found regarding the median age at first child, median age at last child, or the prevalence of miscarriage.

A secondary analysis was performed in order to test the hypothesis whether in mutation carriers ovarian reserve had a stronger negative association with age compared to non-carriers.³⁰ Again, the relative change in AMH levels for mutation carriers was similar to the non-carriers (relative change 0.98, 95% CI 0.76-1.22, $p = 0.76$).

To further evaluate whether there was a difference in ovarian reserve decline between *BRCA1* mutation carriers versus non-carriers and *BRCA2* mutation carriers versus non-carriers, sensitivity analyses were performed. No significant differences were found in the AMH levels of *BRCA1* mutation carriers (2.00 µg/l (range 0.11-19.00)) or *BRCA2* mutation carriers (1.60 µg/l (range 0.11-18.00)) compared to the proven non-carriers (1.80 µg/l (range 0.11-10.00), $p = 0.08$ and $p = 0.64$, respectively). In the multivariate analyses, neither *BRCA1* nor *BRCA2* mutation carriers showed a significant change of serum AMH levels (relative change 1.01, 95% CI 0.78-1.34 and 0.95, 95% CI 0.66-1.20, respectively).

Since the use of two different recruitment approaches could have led to selection bias, a second sensitivity analysis was performed by excluding the 64 participants enrolled by approach B. Again, no difference was found in serum AMH levels between mutation carriers and non-carriers (1.90 µg/l (range 0.11-19.0, $n = 93$) versus 1.85 µg/l (range 0.11-10.0, $n = 98$), respectively, $p = 0.49$). This finding was confirmed by the multivariate analysis (relative change 0.98, 95% CI, 0.73-1.24, $p = 0.69$). With respect

to nulliparity, the same trend as in the primary analysis was found but without reaching significance (56 (62%) versus 45 (48%) in mutation carriers versus non-carriers respectively, $p = 0.07$).

In total, five women with serum AMH levels $\geq 10 \mu\text{g/l}$ were included; four mutation carriers and one non-carrier. All these women had a regular menstrual cycle or a history of a regular menstrual cycle before using hormonal contraceptives. These levels were compared with a previously reported AMH normogram. In three cases AMH levels were categorized between p50 and p90 and in two cases AMH was above the p90.³¹ In a post-hoc analysis we excluded women with AMH levels $\geq 10 \mu\text{g/l}$ in order to further decrease the chance of including women with PCOS. Again, no difference in serum AMH levels between mutation carriers and non-carriers was found (relative change 0.91, 95% CI 0.73-1.14, $p = 0.42$).

Discussion

In this study, no evidence was found for an association between *BRCA1/2* mutation status and a reduced quantitative ovarian reserve, when assessed by serum AMH level.

Four other studies have been published that assessed the association between *BRCA1/2* mutation status and AMH (Supplemental Table S7.1). This study is in line with one study that reported similar AMH levels in healthy *BRCA1/2* mutation carriers compared to age-matched controls from the general population.²¹ However, results from the latter study may have been biased since women with PCOS were not excluded nor adjustments were made for potential confounding factors. Most of the studies with positive findings suffered from considerable methodological issues on population selection (i.e., inclusion of breast cancer cases and/or not excluding PCOS diagnosis)^{32,33} or the lack of appropriate adjustments³⁴ that led to less valid conclusions.^{12,19} Recently, a large well-performed cross-sectional study reported lower AMH levels in *BRCA1* mutation carriers compared to non-carriers.²⁰ Possible explanations for the reported differences compared to Phillips *et al.*²⁰ are: (1) the participants were older, (2) women with irregular cycles were not excluded, introducing the possible skewed inclusion of women with PCOS as it is unknown whether the distribution of PCOS is proportionally distributed among mutation carriers and non-carriers, (3) using plasma instead of serum, and allowing a sampling to storage interval of 48 hours whereby variable effects on the stability of this peptide hormone cannot be excluded, and (4) the usage of a fully automatic AMH assay (Elecys®, Roche Diagnostics) with a lower detection limit and a lower inter-assay variation than our Generation II assay.

With respect to parity and *BRCA* mutation status, contradicting results have been published so far.^{20,22-26,28,35,36} We found that mutation carriers significantly more often reported fertility problems. However, diagnoses were missing in most cases which make this finding less valuable. Furthermore, mutation carriers were more often nulliparous compared to non-carriers, but this result is of limited value due to the cross-sectional study design in a relatively young population in which mutation carriers appeared to be significantly younger.

If the quantitative ovarian reserve status is diminished in *BRCA* mutation carriers, one would expect that the ANM is decreased.¹ Nevertheless, inconsistency also exists in studies that assessed the influence of a *BRCA* mutation on ANM.^{22,28,35,37,38}

The main strengths of the current study apart from the prospective data collection are the large number of mutation carriers included, the fact that proven mutation carriers were compared with proven non-carriers, the exclusion of women with a history of breast cancer and/or PCOS, and that adjustments were made for the most important confounding factors. Furthermore, all AMH measurements were performed in one laboratory.

Our study also has some limitations. The women included were relatively young. Since it has been hypothesized that the *BRCA* mutation effect on ovarian reserve status becomes more apparent in subsequent decades of life, the possible failure to include women suffering from a potentially more severely impaired *BRCA* function into our study may be a hidden source of bias. Nonetheless, selection bias may be present in all clinical studies that assess ovarian reserve status in *BRCA* mutation carriers. Notable, as mutation carriers but not non-carriers have an increased risk of developing cancer or are choosing for a risk reducing salpingo-oophorectomy (RRSO) this will potentially select the more healthy mutation carriers into the cohort studied. Given their young age, it can be hypothesized that women included in our study may not yet display a reduction in ovarian reserve since their *BRCA* function may still be sufficient. However, we did not find an interaction between carrier status and age with respect to ovarian reserve.

The inclusion of women tested within five years before sampling may have led to the inclusion of a relatively healthy exposed subgroup, in which women that meanwhile had developed cancer or had undergone RRSO were not included. This might have slightly biased our results to the null. A sensitivity analysis, excluding women recruited via approach B, revealed results similar to those from the primary analysis, supporting the validity of the overall analysis. Since *BRCA1* and *BRCA2* are different genetic entities with diverse phenotypes, additional sensitivity analyses were performed. No differences were found between *BRCA1* or *BRCA2* mutation carriers versus non-carriers. Following the higher cancer incidence at younger age in *BRCA1* patients, it can be hypothesized that a *BRCA1* mutation may affect ovarian reserve earlier in life than a *BRCA2* mutation. Due to insufficient power to make a distinction between *BRCA1* and *BRCA2* mutation carriers a potential difference between these subgroups

may have remained unnoticed. But since we did not find a trend toward lower serum AMH levels in any of the *BRCA* mutation subgroups, the clinical impact of a remaining difference can be questioned.

In order to exclude women who were likely to have PCOS, we only included women with regular menstrual cycles. Therefore, it is possible that women who had an irregular cycle as an expression of the menopausal transition were excluded. However, we may assume that women heading for early menopause have already lower AMH levels in the life phase where cycles still are strictly regular (Stages of Reproductive Aging Workshop (STRAW)-3).³⁹ We then should have observed a noticeable reduced age specific AMH level in our mutation carriers.

Blood samples were randomly taken in the menstrual cycle, though contradictory results have been reported regarding intra-cycle variability of AMH levels.¹⁶ Potential variation caused by random blood sampling may have influenced the comparison between our groups and may have prevented the detection of small but clinically insignificant differences. None of the other studies on AMH in *BRCA* mutation carriers has provided information regarding blood sampling timing throughout the menstrual cycle.^{12,19-21} Variations in storage time have been comparable for the two groups that have been studied. As such, any effect of long-time storage on AMH concentrations would be present in both the compared groups in the same way. Studies on long-term storage effects on AMH serum concentrations are currently lacking. It would have been most optimal if none of the women included were currently using hormonal contraceptives and were refrained from hormonal contraceptive use for at least three months since that could have influenced their AMH levels. However, such a requirement makes it impossible to perform such a supposedly 'unbiased' study. Hormonal contraceptives were used in both groups, and adjustments were made in the multivariate analyses. Finally, due to medical ethical board restrictions, we were not allowed to ask for reasons of non-participation, or to collect patient characteristics of women who decided not to participate. Therefore, it remains unknown whether non-participating women differ from included women, in terms of reproductive history and/or cancer family history.

Prospective follow-up studies with repeated serum AMH measurements powered on *BRCA1* mutation carriers versus proven non-carriers may give us more insights into whether mutation carriers have indeed reduced AMH levels and whether the *BRCA* mutation effect on ovarian reserve status becomes more apparent in subsequent decades of life. Furthermore, data on infertility, age at last birth, and time to pregnancy could then also be collected.

In our study we have observed no evidence for a reduced ovarian reserve as reflected by a detectable difference in serum AMH levels between participating *BRCA1/2* mutation carriers in comparison to non-carriers. However, limitations of the current analysis and limitations of the studies published so far argue for prospective, well-controlled studies to determine whether mutation carriers, and if so which type of

BRCA mutation carriers, might be at risk of low ovarian reserve and to definitively guide care. Nevertheless, such studies are methodologically challenging due to the occurrence of breast cancer and RRSO events in *BRCA* mutation carriers.

