Ovarian Reserve in Breast Cancer Survivors

by

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Institute of Medical Sciences
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Abstract

The long-term natural history of ovarian reserve after adjuvant chemotherapy for breast cancer has been poorly described. We recruited 52 breast cancer survivors treated with adjuvant chemotherapy before 40 years of age who remained premenopausal after chemotherapy treatment. Twenty (38.5%) were more than five years out from treatment. Ovarian reserve estimates were compared with a control group. Anti-Müllerian hormone (AMH), follicle stimulating hormone and luteinizing hormone demonstrated significant differences consistent with reduced ovarian reserve in breast cancer survivors. Mean AMH was 6.65 pmol/l in survivors compared to 17.43 in controls (p < 0.001). Attained age and age at the time of treatment were correlated with AMH levels in breast cancer survivors.

Conclusion: Ovarian reserve is significantly reduced in young breast cancer survivors. Age is the major predictor of AMH level in survivors. A 35 year old breast cancer survivor has an AMH level similar to a 45 year old control.
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To my husband Ronan and our sons Brendan and Patrick – thank you for your love and laughter. I count my blessings every day.
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<tbody>
<tr>
<td>AC</td>
<td>Doxorubicin and cyclophosphamide</td>
</tr>
<tr>
<td>ACD</td>
<td>doxorubicin, cyclophosphamide and docetaxel</td>
</tr>
<tr>
<td>ACT</td>
<td>doxorubicin, cyclophosphamide and paclitaxel</td>
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<tr>
<td>AFC</td>
<td>Antral follicle count</td>
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<tr>
<td>AMH</td>
<td>Anti-Müllerian hormone</td>
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<tr>
<td>ART</td>
<td>Assisted reproductive therapy</td>
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<tr>
<td>CEF</td>
<td>Cyclophosphamide, epirubicin and 5-fluourouracil</td>
</tr>
<tr>
<td>CMF</td>
<td>Cyclophosphamide, methotrexate and 5-fluourouracil</td>
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<tr>
<td>COCP</td>
<td>combined oral contraceptive pill</td>
</tr>
<tr>
<td>CRF</td>
<td>case record form</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>DCIS</td>
<td>ductal carcinoma in-situ</td>
</tr>
<tr>
<td>EC</td>
<td>epirubicin and cyclophosphamide</td>
</tr>
<tr>
<td>EPR</td>
<td>electronic patient record</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FAC</td>
<td>5-fluourouracil, doxorubicin and cyclophosphamide</td>
</tr>
<tr>
<td>FEC</td>
<td>5-fluourouracil, epirubicin and cyclophosphamide</td>
</tr>
<tr>
<td>FEC-D</td>
<td>5-fluourouracil, epirubicin and cyclophosphamide, docetaxel</td>
</tr>
<tr>
<td>FMP</td>
<td>Final menstrual period</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
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<tr>
<td>GnRH</td>
<td>Gonadotrophon releasing hormone</td>
</tr>
<tr>
<td>HRT</td>
<td>hormone replacement therapy</td>
</tr>
<tr>
<td>IVF</td>
<td>In-vitro fertilization</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>OCP</td>
<td>oral contraceptive pill</td>
</tr>
<tr>
<td>OECD</td>
<td>Organization for Economic Co-operation and Development</td>
</tr>
<tr>
<td>PMH</td>
<td>Princess Margaret Hospital</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>SEER</td>
<td>Survival, Epidemiology and End Results Program</td>
</tr>
<tr>
<td>TC</td>
<td>Docetaxel, cyclophosphamide</td>
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Chapter 1

Literature Review

1 Introduction

Each year, almost 1,000 Canadian women are diagnosed with breast cancer before the age of forty years and 19% of women diagnosed with breast cancer are less than fifty years of age [1-3]. Breast cancer survival rates have improved significantly in the past thirty years resulting in an increasing survivorship population [1]. The long-term side effects experienced by the survivors present a growing clinical and research challenge as survival rates improve.

Premature menopause is a potential long-term complication of systemic chemotherapy. The effects of menopause are many including infertility, vasomotor changes, osteoporosis, psychological effects, genitourinary changes and heart disease [4, 5]. These conditions are associated with significant morbidity and mortality and may be inadequately managed by current clinical care structures. For young women, in particular those diagnosed with breast cancer before the age of 40 years, the impact of chemotherapy on ovarian function is a potentially significant chronic toxicity of treatment. The significance of fertility as an issue for young breast cancer survivors is compounded by changing patterns of childbirth in contemporary society. Canadian women are increasingly delaying childbirth; the pregnancy rate in women over thirty years of age is rising with the largest relative increase in those over forty years of age, in whom the rate has risen by 25% [6]. These trends are also seen in the United States and Europe. An increasing number of couples will rely on assisted reproduction techniques to achieve pregnancy purely due to this delay in childbearing [7]. This societal change will result in more women undergoing treatment for breast cancer at a time when they have not completed their families.

At present, there is a lack of information regarding the potential impact of systemic therapy on subsequent fertility among young breast cancer survivors. Much of the available data employs amenorrhea as a surrogate marker for fertility status. These studies indicate that the majority of women treated with chemotherapy before the age of 40 years experience chemotherapy induced amenorrhea, but will subsequently regain regular menstrual function [8]. However, these women remain at risk of premature menopause years after treatment [9].
Much of the available data relate to older chemotherapy regimens such as CMF (cyclophosphamide, methotrexate, 5-fluorouracil), consisting of cytotoxic drug combinations not considered optimal in the modern adjuvant treatment setting [10]. However, the major drawback of this data is that the presence or absence of amenorrhea is a poor surrogate marker for fertility. A better estimate of the true impact on fertility requires assessment of the remaining ovarian reserve.

Ovarian reserve refers to the quality and quantity of follicles remaining in the ovary. The number of follicles decreases during reproductive life, ultimately culminating in menopause when the supply is exhausted. The use of biochemical and biophysical measures of ovarian reserve is well described in the investigation of female infertility. Anti-müllerian hormone (AMH) or Müllerian Inhibitory Substance and antral follicle count (AFC) in particular are sensitive measures of the natural decline in ovarian reserve seen with age [11-13]. A handful of studies have reported ovarian reserve measures in breast cancer survivors before and after chemotherapy [14-16]. These studies demonstrate significantly reduced ovarian reserve measurements in normally menstruating young breast cancer survivors in comparison with controls. Two studies have identified reduced ovarian reserve in survivors more than one year after chemotherapy treatment [17, 18]. However, most of these data refer to women assessed at a median of 2 years after treatment. The long-term impact of chemotherapy on ovarian reserve in this population remains poorly described.

