Fertility preservation in women: exploring clinical dilemmas
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CHAPTER 1

General introduction
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Discovery of chemotherapy

Curing cancer by chemotherapy is marked as one of the greatest achievements in modern medicine. The first chemotherapeutic agent to be discovered was nitrogen mustard. This discovery had its - ugly - beginnings in World War I when it was observed that soldiers who were gassed with mustard gas had destroyed cell-lines in their bone marrow (Krumbhaar and Krumbhaar, 1919).

During the Second World War, with chemical warfare laying on the lure, an extensive search for information on the effects of war gases was undertaken – mustard gas in particular, because this war gas could circumvent protection by chemical masks. A US fleet, secretly containing a shipment of mustard gas, was sunk after a German raid in Bari in 1943 causing accidental spill of the 100 tons of mustard gas on board. Medical reports showed low white blood cells counts in the blood of the sailors after the attack (Alexander, 1947). The destructive effects of mustard gas on blood cells, already observed in World War I were thereby confirmed.

As part of the US military research program in 1943, Winternitz, who had studied mustard gas in World War I, asked two prominent Yale pharmacologists, Gilman and Goodman, to work together in carrying out experiments to find out whether nitrogen mustard might also halt malignant proliferation of white blood cells that occur in patients with leukemia or lymphoma. This research program was classified as ‘top secret’ because of the secrecy associated with war gas programs so results were not published until 1946. Findings included marked regressions of lymphoid tumor in mice treated with nitrogen mustard and were so convincing that thoracic surgeon Lindskog administered nitrogen mustard to a patient with non-Hodgkin’s lymphoma, resulting in marked regression of disease (Gilman 1946; Goodman et al., 1946; Gilman Science 1946). After these publications, several related alkylating agents, such as busulphan and cyclophosphamide were synthesized and tested.

Discovery of gonadotoxic side effects of chemotherapy

Busulphan, an alkyl sulfonate drug that acts as a bifunctional alkylating agent in the same manner as nitrogen mustard does, was first described as a potentially effective treatment for chronic myeloid leukemia in 1953 (Haddow and Timmis, 1953). Also in 1953, Bollag, a clinician in Switzerland, observed that menses ceased in a 42-year old woman after treatment with busulphan. To further investigate this observation, he performed histological examination in ovaries of rats treated with busulphan, which
revealed atrophic ovaries (Bollag, 1953). Based on these findings, the Czechoslovakian researcher Belohorsky performed histological examination of the ovary of a 32-year old woman whose menses ceased after treatment with busulphan, which showed depletion of follicles (Belohorsky et al., 1960).

**Discovery of combination chemotherapy and its long-term side effects on the menstrual cycle**

Cyclophosphamide, also a nitrogen mustard alkylating agent, was approved by the US Food and Drug Administration (FDA) in 1959 (Emadi, Jones et al., 2009) in an era full of skepticism surrounding the clinical usefulness of chemotherapy because of its harsh side effects and low effectivity in improving survival (Zubrod et al., 1966). Nevertheless, the wealthy philanthropist Mary Lasker was impressed by the data on chemotherapy in childhood leukemia, and had been urging the US Congress to provide funds to set up a cancer research program. She played an important role in the foundation of the National Service Center Cancer Chemotherapy in 1955, a semi-political program that gave rise to a multibillion-dollar cancer pharmaceutical industry.

One of the greatest achievements of the program was the discovery that series of a cyclically administered combination of nitrogen mustard with vincristine, procarbamazapine, and prednisone (MOPP) chemotherapy for Hodgkin’s disease resulted in remission rates of up to 80% (Devita et al., 1970). This led to the basic concept that chemotherapy could cure cancer.

Clinicians began to use combination chemotherapy in advanced breast cancer in the late 1960’s, and the CMF program (cyclophosphamide, methotrexate and 5-fluoracil) showed an impressive overall response rate of over 50% (Canellos et al., 1974). Enthusiasm for clinical use of CMF was further encouraged by positive results of Bonnadonna’s CMF study in the New England Journal of Medicine in 1976 (Bonnadonna et al., 1976), published one year after the announcement that both the wife of the president of the United States, Betty Ford, and the wife of the vice-president, Happy Rockefeller, were diagnosed with breast cancer.

Bonnadonna’s study also reported that half of the premenopausal women in their study ceased menstruating during chemotherapy and they remarked that long-term side effects should be taken into account when administering CMF regimens. This was in line with findings from one year earlier that 14 out of the 22 women who had received cyclophosphamide for non-malignant disease (glomerulonephritis and
rheumatoid arthritis) had symptoms of ovarian failure and that ovarian biopsies in six of them showed absence of primordial follicles (Warne et al., 1973). Several authors at that time emphasized the need to inform women on the risk of premature ovarian insufficiency as an adverse outcome of regimens containing cyclophosphamide (Chapman et al., 1979; Warne et al., 1973), but ways to preserve fertility were remote at that time.