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Supplemental table

Table S7.1 Literature overview of AMH in *BRCA* mutation carriers

Author	Method	Population	Outcome	Adjusted for
Titus, <i>et al.</i> ¹²	Cross-sectional assessment Comparison by ANOVA ELISA, DLS	Breast cancer patients aged 18-42 years 24 <i>BRCA1/2</i> mutation carriers versus 60 proven non-carriers	Mean serum AMH $\pm$ SD (ng/ml): Carriers: 1.22 $\pm$ 0.92 <i>BRCA1</i> : 1.12 $\pm$ 0.73 <i>BRCA2</i> : 1.39 $\pm$ 0.73 Non-carriers: 2.23 $\pm$ 1.56 $p = 0.000^{a,b}$, $p = 0.127^c$	No adjustments reported
		Healthy women aged 18-45 years 62 <i>BRCA1</i> versus 27 <i>BRCA2</i> versus 54 proven non-carriers	Mean serum AMH $\pm$ SD (ng/ml): <i>BRCA1</i> : 1.07 $\pm$ 1.02 <i>BRCA2</i> : 1.33 $\pm$ 1.11 Non-carriers: 1.11 $\pm$ 1.05 $p = 0.679$	*Age and BMI
Wang, <i>et al.</i> ¹⁹	Cross-sectional assessment Linear regression AMH Gen II ELISA	Healthy women aged 25-45 years 172 <i>BRCA1</i> mutation carriers versus 216 proven non-carriers from <i>BRCA1</i> mutated families 147 <i>BRCA2</i> mutation carriers versus 158 proven non-carriers from <i>BRCA2</i> mutated families	Mean adjusted AMH (ng/ml): ^a <i>BRCA1</i> : 0.53 (95% CI 0.33-0.77) ^b <i>BRCA2</i> : 0.73 (95% CI 0.39-1.19) ^c Non-carriers: 1.05 (95% CI 0.76-1.40) $p = 0.026^b$, $p = 0.470^c$	*Age, OCP, BMI, and smoking
			Adjusted AMH levels (pmol/l): ^a <i>BRCA1</i> : exp(8) 0.75 (95% CI 0.58-0.97) ^d <i>BRCA2</i> : exp(8) 0.99 (95% CI 0.77-1.27) ^e $p = 0.27$	
Phillips, <i>et al.</i> ²⁰	Cross-sectional assessment Linear regression Elecys	Healthy women aged ≤ 40 years 41 <i>BRCA1/2</i> mutation carriers versus 324 age-matched general population controls (normal ovulatory cycles)	Mean serum AMH $\pm$ SE (ng/ml) Carriers: 2.71 $\pm$ 0.59 General population: 2.02 $\pm$ 0.12	No adjustments reported
Michaelson-Cohen, <i>et al.</i> ²¹	Cross-sectional assessment Comparison by <i>t</i> test ELISA, DLS			

AMH, anti-Müllerian hormone; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; DLS, diagnostic system laboratory; SD, standard deviation; *BRCA1*, breast cancer gene 1; *BRCA2*, breast cancer gene 2; BMI, body mass index; OCP, oral contraceptives; SE, standard error

^a Total group of mutation carriers compared with non-carriers

^b *BRCA1* mutation carriers compared with non-carriers

^c *BRCA2* mutation carriers compared with non-carriers

^d *BRCA1* mutation carriers compared with non-carriers from *BRCA1* mutated families

^e *BRCA2* mutation carriers compared with non-carriers from *BRCA2* mutated families

Part IV

Addenda

Chapter 8

General discussion

General discussion

Ten years have passed since the first reports were published on preimplantation genetic diagnosis (PGD) for hereditary breast and ovarian cancer (HBOC) syndrome.¹⁻³ In 2006, the first clinical experiences with PGD for *BRCA1* mutations were reported by colleagues of the Universitair Ziekenhuis Brussel (UZ Brussels), Belgium.¹ In the Netherlands, PGD for pathogenic mutations in the *BRCA1* or *BRCA2* gene is performed since the legalization of PGD for hereditary cancer predisposition syndromes in 2008.⁴ Since then, HBOC is one of the indications PGD is most frequently requested for in the Netherlands.⁵ Anno 2017, PGD for HBOC is performed in several countries around the world, among which the United States of America, United Kingdom, and Spain. Worth noticing is that PGD for hereditary cancer predisposition syndromes (including HBOC) is prohibited in other countries, among which Germany. The aim of this thesis is to provide a clinical evaluation of PGD for *BRCA1/2* mutations. In the previous chapters, several important clinical aspects concerning PGD for *BRCA1/2* mutations were addressed. In this last chapter, results are placed into perspective and opportunities for clinical improvements and further research are provided.

The conclusion: PGD is a suitable strategy for couples wanting to avoid transmission of hereditary breast and ovarian cancer syndrome

The main question to answer in this clinical evaluation was whether PGD is a valuable reproductive option for couples affected by a pathogenic *BRCA1* or *BRCA2* mutation. When looking back at the previous years of clinical experience with PGD for HBOC, PGD turned out to be suitable for this group of patients. Due to the relatively large number of referrals and treatments, expertise has been gained in the counseling of couples affected with a *BRCA1/2* mutation at Maastricht University Medical Center (Maastricht UMC+) and her partners in 'PGD Nederland', a collaboration for PGD in the Netherlands (**chapter 2** and **3**). This also applies for the workup in the IVF clinic and the genetic laboratory (**chapter 3** and **4**). Universal single-cell PGD tests have been developed for mutations in the *BRCA1* and *BRCA2* gene, minimizing preparation time and costs for test set-up and validation (**chapter 4**). Pregnancy rates were good in these first years (**chapter 3**). However, some aspects need some reconsidering and provide opportunities for improvement.

Reproductive decision-making in couples carrying a *BRCA1/2* mutation

Deciding on PGD for HBOC, not an easy task

In the first years after the legalization of PGD for HBOC it was noticed in the PGD outpatients clinic of Maastricht UMC+ that the decision whether or not to start with

the treatment was difficult for many couples. Patients were faced with a reproductive dilemma, despite the fact that most of them had the intention to opt for PGD at first. Females who are informed they are at risk of carrying a *BRCA1/2* mutation, either because of cancer in their family, the presence of a *BRCA1/2* mutation in the family, or because of their own oncological history, are forced to make many potential life-changing choices in a relatively short period of time. Decisions which have to be made involve genetic testing, prevention versus surveillance strategies, reproduction, and, in case of cancer, oncological treatments. As a result, the choice whether to opt for PGD is only one important decision among many others, as illustrated in Intermezzo A.

Intermezzo A: A fictitious case illustrating the complexity of decision-making in HBOC

Miss B. is 28 years old. When aged 25 she found out that her father was a carrier of a mutation in the *BRCA1* gene. She knew that she was at 50% risk and that genetic testing could disclose her status. She decided however to postpone genetic testing.

Two months ago miss B. underwent genetic testing. The test revealed that she inherited the familial *BRCA1* mutation. It was a real disappointment to her. The genetic counselor did inform her about surveillance options of the breasts and preventive options to avoid breast and ovarian cancer. She was also informed about the 50% risk of transmitting the mutation to her own offspring. However, she and her partner were not considering to start a family yet and therefore reproductive options were only shortly discussed.

Miss B. is considering to have preventive breast surgery but before making a decision she wants to be informed about the surgical approaches available and the pros and cons of each technique. Within two weeks she has an appointment with a surgeon to discuss the options. She doubts whether this is the right time for a bilateral preventive mastectomy. She is young, in a budding relationship, and busy with her career. How big is the chance that she will get cancer the upcoming years? Could she postpone breast surgery a bit, maybe even until she has been able to breastfeed her prospective children?

She decides to opt for screening of the breasts in the meantime, since she anticipates that it will take her quite some time to make a decision regarding her breast surgery. Unfortunately, her breast MRI and subsequent diagnostics reveal that she already has breast cancer: a triple negative tumor in her left breast. She is referred to a medical oncologist who advises her to opt for neo-adjuvant chemotherapy, followed by a mastectomy and radiotherapy. Regarding the mastectomy, the option of a bilateral mastectomy because of her *BRCA1* mutation is given.

Miss B. is devastated by the news of having breast cancer. She reflects on the suggested treatment strategy with her aunt, who has been treated for breast cancer herself. She doubts whether she should opt for a bilateral mastectomy right away or whether she should postpone this decision. Above all this, the medical oncologists warned her that there was a chance of chemotherapy induced amenorrhea and infertility after the oncological treatment.

Miss B. is given the option to cryopreserve oocytes or embryos. After extensive elaboration with her partner she decides to opt for emergency IVF to cryopreserve oocytes. Although the chance of pregnancy may be slightly smaller in case of cryopreserved oocytes when compared to cryopreserved embryos, the couple does not feel ready to make the decision to try to conceive together. The IVF treatment is exhausting but results in ten cryopreserved oocytes. Although Miss B. is told this is a good yield, she has also understood that on average twenty oocytes are needed to achieve a good chance for a future ongoing pregnancy.

Three years later

Miss B., now 31 years of age, attends the outpatient clinic for PGD. It has been over two years ago since she has finished her oncological treatments. She is doing well and she and her partner would like to start a family. Miss B. and her partner are in doubt whether to conceive without testing and to take the risk of transmitting the *BRCA1* mutation to their prospective children. Perhaps their children would have more diagnostic and therapeutic options in the future, decreasing the need for and the acceptability of reproductive techniques like PGD. Ultimately, they conclude that they want to stop the transmission of the *BRCA1* mutation running in their family and that they want to protect their prospective children from the burden caused by the *BRCA1* mutation. They opt for PGD. All ins and outs of this trajectory are discussed, but there are several additional questions the couple has to think about.

Miss B. decided to have a unilateral mastectomy at the time of oncological treatment. She is worried about a potential adverse effect of the hormones applied for IVF on her risk of breast cancer. She is planned for a preventive contralateral mastectomy and wants to start PGD after her recovery. However, she did not anticipate the fact that the need for at least half a year to fully recover from preventive surgery and to feel physically and mentally ready for a new challenging trajectory as PGD is not an exception. Miss B. starts counting... At the moment she is 31 years old already and she was advised to have her ovaries removed at age 35... She concludes that she has only four years left for her surgery and subsequent recovery, PGD preparations, and child bearing. Ideally, she wants to have two or three children. She does not feel at ease, there is no time to waste...

Regarding the PGD treatment itself, the couple has to decide whether or not to use the cryopreserved oocytes. Miss B.'s ovarian reserve is diminished as a result of her chemotherapy, but her ovarian reserve tests are just above the minimal required limits for IVF/PGD. She can choose for a fresh ovarian stimulation, but the risk of a poor ovarian response is significant. It is expected that a fresh ovarian stimulation will yield only a small number of oocytes. Selection of the embryos based on genetic test results would further reduce the number of embryos available for transfer and thus decrease the chance of pregnancy. The clinical geneticist advises the couple to reconsider their priorities. What is most important: getting a child at all, or getting a child without the familial *BRCA1* mutation?