A detailed description of ovarian reserve in breast cancer survivors after chemotherapy might allow us to predict the time after which the chances of spontaneous or assisted conception decrease significantly. At present the main factors considered when estimating the impact of adjuvant chemotherapy on fertility are the age of the individual and the type of chemotherapy treatment received [19]. The ability to predict the impact of cancer treatment on the ovarian follicular pool in individual cases would allow individualization of fertility counseling and preservation strategies. For young women newly diagnosed with breast cancer, this would provide additional information that may illuminate treatment decisions that will have a long-lasting impact on their physical and mental health.

In this introduction I will review the epidemiology of breast cancer in young women and analyze the current state of knowledge regarding fertility after treatment in this patient population. I will
also review ovarian reserve testing in the setting of natural ageing of the ovary, in cancer survivors and in the prediction of response to assisted reproduction techniques.

1.1 Breast Cancer in Young Women – Epidemiology

Each year over 23,000 women are diagnosed with breast cancer in Canada [1-3]. Breast cancer is the commonest cancer in women accounting for 28% of all cancer cases [2] and Canadian women have a 1 in 9 lifetime risk of developing breast cancer [1]. Nineteen percent of women diagnosed with breast cancer are less than fifty years of age at the time of diagnosis [2] and approximately 1,000 women annually are diagnosed in Canada with breast cancer before they reach 40 years of age [1-3]. Globally, breast cancer is the commonest cancer diagnosis in both the developed and the developing world [20].

The overall incidence of cancer in the Canadian population is rising [2]. The incidence of breast cancer has risen steadily from 1980 to the early 1990’s, largely due to the uptake of mammographic screening [2]. In the same time period, there has been a significant reduction in breast cancer mortality [2]. The age standardized mortality rate for breast cancer has fallen by 35% in the last thirty years, from a peak of 32 per 100,000 in 1986 to 20.7 per 100,000. This is the lowest it has been since 1950. The estimated 5-year relative survival ratio for breast cancer between 2004 – 2006 was 88%. Worldwide, countries with widespread uptake of mammographic screening and access to effective adjuvant therapies have seen sustained decline in mortality rates. In the United States, data from the Survival, Epidemiology and End Results program (SEER) document a 5-year relative survival of 89.1% for 2001 – 2007 [21]. In the United Kingdom, survival has increased significantly over the last thirty years with most recent figures showing 5-year relative survival of 82% (England, 2001 – 2006) [22].

The disease burden associated with breast cancer in Canada is illustrated by direct prevalence estimates, first published in 2009; 1 in 111 females alive on January 1st 2005 had been diagnosed with breast cancer in the preceding 10 years [1]. Women treated for breast cancer form the largest group of cancer survivors in the United States with over 2.5 million women alive with a prior diagnosis of breast cancer on 1st January 2008 [21]. This amounts to almost a quarter of all cancer survivors in the United States.
1.2 Patterns of Childbirth in the Developed World

The widespread availability of reliable contraception has contributed to an increase in the average age of first childbirth and a reduction in the number of children born to each woman in many developed countries [23]. Although the total number of pregnancies in Canada decreased by 9.3% between 1996 and 2005, this decline occurred only in women aged less than thirty years of age [6]. The pregnancy rate for women over the age of 30 years is rising. In the ten years prior to 2005 the pregnancy rates in women aged 35 – 39 years increased by 18%, rising from 42.7 to 51.9 per 1,000 women. The largest relative increase occurred in women over 40 years of age in whom the rate increased from 8.8 to 11 pregnancies per 1,000 women. Seventeen percent of all pregnancies occurred in women aged 35 years or more.

These changes in fertility patterns have also been well described in European countries. Within the Organisation for Economic Co-operation and Development (OECD) the average age at first childbirth rose from 24.1 years in 1970 to 27.1 years in 2000. In Spain and the United Kingdom the age at first childbirth now approaches 30 years, an unprecedented finding. In general the proportion of births in women aged 30 – 34 years is increasing in Canada, the United States and some European countries, while the proportion of births in women aged 20 – 24 years decreases [24].

1.3 Fertility as a concern for breast cancer survivors

Young women are more likely to experience additional and more severe psychosocial problems after cancer diagnosis. For women diagnosed with breast cancer before the age of 40 years, concerns regarding the impact of treatment on future fertility are common and result in significant levels of distress, not experienced by older women for whom fertility is not a concern. Furthermore, reproductive concerns and the impact of treatment induced infertility cause long-lasting adverse effects on quality of life for survivors and their families.

Partridge et al evaluated 657 responses to a retrospective electronic questionnaire designed to determine the prevalence and degree of concern regarding fertility at the time of diagnosis [25]. Eligible participants were premenopausal, less than forty years of age when diagnosed and were members of the Young Survival Coalition, a web-based advocacy group. Forty-eight percent had
experienced at least one live birth prior to diagnosis. Fifty-eight percent recalled wanting a child or more children and 39% were very concerned about the impact of treatment on their future fertility. Avis et al used a standardized tool to assess a wide range of concerns in 204 breast cancer survivors less than 50 years of age when diagnosed who were now up to 3.5 years from treatment. In women to whom they were applicable, the highest scores were recorded for concerns regarding the risk of premature menopause (expressed by 74%) and pregnancy related issues (expressed by 30%) [26]. On multivariate analysis a wish to have more children, no prior pregnancies, a history of prior difficulty conceiving and preferring more information are significantly associated with greater concern regarding fertility after treatment [25, 27]. Interestingly, age, stage of disease and perceived risk of recurrence were not significantly associated with fertility concerns [25].

Overall, 29% of young women stated that fertility concerns impacted on their treatment decisions. However, almost all women who were concerned about fertility reported that these concerns impacted on their treatment decisions [25]. Thewes et al studied 228 women treated for early breast cancer before 40 years of age and found that 71% discussed fertility related issues with a health professional as part of their treatment [27]. Almost three quarters of participants had discussed fertility issues with their physician, with the physician initiating the conversation in 54% of cases. Up to one third of women consulted a fertility specialist. Attending a specialist was the preferred method of obtaining information regarding the impact of treatment on fertility and menopausal status. 51% of respondents felt that their concerns regarding fertility had been adequately dealt with [27]. A substantial minority of 26% reported that their concerns had not been adequately dealt with [25]. The need for accurate information regarding adverse fertility outcomes is highlighted by the fact that a substantial proportion of participants overestimated their risk of becoming postmenopausal [25].