**Discovery of freezing oocytes**

In 1977, Whittingham published a ground-braking paper in which he showed that mice oocytes could be frozen, thawed and fertilized (Whittingham, 1977). One decade later, the world came to hear of the first ongoing human –twin- pregnancy after cryopreservation of oocytes (Chen, 1986). Whittingham nor Chen mentioned the applicability of this technique for women at risk of sterility because of radio- and/or chemotherapy in their key papers.

It was Van Uem et al. who, when reporting on the second live-birth following freezing of oocytes in 1987, wrote that ‘cryopreservation of oocytes might improve the prospects of fertility in young women scheduled to be treated by chemotherapy or radiotherapy for cancer’ (van Uem et al., 1987). This study was the first to mention the concept of fertility preservation for women with cancer. Meanwhile, the struggle to overcome cryobiological problems to make cryopreservation of oocytes a clinically applicable procedure for women with cancer was ongoing.

The first hurdle became manifest at the inception of oocyte freezing, when the mice oocytes frozen with dimethylsulphoxide (DMSO) and stored under liquid nitrogen at − 196 °C, showed lower fertilization rates than those of fresh oocytes (Whittingham, 1977). Membrane damage caused by freezing and thawing was suggested as a possible explanation. It was later confirmed that freezing induced hardening of the zona pellucida, which explained low fertilization rates (Carroll et al., 1990; Johnson et al., 1988; Vincent et al., 1990).

Not only the zona pellucida, but also the meiotic spindle inside the oocyte was targeted by cooling injury (Pickering et al., 1990; Sathananthan et al., 1988). Also, increased polyploidy was found when frozen-thawed oocytes were fertilized (Bouquet et al., 1992; Carroll et al., 1989; Glenister et al., 1987).

Given the low success rates achieved by mature oocyte freezing, the potential benefits of immature oocyte freezing were explored, but this technique was soon abandoned as very low numbers of fertilized oocytes reached developmental stages viable for embryo
transfer (Son et al., 1996; Mandelbaum et al., 1988). These disappointing results forced research groups to re-investigate the problems encountered in mature oocyte freezing. By the mid-1990s, Gook et al. booked success in overcoming the problem of hardened zonae by performing ICSI in slow-frozen metaphase II oocytes using 1,2 propanediol (PROH) as a cryoprotectant (Gook et al., 1995). Porcu et al. followed this approach and were the first -after a hiatus of almost 10 years- to report on a human live birth after performing ICSI on frozen-thawed oocytes (Porcu, Fabbri et al., 1997). For this live birth, 12 oocytes had to be thawed, of which only 4 survived the thawing process (33.3%). Low survival rates after thawing remained the major problem as illustrated by the fact that the same research group obtained only six live births out of more than 700 oocytes thawed (Porcu et al., 1998). Comparable disappointing results were reported by Tucker et al., who had to thaw almost 400 oocytes to achieve one live birth (Tucker et al., 1998).

Despite all these obstacles, Letur-Konirsh et al. were the first to report to have cryopreserved oocytes for a woman with cancer (Letur-Konirsch et al., 1994). In their cohort of 10 women with cancer, 9 women froze embryos and one woman froze oocytes because she had no male partner. The authors fail to mention what type of cancer she had, what type of freezing protocol they used and what the follow up was.

So, clinicians had no options other than to either watch how women started cancer treatment that would leave them sterile, or to offer them cryopreservation of oocytes with its low efficiency.

Towards clinical applicability of freezing oocytes

The need to establish an effective protocol for freezing oocytes was not only pressing because of women with cancer who had to undergo fertility-threatening treatment, but also because of the controversies about frozen embryo’s, including ethical and legal issues (Bankowski et al., 2005; Robertson, 1987). Kuleshova et al. explored ultrarapid cooling of oocytes, a technique called ‘vitrification’. In this procedure, formation of intracellular ice is avoided and the damaging effects of osmosis that occur during cooling and thawing are diminished. A total of 17 oocytes were vitrified in four women who agreed to have their surplus oocytes vitrified instead of their embryos. Out of these 17 oocytes, 11 survived thawing after vitrification (65%) and after ICSI five pronuclear zygotes were obtained. Three embryos were transferred in three women, resulting in one live birth (Kuleshova et al., 1999).