In 2010, we performed an explorative study on the decisions made by 68 couples counseled for PGD because of a *BRCA1/2* mutation. It turned out that half of the couples (34/68) refrained from PGD for HBOC after counseling. An additional 12% of the couples (4/34) refrained after they initially decided to proceed with PGD, thus when the preparations had already been started.⁶ From a clinical point of view it was believed that more insight into the factors influencing decision-making would be valuable for the optimization of patient decisional support.

Chapter 2 provides a qualitative assessment of the perceived (dis)advantages of PGD for HBOC and alternatives leading to a child genetically related to both partners, i.e., prenatal diagnosis (PD) and conception without testing. Interviewed couples had a pathogenic *BRCA1* or *BRCA2* mutation and received extensive counseling on PGD. Subsequently, they personally made a decision upon this reproductive option. The survey provides insight into the transition from PGD intention to actual PGD use. There was a large overlap in motives and considerations between couples opting for PGD and couples refraining from PGD. Regardless the reproductive option chosen, all couples mentioned the same domains and identified few important advantages and a fair number of less important disadvantages of PGD. Obviously, couples weigh these pros and cons differently, leading to a decision in favor or against PGD. In the time since our study, one other report has been published describing the decision-making process in three couples affected with a *BRCA1/2* mutation who have undergone PGD. Additionally, seven other cases are briefly summarized.⁷ This narrative paper illustrates couples' thoughts regarding PGD and the integration of these perceptions into reproductive goals. However, only limited insight is provided in the motives and considerations of refraining couples. Besides, this latter study was executed in the United States of America, where PGD practice differs from the Western European situation at some critical points, such as the organization of PGD practice (often separate units for counseling, IVF, and genetic analysis are involved), health insurance coverage of PGD costs, and the possibility of transferring a *BRCA1/2* mutation positive embryo. Therefore, the results cannot be translated to the Western European PGD setting easily.

A decisive factor in reproductive decision-making: perceived severity of HBOC

The perceived severity of HBOC turned out to be one of the decisive factors in the decision-making process (**chapter 2**). The perception of HBOC severity was influenced by personal and familial cancer history and preventive surgery. This association was described in earlier research⁸ and was found to be important in reproductive decision-making in previous qualitative studies in female *BRCA1/2* mutation carriers of diverse ages and with various levels of knowledge.^{9,10} Our qualitative study showed that a considerable part of the interviewed *BRCA1/2* mutated couples felt serious drawbacks from selection in the form of PD for HBOC, although they did not have difficulties with

termination of pregnancy in general. They owed this to the level of severity they perceived regarding HBOC, which was considered relatively modest compared to other (genetic) disorders. The majority of these couples perceived a moral difference between selection in the embryonic stage of development *in vitro* (in case of PGD) and the termination of an already established pregnancy (in case of PD). The couples felt that the severity of HBOC was insufficient to justify selection by the termination of a pregnancy which already had proceeded to three or four months. However, couples who could be at ease with selection at that point all chose this option. Nevertheless, PD for HBOC is rarely performed in the Netherlands.¹¹

The severity of HBOC was also the main issue in the national debate around the permissibility of PGD for hereditary cancer predisposition syndromes. One may try to objectify the severity of a genetic condition in terms of penetrance, age of onset, and the availability or absence of preventive and therapeutic options. Nevertheless, the real impact of these factors may only be truly considered and put into perspective by persons personally affected with the disease. It turned out that affected couples consider reproductive options very carefully. Therefore, the fear for a slippery slope does not seem to be justified.

Medical developments and the perception of HBOC over time

In the last decennia, important improvements are achieved in the cure of and care for *BRCA1/2* mutation carriers. As a consequence, the morbidity and mortality related to HBOC is decreased and a transition took place in the perception of the condition.¹² From an unpredictable life-threatening familial trait it turned into a modifiable cancer predisposition one can anticipate. With the discovery of the *BRCA1* and *BRCA2* genes in the mid-nineties, genetic testing became available in symptomatic patients as well as predictive testing in family members. At risk persons can opt for genetic testing at any moment appropriate for them. Most asymptomatic men apply for genetic testing either in order to get informed about their reproductive risk or to know whether their (adult) daughters may be affected. In women, disclosure of the genetic status makes it possible to identify individuals at high risk of cancer and to assure others.¹³ Surveillance of the breasts aiming at early detection and therewith leading to improved survival chances is recommended for female *BRCA1/2* mutation carriers and women at 50% risk.¹⁴⁻¹⁶ Preventive options, i.e., bilateral preventive mastectomy and/or risk-reducing salpingo-oophorectomy, are also offered to *BRCA1/2* mutation carriers. Surgical techniques have improved and their efficiency is high.¹⁷⁻²⁰ Studies assessing quality of life of female *BRCA1/2* mutation carriers undergoing preventive surgery show that despite the intensity of these radical measurements psychosocial well-being afterwards is high.²¹ Furthermore, oncological diagnostics and treatment options for breast cancer patients have expanded and become more and more efficient.²²⁻²⁴ For the treatment of *BRCA1/2* mutation carriers with ovarian cancer

PARP inhibitors have become available.²⁵ A substantial number of interviewed couples (**chapter 2**) stated that their confidence in future medical developments for HBOC was a reason not to interfere in the reproductive process.

Embryonic transfer policy in PGD regarding male embryos with the familial *BRCA1/2* mutation

In PGD practice, it can be discussed whether male embryos with the *BRCA1/2* mutation should be considered for transfer. According to Dutch legislations, PGD is allowed in case of an individually increased risk for a child with a severe genetic disorder.⁴ By law it is prohibited to select embryos on gender, unless in cases where gender selection is an established evidence based method to avoid the risk of a severely affected child due to an X-linked genetic condition.²⁶ Although HBOC is not a disorder with X-linked inheritance, transfer of a male embryo affected with a *BRCA1/2* mutation may be defensible since only female carriers face strongly elevated risks of cancer. PGD for the familial mutation can be combined with gender determination in one PCR analysis. There are several transfer policies imaginable. One option is to maintain the current transfer policy: only the familial *BRCA1/2* mutation is analyzed and subsequently only embryos without the familial mutation are transferred. Important advantages of this strategy are that the familial mutation is completely eliminated from the family branch treated, that the oncological risk for offspring of any gender is not increased, and that there is no risk to violate the right of a future child not to know its mutation status. A second option is to analyze the embryos for both the familial mutation and the gender. Subsequently, female and male embryos without the *BRCA* mutation are transferred first and eventually also male embryos with the mutation. By following this strategy more embryos (in theory 75% instead of 50%) may be available for embryo transfer, probably leading to a higher chance of pregnancy. There are several variants possible in the transfer policy: male embryos with the mutation can only be transferred if there are no (good quality) embryos without the mutation, or all male embryos with the *BRCA1/2* mutation are cryopreserved at first and thawed at a later stage in case previous PGD treatments did not result in an ongoing pregnancy. In the day three biopsy strategy the decision regarding the transfer policy has to be made by the couple before the oocyte pick-up, to prevent the need for rushed decisions before a fresh transfer. In the trophectoderm biopsy strategy, which becomes more and more applied in preimplantation genetic screening and probably also in PGD practice the upcoming years, all embryos are frozen after biopsy in anticipation of the genetic results becoming available. In this latter situation there will be enough time to discuss the transfer policy with an individual couple, knowing the genetic results of the frozen embryos. However, transfer of male embryos with a mutation has some serious disadvantages. Firstly, the right of a male born after PGD not to know his *BRCA1/2*

mutation carrier status is violated. Secondly, the male mutation carrier will be confronted with a genetic risk for his offspring and a reproductive dilemma in the future. Thirdly, also a male with a *BRCA1/2* mutation has a (slightly) increased oncological risk. Finally, the fact that the *a priori* chance for a boy or a girl is no longer 50/50 can be perceived as a drawback.

The acceptability of and the need for the inclusion of the gender of the embryo in PGD analysis, the related broadening of the transfer policy, and the way couples can be involved in the decisional process are explored at this moment in our PGD centers, taking into account current legislation. Joint decision-making could increase the couples' autonomy and may be an important step towards more couple-centered care.

Non-invasive prenatal diagnosis for HBOC

In the near future, non-invasive prenatal diagnosis (NIPD) is expected to become available for HBOC.²⁷ Whether this option would become an important alternative for couples deciding on PGD or invasive PD versus no testing is hard to predict. NIPD may provide the advantages of natural conception, a safe and reliable diagnostic method, and selection in a relatively early stage of pregnancy. However, since it is expected that on the short term NIPD techniques generate results not that much earlier than results derived from invasive PD, in both scenarios couples are confronted with the decision for pregnancy termination in a relatively late stage. It can be speculated that couples do not feel much difference between NIPD and PD until NIPD test results will become available much earlier in pregnancy.

Ovarian reserve and reproductive chances

Ovarian reserve in *BRCA1/2* mutation carriers

We took a more profound look at ovarian reserve data of female *BRCA1/2* mutation carriers after a report suggesting a diminished ovarian reserve in *BRCA1* mutation carriers.²⁸ Especially in a PGD setting the number of oocytes obtained after ovarian stimulation and the number of analyzable embryos is important, since embryonic transfer criteria are not primarily based on embryological features but on genetic test results. In theory 50% of the embryos will be untransferrable following the current transfer policy because of the presence of the familial *BRCA1/2* mutation, which is rather high.