Data also suggest that cancer related infertility is associated with long-term distress and reduced quality of life 10 year after diagnosis and beyond. Canada et al conducted interviews with 240 women at least ten years after treatment for cervical cancer, breast cancer or lymphoma, all of whom had been diagnosed before forty years of age. 77 women had wanted a child but did not conceive subsequently – this group showed the highest levels of distress. Women with adopted children or stepchildren showed intermediate levels and those with at least one biological child since diagnosis were the least distressed [28]. Gorman et al found that reproductive concerns
were associated with depressive symptoms in 131 women treated for breast cancer before age 40 years, who participated in the Women’s Healthy Eating and Living Survivorship study [29]. Women were assessed 6 times over an average ten year follow up period. Recalled reproductive concerns were found to be an independent predictor of depressive symptoms after controlling for social support and physical health. Ganz et al used a mailed survey questionnaire to measure quality of life in 577 women who were 2 – 10 year disease free survivors of breast cancer, who had been diagnosed before the age of 50 years [30]. While overall quality of life was good, younger survivors in particular showed evidence of increased adverse emotional effects.

Retrospective cross-sectional studies are subject to selection, recall and responder bias. Many study designs require participants to recall fertility concerns at diagnosis many years after the fact. The study by Partridge et al includes women who are members of a web-based advocacy group, who may not be typical of all young breast cancer patients [25]. Thewes et al note that their sample had a significantly higher educational attainment that the general population [27]. In all of these studies, either all or the vast majority of participants had received adjuvant chemotherapy, therefore a comparison with fertility concerns and outcomes in breast cancer survivors not receiving adjuvant treatment is not possible. In an extensive review published this year, Howard-Anderson et al illustrate consistent findings of increased levels of distress associated with reproductive concerns in young women with resultant long term effects and highlight the need for large scale, longitudinal studies in this group [31].

1.4 Ovarian Reserve

Ovarian reserve refers to the quality and quantity of follicles remaining in the ovary [32]. As the ovary ages both the number and the quality of the remaining follicles progressively decrease, resulting in declining fertility beginning at the end of the third decade as illustrated in figure 1. Ultimately menstrual function ceases and a postmenopausal state is reached. It is not possible to directly measure the number of primordial follicles in vivo. Indirect estimates of ovarian reserve can be obtained using biochemical and biophysical measures. These include follicle stimulating hormone, luteinizing hormone, anti-müllerian hormone, inhibin B and estradiol and ultrasound based measurement of antral follicle count and ovarian volume. These measurements have been used for many years in the investigation of infertility and more recently to examine ovarian
Figure 1 The symmetry of birth rate, follicle number and AMH

Figure 1. Fertility rates, follicle numbers and AMH levels show similar declines with age. The upper graph shows births per 1,000 wives in four historical populations. The central graph shows absolute number of follicles seen in ovarian pathology specimens. The lower graph shows AMH level (datapoints) with predictive model superimposed. Vertical lines show age in years across all three graphs.
reserve in cancer survivors. Anti-müllerian hormone is a novel marker, which provides a more accurate estimate of ovarian reserve than the traditional standard of antral follicle count and hormonal levels. It also possesses several characteristics discussed below, which make it a more practical tool for the assessment of ovarian reserve in cancer survivors. Figure 1 graphically illustrates data from three studies redrawn to demonstrate the parallel decrease in fertility rates, follicle numbers in ovarian pathological specimens and AMH levels.

In this section, I will review the tests used to estimate ovarian reserve with particular reference to AMH, summarize the changes seen with ageing of the ovary and analyze the available results in cancer survivors.

1.4.1 Ovarian Reserve and the Ageing Ovary

Throughout life, follicles leave the primordial follicle pool to enter the growing pool. The initial part of this process is independent of gonadotrophin control with a constant fraction of follicles in the growing cohort. Anti-müllerian hormone is produced by the granulosa cells of primordial and pre-antral follicles at this smaller stage of growth. As the follicles enlarge AMH production decreases, inhibin B is produced and the follicles come under the influence of FSH, which stimulates further growth. One or two of these growing follicles ultimately becomes dominant and ovulates under the influence of FSH and LH.

Ovarian follicles are continuously lost through a process of atresia. With increasing age, the quantity and quality of follicles in the ovary is reduced, manifest clinically as reducing ability to conceive, irregular menstrual patterns and ultimately menopause. It has long been recognized that fertility rates begin to decline rapidly in the third decade of life. Historical data recorded prior to the widespread availability of contraception provide useful information regarding ageing and fertility rates. As far back as the 17th century, remarkably consistent patterns of declining fertility with age are seen across ethnically diverse populations [33]. In modern times, under natural conditions, 75% of women trying to conceive at age 30 years will have a live birth within one year, 66% at age 35 years and 44% at age 40 years [34]. Cessation of fertility occurs at a mean age of 41 years, although menstrual cycles may remain regular for years after this time [35]. A clear association between age and the number of remaining follicles in the ovary has been demonstrated by examination of oophorectomy specimens [36, 37]. The largest series combines data from several authors, providing a report on ovaries from 110 females aged between 0 – 51
years [36]. These studies indicate that the rate of follicle loss is not constant throughout life, mirroring the previously described timeline for reduction in fertility rates. Faddy and Gosden in 1992 proposed a bi-phasic model of follicle loss with the rate of loss more than doubling when a critical level of 25,000 follicles remained, occurring at 37.5 years based on these data [36]. They subsequently revised their mathematical model to a more biologically plausible one which does not incorporate an instantaneous change in the rate of decline [38]. In this model the rate of follicle loss was dependent on the remaining number of follicles, with a variable threshold at which menopause occurred. Based on this method, the predicted age of menopause conformed well to observed data in American women. They postulated that the age of menopause could be predicted with an acceptable degree of precision if a non-invasive tool capable of estimating the remaining number of follicles was developed [38].