By 2005, researchers in Japan reported remarkable high survival- and fertilization rates
of vitrified oocytes of up to 90% using the so-called ‘Cryotop’ method. In the Cryo-
top method, oocytes are washed in vitrification solution after equilibration. Then they
are picked up individually in an extremely small volume of vitrification solution and
placed on top of a polypropylene strip (cryotop), which is attached to a plastic handle.
As soon as the oocyte is placed on top of the cryotop, the oocyte is plunged into liquid
nitrogen (Kuwayama et al., 2005). The technique resulted in high survival and fertilization
rates after thawing and was suggested to resolve the long lasting efficiency problems
of freezing human oocytes (Katayama et al., 2003). Since then, consistently high
survival rates have been reported following vitrification of meta-phase II oocytes and
the available evidence suggests that vitrification is currently the method of choice for
cryopreservation of meta-phase II oocytes (Glujovsky et al., 2014).
Now that there were fertility preserving options for women with cancer who did not
have the option or wish to preserve embryo’s, the American Society for Clinical Oncol-
ogy (ASCO) issued fertility preservation guidelines for health care providers in cancer
in 2006 (Lee et al., 2006) These guidelines mentioned the need to discuss fertility-relat-
ed side effects of cancer treatment and referral to centers where fertility preservation
can be performed.

In conclusion from then till now

Looking back in time, when US president Nixon declared ‘the war on cancer’ in 1971
one could not have envisioned that 35 years later, due to increased survival after cancer
treatment, the focus would shift towards the quality of life for cancer survivors.
With regard to the historic collision of reproduction and cancer survival, one might
notice that the concept of freezing oocytes as a means to preserve fertility for women
with cancer was not designed beforehand, but somehow emerged naturally after the
technical drawbacks of freezing oocytes were overcome. In the same fashion, other
indications for freezing oocytes arose, such as a risk of premature ovarian insufficiency
(POI) because of genetic predisposition or ovarian surgery, or the wish to defer mother-
hood for other so called ‘non-medical’ reasons.

Towards the future

ASCO’s recognition in 2006 that fertility preservation is an integral part of the care for
young patients with cancer can be considered a milestone in the evolution of cancer
treatment. In 2013 the American Society for Reproductive Medicine (ASRM) removed
the experimental connotation of freezing oocytes (Pfeifer et al., 2013). This opened the gate for clinics to offer this procedure to any woman at risk for therapy- or disease-induced POI and for women wishing to defer motherhood for other reasons. As a consequence, we now need studies that investigate the clinical implications of the procedure.

**Background of this thesis**

Cryopreservation of oocytes became available in 2006 in the Netherlands, and was then only performed in the Centre for Reproductive Medicine of the Academic Medical Centre in Amsterdam. Until 2010 cryopreservation of oocytes was only applied for women whose partner had insufficient sperm at the day of ovum pick and sporadically for women with cancer who had to undergo gonadotoxic therapy (de Melker et al., 2010). By 2011 the technique became also available for a new subset of patients, namely women who have to defer motherhood at a time that their fertility is likely to be at threat. This can be due to planned gonadotoxic therapy, ovarian surgery, genetic predisposition for POI or because of age-related decline of fertility or anticipated gamete exhaustion (Pfeifer et al., 2013; Bedoschi and Oktay, 2013; Homburg et al., 2009, Stoop et al., 2014).

The introduction of non-elective freezing oocytes or embryos has brought along new clinical dilemmas. Whereas daily clinical IVF practice is marked as elective care, freezing oocytes requires clinical pathways for acute care. Quality-management projects on how to set-up a program for fertility preservation were lacking, but strongly needed by 2011 as that year is marked by the event of political permission in the Netherlands for freezing oocytes for non-medical reasons and thereby opened the gate for a potentially large influx of women opting for cryopreservation of oocytes. So, we set up a quality management project to establish and evaluate our fertility preservation-program by means of ‘Strenghts, Weaknesses, Opportunities and Threats’ SWOT analysis over a time-period of two years.

We expected women with breast cancer to be the largest group of women who opt for cryopreservation of oocytes in the acute setting, because breast cancer is the most common malignancy in young women (Jemal et al., 2010), and it’s treatment affects fertility in multiple ways. First, breast cancer occurring at reproductive age often requires cyclophosphamide containing chemotherapy regimens, which has gonadotoxic side effects (Bines et al., 1996; Meirow and Nugent, 2001; Sukumvanich et al.,
2010). Second, the medically advised delay of pregnancy until two years after diagnosis (Gwyn and Theriault, 2000; Isaacs, 1995; RCOG, 2011) increases the chances of age-related decline of fertility, and a 5-to 10 year delay during endocrine therapy in case of hormone sensitive breast cancer further amplifies this (Barthelmes and Gateley, 2004; Braems et al., 2011). Third, 5 to 10 % of young women with breast cancer are affected by a BRCA1/2 mutation with a subsequent increased risk of ovarian cancer (Begg et al., 2008), for which women may choose to prophylactically undergo bilateral salpingo-ovariectomy. Therefore, we aimed to answer research questions particularly relevant for women with breast cancer.