We questioned whether *BRCA1/2* mutation carriers would really suffer from a reduced ovarian reserve and whether this would affect their reproductive chances in PGD. We studied ovarian reserve using two different outcome measurements, namely the number of mature oocytes obtained in IVF/PGD (**chapter 6**) and anti-Müllerian hormone (AMH, **chapter 7**). AMH is produced by granulosa cells of ovarian follicles

during early stages of development and thought to be the best currently available test for ovarian reserve.²⁹ AMH is known to be a good predictor of ovarian response to ovarian stimulation in IVF.^{30,31} Ovarian response in terms of the number of oocytes obtained is related to the live birth rate after IVF.³² However, AMH itself does not predict the chance of ongoing pregnancy after IVF treatment.³³ When assessing AMH levels of proven *BRCA1/2* mutation carriers, no differences were observed when compared to a *BRCA* mutation negative control group. The sample size however was too small for reliable subgroup analysis (i.e., *BRCA1* and *BRCA2* mutation carriers versus controls). When studying the number of mature oocytes obtained after ovarian stimulation for IVF with PGD as a proxy variable for ovarian reserve, we did find a decreased number of oocytes in *BRCA1* mutation carriers. Differences between subgroups (*BRCA1* mutation carriers, *BRCA2* mutation carriers, and controls) were small however and the number of mature oocytes were within the normal range for all subgroups.³²

Ovarian reserve and reproductive performance in *BRCA1/2* mutation carriers has extensively been studied the last years using different outcome parameters. However, results are not consistent.^{28,34-49} In the majority of studies reporting a difference between *BRCA* mutation carriers and controls, a negative effect was only present in *BRCA1* mutation carriers. This was also the case in our study (**chapter 6**). The absence of an effect in *BRCA2* mutation carriers in IVF/PGD practice (**chapter 6**) and the lack of a difference in both *BRCA1* and *BRCA2* mutation carriers in our AMH-study (**chapter 7**) may be explained by (1) the fact that ovarian reserve is not reduced in *BRCA2* mutation carriers, or (2) by a subtle effect of *BRCA1/2* mutations on ovarian reserve which (a) could not be detected in our studies due to limitations in the chosen outcome parameters, and/or (b) power issues, and/or (c) the inclusion of (too) young women. A ‘tip of the iceberg’ phenomenon has been proposed in *BRCA1/2* mutations related ovarian dysfunction, including an explanation for the lack of a difference in ovarian reserve between *BRCA1/2* mutation carriers and controls in many studies.⁵⁰ It is hypothesized that a more severe *BRCA* dysfunction (i.e., a ‘severe’ mutation) not only leads to higher risks of cancer, but also leads to a stronger reduction in ovarian reserve. As a result, a lower ovarian reserve may be more prevalent in *BRCA1/2* mutation carriers with early (breast) cancer diagnosis and subsequent chemotherapy, early ovarian cancer diagnosis and subsequent removal of the ovaries, and a significant family history for ovarian cancer at young age, leading to a risk-reducing salpingo-oophorectomy at young age. Following this hypothesis, the possible failure to include women suffering from a more severely impaired *BRCA* function into our studies may have resulted in bias to the null. Additionally, it can be hypothesized that a (subtle) reduction in ovarian reserve only becomes visible with increasing age. Given the young age of the women included in our studies, they may not yet display a reduction in ovarian reserve since their *BRCA* gene dysfunction may not yet have affected their ovarian reserve pool to a critical extent. The same may apply for *BRCA2*

mutation carriers. Despite a role of both *BRCA* genes in DNA double-strand break repair, the role of *BRCA2* is probably less comprehensive.⁵¹ Recently, genotype-phenotype associations have been supposed according to the location of the mutation in both the *BRCA1* and *BRCA2* gene.⁵² It can be hypothesized that the influence of an impaired *BRCA2* gene function on ovarian reserve decline, if present at all, is smaller than the effect of *BRCA1* gene dysfunction and therefore becomes only visible with increasing age. The putative role for the *BRCA1/2* genes in the ovarian aging process may ultimately come to expression only in other reproductive conditions, such as age at natural menopause.⁴⁰⁻⁴²

Results into clinical perspective

Overall, an influence of the *BRCA1* and/or *BRCA2* gene on ovarian reserve is biologically plausible. Several molecular findings support a role of a *BRCA1/2* gene dysfunction in (reproductive) ageing, as described in **chapter 6**.^{37,53-55} However, the effect size is probably too small to be of clinical importance in reproductive medicine. There is currently no evidence for a clinical relevant impact of a *BRCA1/2* mutation on reproductive performance in an IVF/PGD setting, neither for the need to advise female *BRCA1/2* mutation carriers to cryopreserve oocytes or embryos because of premature ovarian ageing. Nevertheless, cryopreservation of oocytes or embryos may be an option to consider for female *BRCA1/2* mutation carriers of reproductive age prior to chemotherapy because of (breast) cancer. Preliminary results from our own clinic show however that the uptake of cryopreserved oocytes or embryos after recovering from cancer treatment is very low. So far, these cryopreserved embryos were only used for PGD. Couples not opting for PGD tried to conceive naturally and succeeded (Maastricht UMC+, unpublished data). The uptake after cryopreservation of oocytes for other reasons has also been shown to be low.^{56,57} Another reason for cryopreservation of gametes is a desire to prolong reproductive lifespan. Due to the advice for (in particular *BRCA1*) mutation carriers to remove the ovaries at young age, the time for family planning is limited. It is possible to transfer embryos and to achieve a pregnancy after removal of the ovaries provided that adequate hormonal substitution is given.⁵⁸ Currently, this has not been practiced on a large scale yet and it is unknown how this option is viewed by *BRCA1/(2)* mutation carriers themselves.

Oncological safety of ovarian stimulation for IVF/PGD

The risk of breast cancer after ovarian stimulation for IVF/PGD

The oncological safety of the IVF treatment needed for PGD was an important factor in the decision-making of many couples interviewed. Women wondered whether it was safe enough to postpone bilateral preventive mastectomy till after the completion of their family for instance in order to be able to breastfeed, or whether it

was more wise to remove their breasts before exposure to ovarian stimulation for IVF/PGD.

In the first years of PGD for *BRCA1/2* mutations we were confronted with two breast cancer diagnoses in *BRCA1* mutation carriers, one at Maastricht UMC+ and one at UZ Brussels (**chapter 3**). The latter woman already had a personal history of breast cancer. Both women were diagnosed shortly after their first ovarian stimulation for IVF/PGD, despite a magnetic resonance imaging (MRI) of the breast without abnormalities shortly before. Although these women had a high *a priori* risk of breast cancer, a potential causality between breast cancer risk and the ovarian stimulation for IVF/PGD treatment was questioned.

Since a causal influence of the exposure to hormonal stimulation could not be ruled out, the centers participating in the PGD consortium in the Netherlands and the PGD center of UZ Brussels agreed to intensify pre-treatment screening and follow-up of *BRCA1/2* mutation carriers undergoing PGD. It was decided to request an MRI of the breasts within three months before every PGD treatment and approximately three months after the last treatment or end of the pregnancy or lactation period. So far, no new cancer cases have been detected (unpublished data). However, a longer follow-up of a larger cohort is needed for more firm conclusions. In the upcoming years it will be evaluated whether this intense screening is still indicated in the future. In expectation of the aforementioned results from the clinic we studied the association between ovarian stimulation for IVF (with or without PGD) and breast cancer in a larger cohort of *BRCA1/2* mutation carriers with a longer follow-up time, on the basis of data in the Dutch HEBON database (Hereditary Breast and Ovarian cancer study, the Netherlands, **chapter 5**). The HEBON study is an ongoing nationwide retrospective cohort among members of *BRCA1/2* mutation families with prospective follow-up. One of the aims of the study is to assess breast cancer risks and potential interactions between genetic and hormonal or lifestyle factors. No association between ovarian stimulation for IVF (with or without PGD) and breast cancer risk was found. However, despite the availability of a national cohort our statistical power was still relatively limited. It is quite complicated to study breast cancer risks in a high risk population like *BRCA1/2* mutation carriers, since a substantial part of these women remove their breasts during life.

Results into clinical perspective

So far, there is no evidence for a clinically relevant rise in cancer risks after exposure to ovarian stimulation for IVF (with or without PGD) in *BRCA1/2* mutation carriers.^{59,60} Based on the currently available literature, there is no reason to exclude female *BRCA1/2* mutation carriers from IVF (with or without PGD). However, women who already have decided to opt for a bilateral preventive mastectomy may consider to let this surgery precede their PGD treatment. Female *BRCA1/2* mutation carriers have a

high *a priori* risk of breast cancer and more limited screening options during pregnancy may delay diagnostics and treatment start-up in case of breast cancer.

Final remarks

Timing of reproduction and prerequisites

A complicating factor for *BRCA1/2* mutation carriers in the decision whether or not to choose for PGD is the limited time frame in which especially female *BRCA1* mutation carriers have to complete their family planning. There is a time window of approximately ten to fifteen years from genetic testing until the advised risk-reducing salpingo-oophorectomy from their mid-thirties. In this period of time many choices have to be made. If the woman chooses to have a bilateral preventive mastectomy before the start of PGD, our clinical experience is that most women need at least half a year for recovery in order to feel physically and mentally prepared for a new intense trajectory as PGD. Often more than one PGD treatment is needed to achieve a pregnancy. Especially in case PGD is not successful and the couple wants to try to conceive naturally after the PGD treatment(s), time becomes an even more pressing issue.

Therefore, timely referral to a specialized clinic for reproductive counseling including PGD is extremely important for female *BRCA1/2* mutation carriers, in order to keep open as many reproductive options as possible. The information to and care for female *BRCA1/2* mutation carriers of reproductive age should be provided in a multidisciplinary setting, in which at least a clinical geneticist, gynecologist, medical oncologist, and on request a psychologist trained in decision-making support are involved. Ideally, the fulfillment of family planning should not be postponed. Cryopreservation of oocytes or embryos may be options to consider for women who (have to) delay motherhood and who did not complete their family at the time it is advised to remove the ovaries. Although for male carriers there are much less physical strains involved in PGD, the complexity of reproductive decision-making should not be neglected and addressed in counseling.

Long-lasting impact of reproductive decision-making

The decision whether or not to opt for PGD may have a substantial long-lasting emotional impact on couples with a *BRCA1/2* mutation (**chapter 2**). This may both be the case in couples deciding to use PGD and in couples refraining from PGD. Couples choosing for PGD anticipated an emotional burden in case PGD would not be effective. The start of PGD can be seen as a point of no return, affecting every prospective reproductive decision. Couples wondered what would be acceptable 'second best options' in case PGD turned out to be unsuccessful. The level of acceptability of conception without testing depended on the outcome of the PGD

trajectory, since it was perceived that a successful PGD treatment for a first child would make it more difficult to make another, perceived as inferior choice for a second child. On the contrary, if PGD would not lead to a live born child couples thought they would be more at ease to make a new reproductive decision, since they felt they had fulfilled their moral duty to protect their child(ren). It is probably impossible for a couple to prepare for these matters at the moment of PGD intake, when practical experience with PGD is lacking and when many couples did not yet encounter pregnancy and/or parenthood. Due to the complexity of the reproductive process and the multiple insecurities involved an efficient coping mechanism might be to take things step-by-step. However, early introduction of these moral dilemmas with which the couple may be confronted later on may have a positive effect on coping mechanisms. Therefore, the potential long-lasting impact of the decision whether or not to choose for PGD should be addressed during the PGD intake.