The number of growing follicles, specifically small antral follicles, provides an indirect estimate of the number of primordial follicles remaining [37-39]. Follicles 2 – 10 mm in size can be visualized by transvaginal ultrasound, with the total number in both ovaries recorded as the total antral follicle count. When measured in this way, the antral follicle count is significantly associated with age as shown in a study recruiting 162 healthy women aged 25 – 46 years with regular menstrual cycles and proven fertility [40]. A bi-phasic model has been suggested to fit these data, with a 4.8% yearly relative rate of decline before the age of 37 years and 11.7% thereafter.

Ovarian volume is estimated by ultrasound and is calculated using the formula for a prolate ellipsoid (height x width x length x 0.523) [41]. Data based on 58,673 observations of ovarian volume in 16,963 women aged 25 – 91 years of age demonstrate a significant decrease in ovarian volume with each decade of life from age 30 to age 70 [41]. Mean ovarian volume in premenopausal women was 4.9 ±0.03 cm³ with an upper limit of 20 cm³. Mean ovarian volume in postmenopausal women was 2.2 ± 0.01 cm³ with an upper limit of 10cm³.

Biochemical measures of ovarian reserve include hormonal indices such as FSH, LH, estradiol and inhibin B. Characteristic changes in hormone levels have been described with increasing reproductive age, as illustrated schematically in figure 2. In the postmenopausal state, FSH and LH levels are significantly increased, while AMH, inhibin B and estradiol levels are significantly reduced [42]. The production of FSH is regulated by a negative feedback loop involving inhibin
B, which is produced by the granulosa cells of larger follicles. Inhibin B levels fall 4 to 5 years prior to menopause [43]. Levels then fall progressively during the various stages of menopause, with consequent increases in FSH levels as the negative feedback mechanism is compromised [44]. While elevated levels of FSH have long been recognized as a marker of the postmenopausal state, FSH elevation is a late occurrence in the menopausal transition. FSH elevation is often accompanied by changes in menstrual patterns such as reduced cycle length and cycle irregularity. FSH therefore, is not a particularly useful marker if one if trying to anticipate the onset of reduced fertility. As discussed in the next section, AMH is the first marker to demonstrate significant changes with increasing age [45, 46]. In these studies, women in their late thirties were found to have lower AMH level than younger women.

Figure 2 Schematic - Changes in menstrual pattern and biochemical markers during late reproductive life

Figure 2. Menstrual pattern is represented by the line of red dots. With increasing age, menstrual periods become irregular and ultimately cease. The three lower arrows indicate the changes in biochemical markers. AMH is the first to show a significant decrease, followed by inhibin B and later FSH and LH changes.
1.4.2 AMH

AMH is a novel marker of ovarian reserve, which provides an earlier and more accurate estimate of ovarian ageing under normal circumstances than other biochemical tests discussed above. Professor Alfred Jost first proposed the existence of AMH in 1947, referring to it as Müllerian Inhibitory Substance and suggesting that its function was to destroy the Müllerian ducts in developing male embryos [47]. AMH was subsequently purified and described as a 140 kDa homodimeric glycoprotein belonging to the Transforming Growth Factor β (TGFβ) family [48, 49]. It is activated by a protease and interacts with two receptors: type II which binds the hormone and type I which initiates downstream signaling [50].

The pattern of AMH expression throughout life differs markedly between males and females. In males, levels rise during the first year of life with the highest levels seen in late infancy [51-53]. AMH levels then fall to reach a basal level at 10 years of age [52], with no AMH detected after 16 years of age [51]. In contrast, levels in females are lowest at birth [52, 53] with undetectable levels in most pre-pubertal females in a large study recruiting 600 male and female participants [53]. With the onset of puberty in females AMH levels rise and demonstrate little variation in regularly cycling women [54] until the mid- to late thirties when AMH levels begin to decline as the ovary ages [45]. In postmenopausal women, AMH is undetectable [45, 55].

In males, AMH is produced by the Sertoli cells of the testis [56]. In females, AMH is produced by the granulosa cells of ovarian follicles up to the early antral stages of development [57-59]. AMH appears to be exclusively produced by the ovaries as evidenced by levels becoming undetectable 3 -5 days after bilateral oophorectomy [55]. Immunohistochemical studies have demonstrated AMH expression in the granulosa cells of 74% of primary follicles with the highest levels seen in secondary, pre-antral and small antral follicles measuring less than 4mm in diameter [59]. At this early stage of development, follicle growth is constant and does not vary with menstrual cycles as illustrated in figure 3. As follicles increase in size AMH production is lost [57, 59]. It is these larger follicles that undergo cyclical recruitment under the influence of FSH.
Figure 3. Primordial follicles are continuously lost, largely through atresia during the maturation process. AMH is produced by smaller follicles at an earlier stage of maturation. It is thought that this part of the follicle maturation process is not under the direct influence of FSH and LH. Inhibin B is produced by slightly larger follicles.

The mechanism governing AMH is thought not to be under the direct influence of FSH [60, 61]. In contrast to estradiol and inhibin B levels, AMH levels do not change significantly in response to increased LH and FSH produced by administration of a gonadotrophins releasing hormone analogue (GnRH) [61]. Consequently, AMH levels do not fluctuate substantially in different phases of the menstrual cycle (see below). Exogenous administration of FSH, likewise had no effect on AMH levels [62]. However, it is possible to induce a gradual decline in AMH during controlled ovarian hyperstimulation for IVF, a process which induces an increased number of dominant follicles [57]. During hyperstimulation, the number of smaller antral follicles declined. AMH levels also decreased, remaining strongly correlated with small antral follicle numbers, reflecting its secretion in the early developmental stages.

AMH is thought to play an inhibitory role in primordial follicle recruitment and decrease the sensitivity of growing follicles to FSH [58, 60, 63, 64]. Although AMH null mice are fertile and can produce a normal litter size, their ovaries contain significantly fewer primordial and pre-antral follicles compared to wild type mice [63]. In 13 month old AMH null mice no primordial follicles were detected indicating premature exhaustion of the primordial follicle pool [64]. The presence of AMH in a mouse ovarian model was associated with 40% less growing follicles compared to control ovaries not exposed to AMH. Examination of ovarian tissue in mice of various ages shows a strong correlation of serum AMH levels with the size of the primordial
follicle pool and with the number of growing follicles, confirming the utility of AMH as an estimate of the quantitative component of ovarian reserve [65].