At the time we started our studies, few studies were at hand that dealt with the issue of controlled ovarian stimulation in women for whom high estrogen exposure could be potentially harmful, as growth of breast tumours can be stimulated by estrogens (Key et al., 2002; Yager and Davidson, 2006; Eliassen et al., 2006). Some studies mentioned the potential beneficial effect of adding letrozole -an aromatase inhibitor- to controlled ovarian stimulation as this would lead to decreased peak estradiol levels (Oktay et al., 2005; Oktay et al., 2010; Reddy and Oktay, 2012), while others report that additional letrozole has counteracting effects on oocyte yield (Revelli et al., 2013). Adding tamoxifen was suggested to be beneficial, as this approach seemed not to compromise oocyte-yield in a small patient series (Oktay et al., 2005). Tamoxifen is a complex drug that undergoes extensive biotransformation to eventually become active as a selective estrogen-receptor modulator. When tamoxifen is used in the adjuvant therapeutic setting of breast cancer for post-menopausal women, ‘efficacy’ of tamoxifen can be expressed when it’s most active metabolite endoxifen reaches a threshold in plasma of 7 ng/ml (Madlensky et al., 2011; Borges et al., 2006; Jin et al., 2005). As it was unknown how efficacy of tamoxifen can be expressed in young women with breast cancer undergoing ovarian stimulation, we performed a pilot study to elucidate how tamoxifen ‘behaves’ in the setting of it being an additional agent during controlled ovarian stimulation.

Adjusted stimulation-protocols with additional tamoxifen or letrozole had found their way into daily clinical practice based on studies with methodologic flaws that concluded that tamoxifen and letrozole can serve a ‘protective’ role in women with breast cancer. In view of this, we thus evaluated these stimulation-protocols in terms of safety and efficiency. First, we conducted a systematic review of literature of these stimulation-protocols in terms of safety. Subsequently, we aimed to fill the knowledge gap of what stimulation protocol (with or without additional tamoxifen or letrozole) serves women with breast cancer best in terms of oocyte yield. We conducted a ran-
domised-controlled trial in which controlled ovarian stimulation plus tamoxifen and controlled ovarian stimulation plus letrozole was compared with standard controlled ovarian stimulation in terms of the number of oocytes retrieved at follicle aspiration. In addition it was unknown what issues are relevant for the women themselves. We therefore aimed to answer the question how women experienced the procedure of freezing oocytes or embryo’s while being recently diagnosed with breast cancer by a qualitative study using phenomenological methodology. By 2013, the question arose what reproductive choices were made after women had previously cryopreserved their oocytes for medical reasons. A follow-up study was performed, in which we collected data on demographics, outcomes of ovarian stimulation, fertility-threatening treatments, menstrual cycle changes, pregnancy attempts and outcomes of these attempts, and women’s intended plans for their cryopreserved oocytes.

**Outline of this thesis**

In **Chapter 2** we describe a quality-management project that took place between 2011 and 2013 in which we present how our Center for Reproductive Medicine organized fertility preservation care by means of a ‘Strengths, Weaknesses, Opportunities and Threats’ (SWOT) analysis.

In **Chapter 3** we report a prospective case-series in which we assessed tamoxifen and tamoxifen metabolite levels (endoxifen) by taking bloodsamples of four women with estrogen receptor-positive breast cancer who underwent controlled ovarian stimulation with additional tamoxifen (60 mg per day) for cryopreservation of oocytes.

In **Chapter 4** we present a systematic review which aimed to assess the effects of adding tamoxifen or letrozole to standard controlled ovarian stimulation protocols on the breast cancer free interval in young women with estrogen receptor-positive breast cancer who banked oocytes or embryos.

In **Chapter 5** we present the study-protocol of the STIM-trial (trial register number: NTR4108): “Stimulation of the ovaries in women with breast cancer undergoing fertility preservation: alternative versus standard stimulation protocols”. This is a multicentre randomised open-label controlled trial. We compared COS-alone with
COS plus tamoxifen 60 mg with COS plus letrozole 5 mg. Primary outcome is the number of oocytes retrieved at follicle aspiration. Secondary outcomes are number of mature oocytes retrieved, number of oocytes or embryos banked and peak E2 levels during COS.

In Chapter 6 we present a qualitative phenomenological study investigating the lived experience of women undergoing fertility preservation while being newly diagnosed with breast cancer.

In Chapter 7 we report a follow-up study on the reproductive choices and outcomes of 68 women after cryopreservation of their oocytes for medical reasons.

In Chapter 8 we provide a general discussion of the findings of this thesis and provide suggestions for future research.

In Chapter 9 we provide a summary of the data presented in this thesis.
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