Future perspectives and opportunities for further research

The research described in this thesis addresses three subdomains within the clinical practice of PGD for HBOC, namely an evaluation of the reproductive decision-making, ovarian reserve in *BRCA1/2* carriers, and oncological safety. In all three subdomains some questions have been answered, while other questions remain or have arisen.

Our qualitative exploration of the motives and considerations involved in decision-making regarding PGD for HBOC illustrated the complexity of this process. A quantitative assessment has been carried out to confirm our results and to put them into a broader perspective. In order to facilitate reproductive decision-making and to increase couples' commitment and decisional satisfaction, a digital decision aid is currently in development. Additionally, a study has been carried out to gain more insight into the knowledge of and attitude towards PGD for HBOC of potential referrers and to instruct them where appropriate, in order to warrant timely referral of couples who may benefit from PGD.

Monitoring the reproductive outcome and follow-up of couples undergoing PGD for HBOC remains important. The opinion of *BRCA1/2* mutation carrying couples regarding the transfer of male embryos with the familial mutation should be explored and discussed by policy-makers. If transfer policies will be broadened, this item should be addressed in the decision aid.

The question whether a *BRCA1/2* mutation may harm ovarian reserve is still not unraveled. Prospective studies for instance repeatedly assessing AMH levels in large groups of *BRCA1* and *BRCA2* mutation carriers may provide more insight, especially if women could be followed until older age as a result of delayed oophorectomy in combination with preceding risk-reducing salpingectomy.^{61,62} Molecular studies on the association between *BRCA1/2* mutations, DNA damage, apoptosis, and embryo quality can also be valuable.³⁷

Regarding the safety of ovarian stimulation for IVF (with or without PGD) in *BRCA1/2* mutation carriers, international studies including larger groups of *BRCA1* and *BRCA2* mutation carriers with a longer follow-up are needed in order to confirm the absence of a (clinically relevant) adverse effect on breast cancer risks. In the meantime, screening of the breasts pre- and post-treatment in female *BRCA1/2* mutation carriers undergoing PGD may be beneficial.

In conclusion, PGD for HBOC is a suitable reproductive strategy for *BRCA1/2* mutation carrying couples, when practiced in a specialized center in a multidisciplinary setting. Concerns regarding a reduced ovarian reserve in *BRCA1/2* mutation carriers have not been confirmed and reproductive chances are good. There is no evidence for a (clinically relevant) rise in breast cancer risk after exposure to ovarian stimulation for IVF in female *BRCA1/2* mutation carriers. However, screening of the breasts before and after IVF (with or without PGD) treatment is advisable, given the high *a priori* risk for breast cancer in these women and more limited screening options during pregnancy.

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Valorization

Valorization

Introduction

Breast cancer is the third most prevalent cancer in the Netherlands.¹ In 2016, 16,640 persons (including 129 men) were diagnosed with breast cancer. This corresponds to 14% of all cancer diagnoses. In addition, 1,200 women are diagnosed with ovarian cancer yearly.²

Approximately 5-10% of all breast cancer cases and 10% of ovarian cancer cases are caused by a genetic predisposition, predominantly mutations in the *BRCA1* and *BRCA2* gene. The prevalence of *BRCA1/2* mutations has been estimated at 0.25-0.5%.³ With approximately 17 million inhabitants, this can be translated to 42,500-85,000 people with a *BRCA1* or *BRCA2* mutation in the Netherlands.

Women with a pathogenic mutation in the *BRCA1* or *BRCA2* gene have a strongly increased risk of breast cancer overall and especially of breast cancer at young age. Breast cancer leads to a significant physical, psychological, and social-emotional burden on individual basis, but has also an important societal and economic impact. The available preventive options to reduce cancer risks for female carriers of a *BRCA1/2* mutation are rigorous and in particular prophylactic breast surgery is not a first-choice option for many women.⁴ Surveillance strategies in order to detect breast cancer at an early stage can be very stressful and, of course, cannot prevent breast cancer. So regardless of the risk management strategy chosen, there is a burden to the patient. Female carriers of a *BRCA1/2* mutation also face an elevated risk for ovarian cancer. Ovarian cancer is often diagnosed in an advanced stage, resulting in an overall 5-year survival rate of only 40%.² As a consequence, ovarian cancer is referred to as 'the silent lady killer'. A risk-reducing salpingo-oophorectomy (RSO) decreases the risk for ovarian cancer significantly in female mutation carriers when performed between the age of 35 and 45. However, this strategy induces menopause and associated health risks are the price to pay. The feasibility of a risk-reducing salpingectomy followed by a delayed oophorectomy is currently under investigation in the Netherlands (TUBA-study, Radboud University Medical Center Nijmegen).

For couples with hereditary breast and ovarian cancer (HBOC) syndrome, there are nowadays two preventive reproductive options leading to a child genetically related to both partners. Prenatal diagnosis for *BRCA1/2* mutations with pregnancy termination in case of a (female) child with the mutation is emotionally very burdensome and ethically controversial. As a consequence, it is seldomly performed in the Netherlands.⁵ In the last decade, preimplantation genetic diagnosis (PGD) has become available as an alternative. Until 2016, 98 couples underwent PGD for a *BRCA1/2* mutation.⁶

Relevance of scientific results for clinical practice

Initially, PGD was only carried out for fully penetrant monogenic diseases with a young age at onset and a severe course, resulting in major burden in terms of inexorable physical disease and/or impairment and/or a significant reduction in life expectancy.⁷ In subsequent years PGD was also carried out for genetic conditions leading to an increased risk of signs and symptoms later on in life, so called late onset disorders. Since 1998 PGD for Huntington's disease has been applied, a severe neurological disease with onset at adult age. The legalization of PGD for *BRCA1/2* mutations in 2008 was preceded by an intense political debate. Since the start however HBOC is one of the disorders PGD is most often applied for.⁶ Both couples with a male and/or female mutation carrier can apply for PGD. Also for other cancer syndromes, such as hereditary colorectal cancer, PGD is applied regularly. This development raised issues, not only among medical professionals and third parties (e.g., politics, media) but also in affected couples. "Are hereditary cancer predisposition syndromes severe enough to justify genetic selection as applied in PGD?" "Is this the beginning of a slippery slope?" As a consequence of the shift of the application of PGD from severe early onset towards 'less severe' late onset diseases, it became more and more important that the pros of PGD prevailed the cons. In clinical practice we noticed that for many couples with a *BRCA1/2* mutation it was hard to decide whether or not to opt for PGD. In order to be able to provide more support in the challenging decision-making process, we explored the motives and considerations involved (**chapter 2**). Beside decisive intrinsic factors as the perceived severity of the condition and the couples' moral views regarding selection, several extrinsic factors were taken into account. Important extrinsic factors were the success chance of the procedure, the safety of the *in vitro* fertilization (IVF) treatment needed for PGD in terms of breast cancer risks for female mutation carriers, and the timeline of PGD and its compatibility with prophylactic surgeries in case of a female mutation carrier. Shortly after the start of PGD for HBOC a universal test for PGD of *BRCA1/2* mutations based on haplotyping was set up in our laboratory (**chapter 4**). This universal test can be applied in approximately 90% of the couples requesting PGD for a *BRCA1* or *BRCA2* mutation. For these couples, there is no longer need to develop a mutation-specific protocol. The universal PGD test enables us to offer a test within 1-2 months with a robustness conform European requirements.⁸ Its availability has limited PGD work-up time and costs for test set-up and validation.

We assessed the clinical suitability of PGD for *BRCA1/2* mutations by studying (1) ovarian reserve of female mutation carriers and (2) oncological safety in terms of breast cancer risk in female mutation carriers.

Ovarian reserve is an important parameter in the prediction of the chance of pregnancy after an IVF treatment, whether or not combined with PGD.⁹ Especially in PGD practice a sufficient ovarian reserve is vital since a surplus of oocytes is needed

because selection not only takes place based on embryological terms but also based on genetic test results. Several studies suggested a negative impact of a mutation in the *BRCA1* and perhaps the *BRCA2* gene on ovarian reserve. Our studies do, however, not provide evidence for a clinically relevant reduction in ovarian reserve in *BRCA1/2* mutation carriers (**chapter 6 and 7**). When assessing results of PGD treatments for *BRCA1/2* mutations in terms of pregnancy rates, these are not lower than expected in women carrying a *BRCA1/2* mutation (**chapter 3**). Consequentially, a *BRCA1/2* mutation itself should not be a reason to reject a woman from IVF (with or without PGD) treatment. Another possibility to consider for (in particularly *BRCA1*) mutation carriers may be the freezing of oocytes or embryos preceding a RRSO. The need for this option is yet unclear however and needs further investigation.

The oncological safety of the IVF treatment necessary for PGD was studied as the risk of breast cancer. We hypothesized that if there would be an adverse oncological effect, it most likely would concern the risk of breast cancer because of the involvement of estrogens in the pathophysiology of breast cancer. The association between exposure to ovarian stimulation for IVF and the incidence of breast cancer was studied in a large nationwide cohort of women with a *BRCA1* or *BRCA2* mutation. No increased risk of primary breast cancer after IVF was found (**chapter 5**). For now, there are no oncological terms on which female *BRCA1/2* mutation carriers should be dissuaded from IVF treatment (with or without PGD). However, because of the high *a priori* risk of breast cancer in these women and more limited screening options during pregnancy it is considered wise to perform additional breast screenings before the start of a new IVF treatment (with or without PGD) in female mutation carriers with breast tissue in situ.