The association of AMH with smaller follicles growing in a non-cyclic manner should result in reduced variability in AMH levels and this has in fact been borne out by findings of consistent levels of AMH between menstrual cycles [54] and within menstrual cycles [66-68]. While different individuals may have large numerical differences in AMH levels, the AMH level for one individual is significantly more consistent between cycles than FSH, inhibin B, estradiol or antral follicle count [54, 66]. Furthermore, a single AMH measurement provided acceptable reliability, while FSH and estradiol required measurement during 3 and 14 consecutive menstrual cycles respectively to attain the same level of reliability [69].

Studies largely recruiting healthy women in their twenties and thirties with regular cycles, in which ovarian reserve indices were measured on at least 12 occasions during a single cycle, have consistently demonstrated that AMH levels do not vary significantly while FSH, LH, estradiol and inhibin B levels vary according to cycle phase [66-68]. The largest study recruited 44 healthy volunteers with proven fertility and found an average deviation of 17.4% from the cycle mean in individual AMH levels [66]. This deviation was considered small given that a 50-fold difference in AMH levels between different individuals was recorded. Some studies have demonstrated small but statistically significant changes in AMH levels around the time of ovulation [70, 71]. These studies are based on smaller sample sizes of 10 – 20 women and the AMH level was measured 3 – 7 times. The fluctuation seen was not considered relevant as far as AMH measurement for clinical practice was concerned [71].

The non-cycling smaller follicles are dependant on the primordial follicle pool – as this reserve decreases with age we would expect to see a decrease in AMH. AMH levels are in fact correlated with the number of follicular structures seen at biopsy [72], with ultrasound counts of early antral follicles [45] and with age [45, 46]. A prospective, longitudinal study of 41 healthy volunteers (mean age 29 years) undergoing ovarian reserve estimation on two occasions an average of 2.6 years apart found a significant decrease in mean AMH from 2.1 to 1.3 µgm/L (p<0.001) [45]. No significant difference was seen in any other ovarian reserve markers in the same timeframe. In a study of similar design recruiting 81 normo-ovulatory women (mean age 39.6 years) attending an average of 3.9 years apart, AMH levels decreased from 1.2 to 0.5 µgm/L (p<0.001) [46]. AMH
was the only marker showing a mean longitudinal decline over time in participants of all ages. Changes in other indices such as FSH, inhibin B and estradiol were only seen in those more than 40 years of age at entry to the study. A recent abstract publication reports age specific AMH levels from >15,000 randomly selected samples analyzed in a single reference laboratory in North America employing the same assay in all cases [73]. AMH levels declined in a linear fashion in women aged 25 – 48 years and were highly correlated with age ($r^2 = 0.97$). This characteristic of AMH has also been demonstrated in breast cancer survivors, with significantly lower levels seen in survivors >40 years of age compared to those < 35 years of age [14]. In this study no significant rise in FSH levels was seen until survivors reached the age of 50 years, underlining the potential utility of AMH in the early detection of reduced ovarian reserve in women treated for breast cancer before the age of 40 years.

AMH levels from 144 healthy volunteers were used to predict the age of menopause, showing good conformity with observed age of menopause in a population based sample [74]. A longitudinal study following 50 women over six years in the pre and peri-menopausal stages through to final menstrual period (FMP) found that AMH declined to a low point five years before the FMP. Furthermore, AMH level was predictive of time to FMP and age at FMP. Inhibin B levels followed a similar pattern but were less predictive of time to FMP and age at FMP [43]. A single measurement of AMH is suggested as sufficient to predict the age of menopause with only one in ten women in their forties with an AMH level > 0.39 ng/ml reaching menopause within six years of testing [12].

Serum samples for estimates of standard ovarian reserve hormones such as FSH, must be taken during the early follicular phase of the menstrual cycle for valid interpretation, a characteristic which makes their use in general oncology or late effects clinics cumbersome if not entirely impractical. Furthermore, interpretation of these tests is complicated by pregnancy and current hormonal therapy including the oral contraceptive pill and hormone replacement therapy, among other factors [75]. AMH is a more attractive tool in practical terms, given that it provides a useful estimate of ovarian reserve as a single random measurement during one menstrual cycle, facilitating its incorporation into routine clinical follow-up. Use of the oral contraceptive pill does not seem to affect AMH levels [42, 76, 77]. It also has clear practical advantages over transvaginal ultrasound in terms of patient preference and resource utilization [78].
1.5 Fertility after Breast Cancer Treatment

Younger age at the time of diagnosis of breast cancer, in particular age <35 years, is an independent predictor of poorer outcome [79]. Intensive combination chemotherapy regimens have become the treatment of choice for these women [80]. All of the chemotherapeutic regimens in current use may cause temporary or permanent amenorrhea [81-83].

1.5.1 Pregnancy after breast cancer

Partridge et al, again in partnership with the Young Survival Coalition mentioned above conducted a second study reporting on 440 young survivors of early breast cancer who responded to an electronic survey regarding fertility outcomes after treatment [84]. The majority of participants had received chemotherapy. 67% were premenopausal, while 8% continued on therapeutic ovarian suppression. Of great interest is that fact that 57% of the 60 women who tried to become pregnant after treatment were successful. Overall, 16% had been pregnant at least once since diagnosis, with 9% having at least one live birth. Of those who reported wanting a future pregnancy at the time of diagnosis 24% subsequently became pregnant.

Ganz et al surveyed 577 women to ascertain menopausal and fertility outcomes up to 10 years after breast cancer treatment [30]. All participants were treated for breast cancer when ≤ 50 years of age over a range of 2 – 10 years previously. 5% reported a live birth occurring after breast cancer treatment. Two thirds of women had proven fertility with a live birth prior to treatment. 61 (11%) women considered having a child after treatment and 10 of these had become pregnant. Although some were currently trying to conceive and / or pursuing fertility treatment options, the majority of these women were not now actively trying to become pregnant. A variety of reasons were given including physicians advising against pregnancy, concerns regarding recurrence and age.

The bulk of the literature relating to pregnancy after breast cancer treatment was designed to evaluate the impact of pregnancy on breast cancer prognosis. Unfortunately the design of these studies does not allow us to derive useful information regarding pregnancy outcomes in women who wish to have children after treatment. Many are linkage studies between cancer registries and birth registries, which capture only live births, thus missing information regarding pregnancy resulting in miscarriage, termination or still birth. Potential confounders such as fertility of the
partner are not recorded. Time to pregnancy, which has been assessed prospectively in other survivor groups [85] is not available for breast cancer survivors.

In a recent review endorsed by several international breast cancer research groups, the authors conclude that pregnancy is safe and feasible in women at low risk of recurrence [86]. They highlight however, that the available data are incomplete and frequently not applicable to the general population. To address this deficit, the Breast International Group and North American Breast Cancer Group are currently planning an international prospective study assessing disease and pregnancy outcomes in women with endocrine responsive breast cancer who discontinue hormonal treatment to achieve pregnancy.

1.5.2 Chemotherapy and fertility

**Adjuvant therapy for breast cancer**

Adjuvant chemotherapy improves overall survival in women with high-risk node negative and node positive breast cancer with the greatest absolute benefit seen in women less than 50 years of age [87]. One of the first regimens to demonstrate efficacy was a combination of cyclophosphamide, methotrexate and fluorouracil (CMF), which was widely used in the past [88, 89]. The addition of taxanes to anthracycline-based chemotherapy has further improved overall survival outcomes [90, 91]. The Early Breast Cancer Trialists’ Collaborative Group have recently updated their meta-analysis comparing different polychemotherapy regimens, confirming the benefit of more dose intensive anthracycline containing regimens and the addition of taxanes to AC type regimens [92]. The dosing schedules for commonly used treatment combinations are listed in table 1.

The discovery of the Her2-neu receptor led to the development of the monoclonal antibody traztusumab. Approximately 25% of women with breast cancer have tumors that overexpress the Her2-neu receptor. Randomised trials have demonstrated significant overall and disease free survival benefit from the addition of traztusumab to standard chemotherapy in women with Her2-neu positive tumours. [93-95].
Endocrine responsive breast cancer expresses estrogen and progesterone receptors (ER / PR receptors). Women with this type of breast cancer experience improved outcomes with adjuvant endocrine therapy (tamoxifen or aromatase inhibitor) [96]. In general these therapies are given for a period of 5 years, although the optimal treatment combination and duration is the subject of ongoing research. In premenopausal women, complete suppression of ovarian function may also be beneficial. Permanent ovarian ablation may be achieved by surgical removal or radiotherapy treatment. Temporary, reversible ovarian suppression is achieved by administration of gonadotrophin releasing hormone analogues. Although meta-analysis in the modern era has shown a benefit for ovarian ablation in premenopausal women [97], it is not a standard component of treatment and the optimal combination of ovarian ablation / suppression with other hormonal and chemotherapeutic interventions remains a topic of ongoing research.

**Chemotherapy induced amenorrhea**

Amenorrhea has been widely used as a surrogate marker of primary ovarian failure induced by chemotherapy [8]. Accepting that women are born with a finite number of primordial follicles, one could speculate that apoptotic follicle death induced by chemotherapy will result acutely in permanent or reversible amenorrhea and may result in early menopause in the long term. Younger women, who have a larger follicle reserve at the time of treatment, would therefore be less likely to experience permanent amenorrhea. In fact, this has been observed clinically as discussed below. The reported incidence of chemotherapy induced amenorrhea ranges from 0 – 100% as a result of different definitions of menstrual status, varying age distributions, differences in treatment regimens and diverse follow up times. Some authors provide no formal definition of menopausal status [98-102]. Premenopausal status may be variably defined as having a period within the last 3, 6 or 12 months [89, 103, 104]. Some authors stipulate the use of follicle stimulating hormone and luteinizing hormone levels to establish menopausal status if necessary but do not report results of these tests [103, 105, 106].

The specific impact on fertility of individual chemotherapeutic agents, cumulative dose and dose-density are frequently confounded by the inclusion of various agents at differing doses in most regimens. Three extensive review articles on the topic of chemotherapy-induced amenorrhea in breast cancer patients contain a majority of articles referring to CMF type
chemotherapy, highlighting the relative lack of data on the more modern regimens now recommended for the treatment of young women with breast cancer [81, 83, 107]. Studies expressly designed to record menstrual data employ multivariate regression techniques to analyze the significance of chemotherapy regimens while controlling for known predictors of amenorrhea such as patient age [82, 104, 108, 109].

In this section I will review the impact of adjuvant chemotherapy on acute and late menstrual status with particular emphasis on data related to women less than forty years of age treated with modern chemotherapy regimens, when such data are available.

**Alkylating agents**

Alkylating agents are associated with well established adverse effects on fertility in both males and females. Cyclophosphamide is the alkylating agent commonly used in the treatment of breast cancer. Receiving cyclophosphamide is a significant risk factor for amenorrhea in cancer survivors with an odds ratio of 3.98 [110]. Younger women are consistently less likely to experience amenorrhea [81, 83, 88, 89, 100, 102, 104, 111]. Rates of amenorrhea range from 18% to 61% in younger women with an average incidence of 40% among studies and from 61% to 97% in older women with an average incidence of 73% among studies [81, 83]. Cyclophosphamide monotherapy and CMF may produce similar rates of amenorrhea underlining the key role of cyclophosphamide in ovarian toxicity. [112]. In multivariate analysis, age at the time of treatment, systemic chemotherapy and tamoxifen in addition to chemotherapy were significant predictors of amenorrhea [108].

Higher cumulative doses of cyclophosphamide result in higher rates of amenorrhea [88, 102, 108, 111, 113]. Combined data from three randomized controlled trials demonstrates amenorrhea in 62% of women <40 years of age after 12 cycles of CMF (cumulative dose 16.8 gm/m²) compared to 29% after 6 cycles of CMF (cumulative dose 8.4 gm/m²) [88]. A randomized trial with 2,628 participants evaluating no adjuvant chemotherapy, one cycle of peri-operative CMF and 6 cycles of adjuvant CMF demonstrated amenorrhea in 21%, 31% and 68% of women respectively [113]. These findings were confirmed in a systematic review of 13 trials including 3,929 patients [107]. The impact of dose intensity on rates of amenorrhea cannot be assessed in these studies as higher cumulative doses are generally given in prolonged schedules.
Anthracycline based regimens