From research to clinical practice

The research described in this thesis is not only relevant for patients faced with this matter and medical professionals involved in the care for and cure of patients with a *BRCA1/2* mutation, but also for other stakeholders such as politics. The publication of study aims and results have contributed to the awareness and knowledge of PGD for HBOC among these parties. The fact that our publication regarding the results of the first five years of clinical experience with PGD for *BRCA1/2* mutations (**chapter 3**) was selected for press release during the 28th annual meeting of the European Society for Human Reproduction and Embryology (ESHRE) in Istanbul, 2012, illustrates the newsworthiness of the findings. As a result of the growing number of professionals aware of the availability and, crucially, suitability of PGD for *BRCA1/2* mutations, patients are more often counseled about this reproductive option and, if requested, timely referred to a specialized PGD center. The insights gained into motives and considerations playing a role in the decision-making process are supportive for future couples facing this quandary. Importantly, the results are also assuring for third

parties. Although a slippery slope was feared at first, it turned out that predisposed couples do not take up PGD easily. The questions media and politicians asked in the past, are the same questions patients ask themselves. The final decision whether or not to opt for PGD is a very well-considered one in the vast majority of couples. The high number of requests of PGD for HBOC can partly be explained by the prevalence of *BRCA1/2* mutations, but also shows that predisposed couples are in need of a reproductive strategy less rigorous than prenatal diagnosis. Probably, the justification of PGD as a reproductive option for couples with a hereditary cancer predisposition syndrome can only be judged by predisposed couples themselves, since the perceived severity of the condition is one of the decisive factors in the decision-making process. When evaluating the suitability of PGD for *BRCA1/2* mutations, our data have shown that the treatment leads to a good chance of pregnancy while the risk of breast cancer for predisposed women does not seem to increase. There is currently no need for concern in this particular group of patients.

Outcomes have been or will be published in scientific medical journals and presented at national and international congresses and expert meetings. Where appropriate, outcomes will also be presented at patient organization meetings. The conclusions will be incorporated in a decision aid (see 'Remaining questions and future plans') and discussed during PGD counseling.

Remaining questions and future plans

Our qualitative study explored motives and considerations taken into account in the decision-making process. Currently these results are further studied in a quantitative approach. Based on these results, a decision aid is developed and will be implemented in clinical practice in the near future. This digital decision aid provides information on pros and cons of PGD, prenatal testing, and conception without testing. It aims to support couples in their reproductive decision by weighing the perceived importance of each item and to uncover possible different views between both partners.

There is no convincing evidence for a clinical relevant reduction in ovarian reserve in *BRCA1/2* mutation carriers, but prospective studies on ovarian response of *BRCA1/2* mutation carriers in an IVF setting have been missing so far. A prospective study on ovarian response to stimulation for IVF/PGD is currently ongoing in our centers. Additionally, fundamental studies assessing the effect of a *BRCA1/2* mutation on oocyte and embryo quantity and quality as well as on apoptosis, as indicators for ovarian reserve are now executed in our center. It is important that future studies have the power to distinguish between *BRCA1* and *BRCA2* mutations, since these are different genetic entities with different cancer risks which may have different effects on ovarian reserve. Furthermore, the need of cryopreservation of oocytes or embryos of female *BRCA1/2* mutation carriers is presently studied.

Although the first results regarding the oncological safety of IVF in female *BRCA1/2* mutation carriers in terms of breast cancer risks are reassuring, the level of evidence is suboptimal due to study design and relatively low power. Oncological safety is further addressed by the performance of additional breast screenings before subsequent PGD treatments in female mutation carriers who still have breast tissue in situ. The necessity of these additional check-ups will be evaluated the upcoming years.

In conclusion, the research described in this thesis contributes to a responsible application of PGD for HBOC. Results may also be applicable to other hereditary cancer syndromes with a serious tumor predisposition and a high risk for offspring.

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Summary

Hereditary breast and ovarian cancer is a cancer predisposition syndrome caused by a mutation in the *BRCA1* or *BRCA2* gene. Women with a mutation in one of these genes face elevated risks of breast, fallopian tube, and ovarian cancer. Men with a mutation in the *BRCA1* or *BRCA2* gene have an increased risk of breast and prostate cancer. Female *BRCA1* mutation carriers may be susceptible for serous(-like) endometrial cancer and both male and female *BRCA2* mutation carriers are prone for pancreatic cancer and possibly melanoma. Surveillance programs targeting at breast cancer are available for female mutation carriers and prophylactic breast and ovarian surgery is offered to them. Since the *BRCA1* and *BRCA2* gene have an autosomal dominant inheritance mode, both male and female mutation carriers have a 50% risk to transmit the mutation to their offspring.

Nowadays, mutation carriers who wish to avoid transmission of the genetic predisposition to their children have two reproductive options that lead to offspring genetically related to both prospective parents, namely prenatal diagnosis (PD) and preimplantation genetic diagnosis (PGD). For PGD *in vitro* fertilization (IVF) is performed, followed by genetic testing of the embryos for the presence of a familial mutation. In the Netherlands, PGD is carried out for severe monogenic, fully penetrant diseases since 1995. Maastricht University Medical Center (Maastricht UMC+) is the only licensed center for PGD. PGD for hereditary cancer predisposition syndromes, including *BRCA1/2* mutations, was legalized after an intense political debate in 2008. Since then, *BRCA1/2* mutations are one of the most prevalent indications for PGD in the Netherlands. In Belgium, PGD for hereditary breast and ovarian cancer syndrome was started in 2006. Anno 2017, PGD for *BRCA1/2* mutations is provided in several countries around the world, among which the United States of America, United Kingdom, and Spain.

In this thesis the suitability of PGD as a reproductive technique for couples with a *BRCA1/2* mutation is appraised. The aim is threefold: 1. to evaluate current practice (**Part I**), 2. to assess oncological safety of IVF (with or without PGD) for female *BRCA1/2* mutation carriers (**Part II**), and 3. to study ovarian reserve of *BRCA1/2* mutation carriers (**Part III**). In the **preface** the origin of the thesis is documented. **Chapter 1** provides an introduction in the genetics and clinical presentation of *BRCA1/2* mutations, the reproductive options for mutation carriers, and the organization of PGD in the Netherlands. Additionally, the aims of the thesis are presented.

In **Part I** the reproductive decision-making process of couples deciding on PGD for a *BRCA1/2* mutation is studied as well as the clinical and technical feasibility of PGD for *BRCA1/2*. In **chapter 2** a qualitative study is presented aiming to identify factors influencing reproductive decision-making. Eighteen *BRCA1/2* mutation carrying couples of reproductive age who received extensive counseling regarding PGD and PD

were interviewed to explore their motives and considerations regarding PGD, PD, and conception without testing. It turned out that couples primarily classified PGD and PD as acceptable reproductive options based on perceived severity of the condition and according to their moral beliefs. Then, they outweigh perceived advantages and disadvantages. There was a large overlap in motives and considerations regarding PGD between PGD users, PD users, and couples who decided to conceive without testing. All couples mentioned few important advantages of PGD (e.g., protecting the child and family from the mutation) and a fair number of less important disadvantages (e.g., the necessity of IVF and the relatively low chance of pregnancy after IVF/PGD). Additionally, female mutation carriers indicated the safety of ovarian stimulation and the compatibility of PGD with prophylactic surgeries as important factors in decision-making. It turned out that the emotional impact of the decision-making can be long-lasting. Especially non-users may experience feelings of doubt about the moral justness of their decision.

Subsequently, the suitability of PGD for *BRCA1/2* mutations was evaluated by taking a look at treatments performed in the first years since the legalization. In **chapter 3** an overview of clinical practice is provided, including gynecological and oncological screening procedures and PGD techniques. The results of 145 PGD treatments performed in 70 couples carrying a *BRCA1/2* mutation in the Netherlands and Universitair Ziekenhuis Brussel, Brussels, Belgium, are provided. Among these couples were 42 female mutation carriers (59.2%), of whom six had a history of breast cancer (14.3%). Of 142 fresh IVF/PGD cycles started, 34 (23.9%) resulted in a clinical pregnancy (clinical pregnancy rates 27.9% per oocyte pick-up and 39.1% per embryo transfer). In addition, two out of three PGD cycles performed on embryos cryopreserved before chemotherapy led to the delivery of a healthy child. Conversely, two female *BRCA1* mutation carriers were diagnosed with breast cancer shortly after IVF/PGD treatment. Both women had a magnetic resonance imaging (MRI) of the breast without abnormalities shortly before IVF/PGD treatment. It was concluded that PGD is a suitable reproductive option for couples affected by a *BRCA1/2* mutation, yielding good pregnancy rates for both asymptomatic male and female mutation carriers and female breast cancer survivors. However, it was unclear whether ovarian stimulation for IVF (with or without PGD) had an impact on cancer risks in female *BRCA1/2* mutation carriers. Therefore, the oncological safety of the procedure needed further investigation.

In **chapter 4** more insight is given into the transition from mutation-specific PGD protocols with one or two markers, to universal single-cell PGD tests for *BRCA1* and *BRCA2* mutations (laboratory clinical genetics, Maastricht UMC+). These universal PGD tests are based on haplotyping in a multiplex polymerase chain reaction (PCR) analysis, including six microsatellite markers for *BRCA1* and eight microsatellite markers for *BRCA2*. The universal tests can be applied in 90% of the couples

requesting PGD for a *BRCA1/2* mutation, minimizing preparation time and costs for test set-up and validation.

Because of two cases of breast cancer in *BRCA1* mutation carriers shortly after IVF/PGD (**chapter 2**), the association between ovarian stimulation for IVF and the risk of breast cancer in female *BRCA1/2* mutation carriers was studied (**Part II, chapter 5**). Data of female *BRCA1/2* mutation carriers enrolled in the nationwide HEBON study (Hereditary Breast and Ovarian Cancer study, the Netherlands) combined with female *BRCA1/2* mutation carriers who had undergone IVF/PGD were used to study the association between exposure to ovarian stimulation for IVF and the incidence of breast cancer. Data of 1,550 *BRCA1* and 964 *BRCA2* mutation carriers were analyzed using time-dependent Cox-regression models with age as the timescale, stratified for birth cohort and adjusted for infertility. Observation time started at birth and ended at diagnosis of invasive breast cancer, other invasive cancer diagnosis, or bilateral prophylactic mastectomy, whichever was first. In case these events did not take place before the moment the questionnaire was filled out or the last PGD contact took place, follow-up ended at the age of questionnaire completion (HEBON subgroup) or last PGD contact (PGD subgroup). No association was found between exposure to ovarian stimulation for IVF and breast cancer risk in *BRCA1* and *BRCA2* mutation carriers combined (HR 0.79, 95% CI 0.46-1.36), nor in *BRCA1* mutation carriers alone (HR 1.12, 95% CI 0.60-2.09). There was also no association concerning infertile women (HR 0.73, 95% CI 0.39-1.37). Female age at first IVF and the time since the first IVF treatment were also both not associated with breast cancer risk.