Anthracyclines such as doxorubicin and epirubicin are usually administered in combination with cyclophosphamide, which confounds the analysis of the relative contribution of the anthracycline to amenorrhea [104, 108, 109]. Comparisons of amenorrhea after CMF and CEF (cyclophosphamide, epirubicin and fluorouracil) demonstrate inconsistent results and are difficult to interpret because the cumulative dose of cyclophosphamide is higher in the standard Bonadonna CMF regimen (8.4 gm / m\(^2\) for 6 cycles CMF, 6.3 gm / m\(^2\) for 6 cycles CEF). The National Cancer Institute of Canada Clinical Trials Group (NCIC CTG) conducted a randomized trial comparing CMF with CEF and found no significant difference in the rates of amenorrhea at 12 months [104]. Prospectively gathered data on a pilot cohort demonstrated a higher rate of amenorrhea in those receiving CMF versus CEF (55.6% versus 64.6%) but there was no significant difference in the risk associated with either regimen on multivariate analysis controlling for age among other factors [108]. It should be noted that only 25 women (14%) received the CEF regimen in this report. A higher rate of amenorrhea was reported with CMF in comparison with doxorubicin and cyclophosphamide (AC) with or without a taxane component [109]. At one year from completion of treatment 37% were amenorrheic after AC, while 68% were amenorrheic after CMF. Again, this comparison is complicated by differing doses of cyclophosphamide. The AC based regimens delivered a median total dose of 4 – 4.2 gms compared with 7.92 gms of cyclophosphamide in the CMF regimen.

Patient age at the time of treatment is a consistent predictor of the occurrence of amenorrhea for anthracycline-based regimens [8, 99, 100, 103, 105, 109]. Women aged <40 years at the time of treatment are significantly more likely to continue menstruating during chemotherapy or to regain menstrual function after chemotherapy, when compared to women over 40 years of age the time of treatment [8]. Trials evaluating increased cumulative dose and dose-dense regimens have reported conflicting results in terms of rates of amenorrhea [83]. A retrospective analysis of a randomized controlled trial with 1,550 participants comparing doxorubicin dose in combination with fluorouracil and cyclophosphamide (FAC) found no difference in rates of amenorrhea in women receiving 30 mg/m\(^2\), 40 mg/m\(^2\) and 60 mg/m\(^2\) [114]. A clinical trial evaluating increased doses of epirubicin and cyclophosphamide (EC) demonstrated higher rates of amenorrhea in the high dose arm [105]. This toxicity was marked, even in women less than 40 years of age, who experienced permanent amenorrhea in 61% of cases. As previously mentioned, these
comparisons are confounded by increased doses of cyclophosphamide in the high dose treatment arm.

**Taxane containing regimens**

As with other chemotherapy regimens, amenorrhea induced by taxane containing regimens is more likely to occur in older women, who are less likely to regain menstrual function [82]. Reports based on large prospective clinical trials have demonstrated higher rates of amenorrhea with the addition of a taxane [82, 109, 115, 116], although there are conflicting reports based on retrospective data [117]. A report from the National Surgical Adjuvant Breast and Bowel Project found 83% of women receiving four cycles of AC followed by four cycles of docetaxel experienced amenorrhea for at least six months [82]. Three cycles of FEC followed by three cycles of docetaxel resulted in amenorrhea in 64.8% of women in a randomized trial in which almost half of the women recruited were premenopausal and less than 50 years of age [116]. Prospective data indicates that the addition of either docetaxel or paclitaxel to AC chemotherapy increased the rate of amenorrhea at one year (35% for AC, 47% for ACT, 74% for ACD) [109]. Of note, at three years from treatment, rates of amenorrhea remained higher in those who received a taxane, but there was little difference in the figures for ACT and ACD treatments (47% for AC, just over 60% for both taxane regimens).

**Trastuzumab**

Women with Her2-neu expressing tumors may undergo treatment with trastuzumab. There are no published data on the long-term ovarian impact of trastuzumab therapy.

**Endocrine therapies**

Multivariate analysis has demonstrated that tamoxifen therapy in addition to chemotherapy increases the risk of amenorrhea in the first year after treatment [82, 108, 109]. A large prospective trial demonstrated that women receiving tamoxifen were significantly more likely to be amenorrheic at one year after completion of chemotherapy, with an odds ratio of 0.50 (95% CI 0.37 – 0.67). On long term follow up at three years however, this effect became non-significant [82].
**Long term menstrual patterns after chemotherapy for breast cancer**

There is a distinct difference in the pattern of menstrual function over time after CMF type chemotherapy compared to anthracycline based chemotherapy, with CMF demonstrating a less pronounced immediate effect but continuous increase in rate of amenorrhea to three years and beyond, while AC based regimens result in higher rates of acute amenorrhea followed by significant recovery of function and a plateau [99, 109]. A large prospective study designed to evaluate the impact of several chemotherapy regimens on menstrual function recruited 595 women who completed daily bleeding records for up to five years [109]. Participants were aged 20 – 45 years and entered the study within 8 months of their breast cancer diagnosis. Immediately after starting chemotherapy, 75% of women receiving AC were amenorrheic compared to 50% of those receiving CMF. One year after completion of chemotherapy, there was evidence of recovery of function in those receiving AC (37% amenorrheic after AC, 68% after CMF). Those receiving CMF experienced a continuous increase in rates of amenorrhea with only 20% retaining menstrual function at 3 years compared to over half of those receiving AC.

Ten year follow up of International Breast Cancer Study Group trials comparing one peri-operative cycle of CMF or no CMF with 6 or 7 cycles of CMF demonstrate that young women remain at long-term risk of premature menopause [9]. A Cox proportional hazards analysis based on 767 participants found that 6 or 7 cycles of CMF and occurrence of temporary amenorrhea were predictors of menopause. Logistic regression predicted that 91% of women treated with 6 or 7 cycles of CMF at age 35 years would be menopausal at age 45 years and 84% of those treated at age 30 years would be menopausal at age 40 years.