In **Part III** ovarian reserve of *BRCA1/2* mutation carriers was examined. We studied ovarian reserve using two different outcome measurements, namely the number of mature oocytes obtained after IVF/PGD (which is related to the life birth rate in IVF, **chapter 6**) and anti-Müllerian hormone (AMH, a predictor of ovarian response in IVF, **chapter 7**). In **chapter 6** outcome data are presented of first PGD cycles performed in 20 female *BRCA1* and 23 female *BRCA2* mutation carriers. The median number of mature oocytes was 6.5 (IQR 4.0-8.0) in *BRCA1* mutation carriers, 7.5 (IQR 5.5-9.0) in *BRCA2* mutation carriers, and 8.0 (IQR 6.0-11.0) in controls. After adjustment for potential confounders, a statistically significant lower yield of mature oocytes was detected in the *BRCA1* subgroup (*BRCA1* mutation carriers versus controls $p = 0.02$, *BRCA2* mutation carriers versus controls $p = 0.50$).

When evaluating ovarian reserve in terms of serum AMH levels, no adverse outcome was found in females carrying a *BRCA1/2* mutation (**chapter 7**). AMH levels of 124 *BRCA1/2* mutation carriers were compared to AMH levels of 131 proven non-carriers in a multicenter cross-sectional study. There was no difference in AMH level between *BRCA1/2* mutation carriers (median 1.90 $\mu\text{g/l}$, range 0.11-19.00 $\mu\text{g/l}$) and non-carriers (1.80 $\mu\text{g/l}$, range 0.11-10.00 $\mu\text{g/l}$, $p = 0.34$). Adjusted linear regression analysis revealed no reduction in AMH levels in the mutation carriers (relative change = 0.98 (95% CI 0.77-1.22), $p = 0.76$). When considering the results of both studies on

ovarian reserve, it was concluded that there is no evidence for a clinically relevant impact of the presence of a *BRCA1* or *BRCA2* mutation on ovarian reserve.

A reflection on all study results is provided in **chapter 8**. Outcome data are placed into perspective and additionally several contemporary challenges in the care for *BRCA1/2* mutation carriers (e.g., transfer policies of *BRCA1/2* mutation positive male embryos in PGD) are discussed. Finally, opportunities for future research are presented.

Samenvatting

Erfelijke borst- en eierstokkanker is een tumorsyndroom dat veroorzaakt wordt door een mutatie in het *BRCA1* of *BRCA2* gen. Vrouwen met een mutatie in een van deze genen hebben een verhoogd risico op borst-, eileider- en eierstokkanker. Mannen met een mutatie in het *BRCA1* of *BRCA2* gen hebben een verhoogd risico op borst- en prostaatkanker. Vrouwen met een mutatie in het *BRCA1* gen hebben tevens mogelijk een verhoogd risico op een bepaald type baarmoederkanker (sereus carcinoom) en zowel mannen als vrouwen met een *BRCA2* mutatie hebben een verhoogd risico op alvleesklierkanker en mogelijk melanoom. Aan vrouwelijke mutatiedraagsters worden controles van de borsten vanaf 25-jarige leeftijd aangeboden om borstkanker in een zo vroeg mogelijk stadium op te sporen. Daarnaast kunnen zij kiezen voor preventieve chirurgie van de borsten en/of eierstokken. De *BRCA* genen erven autosomaal dominant over, d.w.z. dat zowel mannen als vrouwen met een *BRCA1/2* mutatie 50% risico hebben de erfelijke aanleg voor borst- en eierstokkanker door te geven aan hun kinderen. Zowel zonen als dochters kunnen de aanleg erven.

Tegenwoordig hebben personen met een *BRCA1* of *BRCA2* mutatie twee mogelijkheden om te voorkomen dat zij de erfelijke aanleg voor borst- en eierstokkanker doorgeven aan hun biologische kinderen, namelijk prenatale diagnostiek (PND) en preïmplantatie genetische diagnostiek (PGD). Voor PGD is een *in vitro* fertilisatie (IVF) behandeling nodig. De embryo's die ontstaan na IVF kunnen genetisch onderzocht worden op de aanwezigheid van de bij (een van de) ouders voorkomende *BRCA1/2* mutatie. Vervolgens komen alleen embryo's zonder de bij (een van de) ouders voorkomende *BRCA1/2* mutatie in aanmerking voor terugplaatsing in de baarmoeder. In Nederland wordt PGD voor ernstige, volledig penetrante monogene aandoeningen toegepast sinds 1995. Het Maastricht Universitair Medisch Centrum (Maastricht UMC+) is het enige centrum met een vergunning voor PGD. PGD voor erfelijke kankersyndromen, waaronder mutaties in het *BRCA1* of *BRCA2* gen, werd toegestaan na een hevig politiek debat in 2008. Sindsdien is de erfelijke aanleg voor borst- en eierstokkanker een van de meest voorkomende indicaties voor PGD in Nederland. In België werd PGD voor erfelijke borst- en eierstokkanker voor het eerst toegepast in 2006. Anno 2017 zijn er verschillende landen wereldwijd waar PGD voor erfelijke borst- en eierstokkanker wordt verricht, waaronder de Verenigde Staten, het Verenigd Koninkrijk en Spanje.

In dit proefschrift wordt de toepasbaarheid van PGD voor *BRCA1/2* mutaties onderzocht. De doelstelling is driedelig: 1. het evalueren van de huidige praktijk (**Deel I**), 2. het onderzoeken van de oncologische veiligheid van een IVF behandeling (met of zonder PGD) voor vrouwen met een *BRCA1/2* mutatie (**Deel II**), en 3. het bestuderen van de ovariële reserve van *BRCA1/2* mutatiedraagsters (**Deel III**). In het **voorwoord** wordt de aanleiding tot het onderzoek toegelicht. **Hoofdstuk 1** biedt een introductie in de genetica en de klinische presentatie van *BRCA1/2* mutaties, de reproductieve

mogelijkheden voor mannen en vrouwen belast met een *BRCA1/2* mutatie en de organisatie van PGD in Nederland. Tenslotte worden de doelstellingen van het onderzoek uiteengezet.

In **Deel I** wordt het reproductieve keuzeproces omtrent PGD voor erfelijke borst- en eierstokkanker bestudeerd, alsmede de klinische en technische toepasbaarheid van PGD voor *BRCA1/2* mutaties. In **hoofdstuk 2** wordt een kwalitatieve studie gepresenteerd naar factoren die een rol spelen in het keuzeproces omtrent PGD voor erfelijke borst- en eierstokkanker. Achttien met een *BRCA1/2* mutatie belaste paren van reproductieve leeftijd werden geïnterviewd. De paren waren in het verleden uitgebreid gecounseld over PGD en PND en werden bevraagd over hun motieven en overwegingen met betrekking tot PGD, PND en het nastreven van een zwangerschap zonder genetisch onderzoek bij het kind naar de bij een van hen voorkomende *BRCA1/2* mutatie. Het bleek dat paren PGD en PND al dan niet als reële reproductieve opties zagen afhankelijk van hoe ernstig zij de genetische aanleg voor borst- en eierstokkanker vonden, alsmede hun morele visie op selectie en zwangerschapsafbreking. Vervolgens wogen zij de door hen ervaren voor- en nadelen af. Er was een grote overlap in de motieven en overwegingen ten opzichte van PGD van paren die voor PGD, PND en een zwangerschap zonder genetisch onderzoek kozen. Alle paren noemden een beperkt aantal voor hen zwaarwegende voordelen van PGD (bijvoorbeeld het beschermen van het toekomstige kind en verdere familie voor de *BRCA1/2* mutatie) en een groter aantal minder zwaarwegende nadelen (bijvoorbeeld de noodzaak van IVF en de relatief lage kans op zwangerschap na IVF/PGD). Voor vrouwelijke mutatiedraagsters was daarnaast de veiligheid van de voor PGD noodzakelijke IVF behandeling voor henzelf een belangrijke factor in het keuzeproces, alsmede de planbaarheid van IVF/PGD ten opzichte van preventieve operaties. Een deel van de paren gaf aan dat de gemaakte keuze en het proces dat daaraan vooraf ging een grote emotionele impact op hen had op de langere termijn. Vooral paren die voor een zwangerschap zonder genetisch onderzoek hadden gekozen twijfelden of zij de juiste beslissing hadden genomen.

De toepasbaarheid van PGD voor *BRCA1/2* mutaties werd geëvalueerd aan de hand van de resultaten van de behandelingen die uitgevoerd werden in de eerste jaren na de invoering van PGD voor erfelijke borst- en eierstokkanker. In **hoofdstuk 3** wordt een overzicht van de klinische praktijk gegeven inclusief de gynaecologische en oncologische screeningsprocedures en PGD technieken. De uitkomsten van 145 PGD behandelingen uitgevoerd onder 70 met een *BRCA1/2* mutatie belaste paren in Nederland en het Universitair Ziekenhuis Brussel, België, worden gepresenteerd. Onder deze paren waren 42 vrouwelijke mutatiedraagsters (59.2%), van wie er zes in het verleden waren behandeld voor borstkanker (14.3%). Er werden 142 ovariële stimulaties voor IVF/PGD gestart, waarvan er 34 (23.9%) leidden tot een klinische zwangerschap (27.9% van de behandelingen waarin een eicelpunctie werd verricht en 39.1% van de behandelingen waarin een embryo werd teruggeplaatst). Er werd tevens

driemaal PGD uitgevoerd op embryo's die waren ingevroren voorafgaand aan chemotherapie. Twee van deze behandelingen leidden tot de geboorte van een gezond kind. Twee vrouwen met een *BRCA1* mutatie werden gediagnosticeerd met borstkanker kort na hun ovariële stimulatie voor IVF/PGD. Kort voorafgaand aan hun IVF/PGD behandeling hadden beide vrouwen een normale MRI van de borsten. Er werd geconcludeerd dat PGD toepasbaar is voor paren belast met een *BRCA1/2* mutatie: de behandeling leidt tot een goede kans op zwangerschap, zowel in geval van een mannelijke mutatiedragers als in geval van een vrouwelijke mutatiedraagster met of zonder voorgeschiedenis van borstkanker. Omdat het niet duidelijk was of de ovariële stimulatie die nodig is voor IVF/PGD van invloed was geweest op de borstkanker bij de twee voorgenoemde vrouwen met een *BRCA1* mutatie werd verder onderzoek naar de oncologische veiligheid van IVF (met of zonder PGD) voor vrouwen met de erfelijke aanleg voor borst- en eierstokkanker noodzakelijk geacht.

In **hoofdstuk 4** wordt meer inzicht gegeven in de overgang van mutatie-specifieke PGD testen, gebaseerd op mutatiedetectie gecombineerd met een of twee microsatelliet markers, naar universele PGD testen voor mutaties in het *BRCA1* of *BRCA2* gen (laboratorium klinische genetika, Maastricht UMC+). Deze universele PGD testen zijn gebaseerd op haplotypering in een multiplex polymerase ketting reactie (polymerase chain reaction, PCR): in geval van een *BRCA1* mutatie wordt er gebruik gemaakt van zes microsatelliet markers in en rond het *BRCA1* gen, in geval van een *BRCA2* mutatie van acht microsatelliet markers rond het *BRCA2* gen. De universele testen kunnen gebruikt worden voor 90% van de paren die PGD voor een *BRCA1/2* mutatie vragen. Hiermee worden de kosten voor de testontwikkeling en -validatie alsmede de voorbereidingstijd beperkt.

Naar aanleiding van de twee vrouwen met een *BRCA1* mutatie die gediagnosticeerd werden met borstkanker kort na hun IVF/PGD behandeling (**hoofdstuk 2**) werd de associatie tussen ovariële stimulatie voor IVF (met of zonder PGD) en het risico op borstkanker bij vrouwen met een *BRCA1/2* mutatie bestudeerd. De resultaten worden weergegeven in **Deel II, hoofdstuk 5**. Gegevens van vrouwelijke *BRCA1/2* mutatiedraagsters die deelnamen aan de landelijke HEBON studie (Hereditair Borst- en eierstokkanker Onderzoek Nederland) werden gecombineerd met gegevens van vrouwen met een *BRCA1/2* mutatie die PGD hadden ondergaan. Data van 1.550 vrouwen met een *BRCA1* mutatie en 964 vrouwen met een *BRCA2* mutatie waren beschikbaar en werden geanalyseerd middels leeftijdsafhankelijke Cox regressie modellen, gestratificeerd voor geboortecohort en gecorrigeerd voor infertiliteit. De observatietijd startte bij de geboorte en eindigde op het moment waarop borstkanker gediagnosticeerd werd, dan wel een andere invasieve kanker vastgesteld werd of een preventieve borstamputatie beiderzijds plaatsvond. Indien geen van deze gebeurtenissen zich voordeed, eindigde de follow-up op het moment dat de HEBON vragenlijst ingevuld werd (HEBON subgroep) dan wel het laatste contact in het kader van de PGD behandeling plaatsvond (PGD subgroep). Er werd geen associatie

gevonden tussen blootstelling aan ovariële stimulatie voor IVF en borstkankerrisico in de groep *BRCA1* en *BRCA2* mutatie draagsters gecombineerd (HR 0.79, 95% CI 0.46-1.36), noch in de subgroep *BRCA1* mutatie draagsters (HR 1.12, 95% CI 0.60-2.09), noch in de subgroep infertiele vrouwen (HR 0.73, 95% CI 0.39-1.37). Er was eveneens geen associatie tussen de leeftijd van de vrouw ten tijde van de eerste IVF behandeling en het risico op borstkanker, en ook niet tussen de tijd die verstreken was sinds de eerste blootstelling en het risico op borstkanker.

In **Deel III** worden studies naar de ovariële reserve van vrouwen met een *BRCA1/2* mutatie gepresenteerd. Hun ovariële reserve werd onderzocht gebruikmakend van twee verschillende uitkomstmaten, namelijk het aantal eicellen dat verkregen werd na ovariële stimulatie voor IVF/PGD (hetgeen gerelateerd is aan de kans op een levend geboren kind na IVF, **hoofdstuk 6**) en het anti-Müllerian hormoon (AMH, een voorspeller van de ovariële response bij IVF, **hoofdstuk 7**). In **hoofdstuk 6** wordt de ovariële response van 20 *BRCA1* en 23 *BRCA2* mutatie draagsters op een eerste ovariële stimulatie voor IVF/PGD vergeleken. Het mediane aantal mature eicellen was 6.5 (IQR 4.0-8.0) in de groep vrouwen met een *BRCA1* mutatie, 7.5 (IQR 5.5-9.0) in de groep vrouwen met een *BRCA2* mutatie en 8.0 (IQR 6.0-11.0) in de controlegroep. Na correctie voor mogelijke confounders werd een statistisch significant lager aantal mature eicellen gevonden in de *BRCA1* subgroep (*BRCA1* mutatie draagsters versus controles $p = 0.02$, *BRCA2* mutatie draagsters versus controles $p = 0.50$).

In onze studie naar ovariële reserve met AMH waarde als uitkomstmaat werden geen aanwijzingen gevonden voor een verminderde ovariële reserve in vrouwen met een *BRCA1/2* mutatie (**hoofdstuk 7**). In een multicenter, cross-sectionele studie werden de AMH waarden van 124 vrouwen met een *BRCA1/2* mutatie vergeleken met de AMH waarden van 131 vrouwen zonder *BRCA1/2* mutatie. Er was geen verschil in AMH waarden tussen vrouwen met een *BRCA1/2* mutatie (mediaan 1.90 $\mu\text{g/l}$, range 0.11-19.00 $\mu\text{g/l}$) en vrouwen zonder *BRCA1/2* mutatie (mediaan 1.80 $\mu\text{g/l}$, range 0.11-10.00 $\mu\text{g/l}$, $p = 0.34$). Ook in een voor mogelijke confounders gecorrigeerde lineaire regressie analyse werd geen verlaagd AMH gevonden bij mutatie draagsters in vergelijking met controle vrouwen (relative change = 0.98 (95% CI 0.77-1.22), $p = 0.76$). Beide studies naar ovariële reserve in acht nemende wordt geconcludeerd dat er geen aanwijzingen zijn voor een klinisch relevante invloed van een *BRCA1* of *BRCA2* mutatie op de ovariële reserve.

In **hoofdstuk 8** wordt gereflecteerd op de studieresultaten. De uitkomsten worden in perspectief geplaatst en uitdagingen in de huidige zorg voor zowel vrouwen als mannen belast met een *BRCA1/2* mutatie worden bediscussieerd. Een voorbeeld is het terugplaatsen van mannelijke embryo's met de erfelijke aanleg voor borst- en eierstokkanker na PGD. Tenslotte worden aanbevelingen gedaan voor toekomstig wetenschappelijk onderzoek op dit gebied.

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Dankwoord

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Curriculum Vitae

Curriculum Vitae

Inge A.P. Derks-Smeets werd geboren op 3 juni 1984 in Schipperskerk, een klein dorp dat thans deel uitmaakt van de gemeente Sittard-Geleen. In 2002 behaalde zij cum laude haar gymnasium diploma aan de Trevianum Scholengroep te Sittard. Inge werd via de decentrale selectie procedure toegelaten tot de opleiding geneeskunde aan de Vrije Universiteit Amsterdam. In 2006 participeerde zij in het kader van haar wetenschappelijke stage in een studie naar delirium op de pediatrische intensive care unit (PICU) in het Maastricht Universitair Medisch Centrum (Maastricht UMC+, stagebegeleider dr. J.N.M. Schievelde, kinderpsychiater). Deze stage wekte haar interesse voor wetenschappelijk onderzoek en resulteerde in verscheidene (mede-)auteurschappen van wetenschappelijke publicaties. In 2009 behaalde Inge het artsexamen. Datzelfde jaar deed zij klinische ervaring op als arts-assistent (ANIOS) gynaecologie en verloskunde in achtereenvolgens het Máxima Medisch Centrum te Veldhoven en het Catharina Ziekenhuis te Eindhoven.

Vanaf 2010 werkte Inge als arts preïmplantatie genetische diagnostiek (PGD) op de afdeling klinische genetica van het Maastricht UMC+. In 2011 startte zij met wetenschappelijk onderzoek naar PGD voor erfelijke borst- en eierstokkanker, onder begeleiding van prof. dr. C.E.M. de Die-Smulders, prof. dr. V.C.G. Tjan-Heijnen, dr. R.J.T. van Golde (respectievelijk verbonden aan de afdelingen klinische genetica, medische oncologie en voortplantingsgeneeskunde van het Maastricht UMC+) en prof. dr. W. Verpoest (centrum voor reproductieve geneeskunde, Universitair Ziekenhuis Brussel, België). Voor een groot gedeelte van het onderzoek verkreeg zij een persoonlijke onderzoeksbeurs van KWF Kankerbestrijding. De resultaten van dit onderzoek vormden de basis voor dit proefschrift.

Inge is gehuwd met Donné Derks en met hun kinderen Brenn (2013), Ward (2015) en Ava (2017) wonen zij in Spaubeek.

List of publications

List of publications

Derks-Smeets IAP[‡], Schrijver LH[‡], De Die-Smulders CEM, Tjan-Heijnen VCG, Van Golde RJT, Smits LJ, Caanen B, Van Asperen C, Ausems M, Collée M, Van Engelen K, Kets CM, Van der Kolk L, Oosterwijk JC, Van Os T, HEBON, Rookus MA, Van Leeuwen FE^{‡‡}, Gómez García EB^{‡‡}. Ovarian stimulation for IVF and risk of primary breast cancer in BRCA1/2 mutation carriers. *Submitted for publication*.

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