These findings have also been noted in very young breast cancer survivors. A retrospective review of 160 women treated before the age of 35 years found a slightly higher rate of temporary amenorrhea in women receiving AC (34% for AC, 25% for CMF), although rates of permanent amenorrhea were higher after CMF (3.8% for AC, 6.8% for CMF) with a median follow up of 54 months [99]. These figures also illustrate the protective effect of younger age at the time of treatment.
### Table 1: Chemotherapy Regimens Used to Treat Breast Cancer

<table>
<thead>
<tr>
<th>Chemotherapy regimen</th>
<th>Schedule</th>
<th>Constituent Drugs</th>
<th>Cumulative doses for complete course</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CMF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bonadonna et al 1995</td>
<td>6 cycles, every 28 days</td>
<td>Cyclophosphamide 100 mg / m² PO days 1 - 14 Methotrexate 40 mg / m² IV days 1 and 8 Fluorouracil 600 mg / m² IV days 1 and 8</td>
<td>8,400 mg / m² 480 mg / m² 7,200 mg / m²</td>
</tr>
<tr>
<td><strong>AC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fisher et al 1990</td>
<td>4 cycles, every 21 days</td>
<td>Doxorubicin 60 mg / m² IV day 1 Cyclophosphamide 600 mg / m² IV day 1</td>
<td>240 mg / m² 2,400 mg / m²</td>
</tr>
<tr>
<td><strong>ACD (docetaxel)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sparano et al 2008</td>
<td>4 cycles AC, every 21 days</td>
<td>Doxorubicin 60mg / m² IV day 1 Cyclophosphamide 600mg / m² IV day 1 Docetaxel 100mg / m² IV on day 1</td>
<td>240 mg / m² 2,400 mg / m² 400 mg / m²</td>
</tr>
<tr>
<td><strong>ACT (paclitaxel)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sparano et al 2008</td>
<td>4 cycles of AC, every 21 days</td>
<td>Doxorubicin 60mg / m² IV day 1 Cyclophosphamide 600mg / m² IV day 1 Paclitaxel 175mg / m² IV on day 1</td>
<td>240 mg / m² 2,400 mg / m² 700 mg / m²</td>
</tr>
<tr>
<td><strong>Dose dense ACT (paclitaxel)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citron et al 2003</td>
<td>4 cycles AC, every 14 days</td>
<td>Doxorubicin 60mg / m² IV day 1 Cyclophosphamide 600mg / m² IV day 1 Paclitaxel 175mg / m² IV on day 1</td>
<td>240 mg / m² 2,400 mg / m² 700 mg / m²</td>
</tr>
<tr>
<td><strong>CEF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levine et al 1998</td>
<td>6 cycles every 28 days</td>
<td>Cyclophosphamide 75 mg / m² PO days 1 - 14 Epirubicin 60 mg / m² IV days 1 and 8 Fluorouracil 500 mg / m² IV days 1 and 8</td>
<td>6,300 mg / m² 720 mg / m² 6,000 mg / m²</td>
</tr>
<tr>
<td><strong>FEC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roche et al 2006</td>
<td>6 cycles, every 21 days</td>
<td>Fluorouracil 500 mg / m² IV day 1 Epirubicin 100 mg / m² IV day 1 Cyclophosphamide 500 mg / m² IV day 1</td>
<td>3,000 mg / m² 600 mg / m² 3,000 mg / m²</td>
</tr>
<tr>
<td><strong>TC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jones et al JCO 2009:29:1177-1183</td>
<td>TC x 4, every 21 days</td>
<td>Docetaxel 75 mg / m² IV day 1 Cyclophosphamide 600 mg / m² IV day 1</td>
<td>300 mg / m² 2,400 mg / m²</td>
</tr>
<tr>
<td><strong>FEC – D</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roche et al 2006</td>
<td>FEC x 3, Docetaxel x 3, given every 21 days</td>
<td>Fluorouracil 500 mg / m² IV day 1 Epirubicin 100 mg / m² IV day 1 Cyclophosphamide 500 mg / m² IV day 1 Docetaxel 100 mg / m² IV day 1</td>
<td>1,500 mg / m² 300 mg / m² 1,500 mg / m² 300 mg / m²</td>
</tr>
</tbody>
</table>
1.5.3 Ovarian reserve in cancer survivors

A systematic literature search for articles describing the measurement of ovarian reserve in cancer survivors published after 1990 identified 15 relevant articles as summarized in table 2. The search was limited to articles reporting biochemical and / or biophysical measures of ovarian reserve in the setting of chemotherapy treatment for malignancy. Only those studies providing a comparison with a control group or comparing the same cohort before and after chemotherapy treatment were included. In an effort to find data related to modern chemotherapy approaches, articles published before 1990 were also excluded.

All but one of the studies examined ovarian reserve in breast cancer survivors or survivors of childhood cancer. Twelve of the fifteen studies (80%) were relatively small with sample sizes of fifty or less. Five studies examine the immediate effect of chemotherapy treatment, comparing ovarian reserve before and after treatment. Four relate to adjuvant treatment for breast cancer, one examines the effect of bone marrow transplant in the treatment of haematological malignancy. The results of these studies are summarized in table 3. Ten studies examine the long-term impact of cancer treatment on ovarian reserve. Those with longest follow up relate to survivors of childhood cancer, treated with a variety of chemotherapy and/or radiotherapy regimens. The results of these studies are summarized in table 4.

<table>
<thead>
<tr>
<th>Survivorship Population</th>
<th>No. of studies</th>
<th>No. of subjects (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast Cancer</td>
<td>6</td>
<td>272 (39%)</td>
</tr>
<tr>
<td>Haematological malignancies</td>
<td>2</td>
<td>40 (6%)</td>
</tr>
<tr>
<td>Childhood cancer</td>
<td>6</td>
<td>356 (51%)</td>
</tr>
<tr>
<td>Childhood Hodgkin’s Disease</td>
<td>1</td>
<td>30 (4%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>15</strong></td>
<td><strong>698 (100%)</strong></td>
</tr>
<tr>
<td>Study</td>
<td>Survivors n</td>
<td>Controls n</td>
</tr>
<tr>
<td>------------------------</td>
<td>-------------</td>
<td>------------</td>
</tr>
<tr>
<td>Yu 2010 [16]</td>
<td>26</td>
<td>134</td>
</tr>
<tr>
<td>Anderson 2006 [14]</td>
<td>50</td>
<td>None</td>
</tr>
<tr>
<td>Anders 2008 [120]</td>
<td>44</td>
<td>None</td>
</tr>
<tr>
<td>Chatterjee 1994 [121]</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Study</td>
<td>Survivors n</td>
<td>Controls n</td>
</tr>
<tr>
<td>---------------------</td>
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</tr>
<tr>
<td>Su 2010 [18]</td>
<td>110</td>
<td>110</td>
</tr>
<tr>
<td>Partridge 2009 [17]</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>van Beek 2007 [78]</td>
<td>30</td>
<td>41</td>
</tr>
<tr>
<td>Bath 2003 [62]</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td>Bath 2001 [125]</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>Muller 1996 [126]</td>
<td>21</td>
<td>23</td>
</tr>
</tbody>
</table>

[^1,2]: Smaller study is a subset of the larger study. Same control group used in both studies.
Immediate effect of chemotherapy

Biochemical markers – AMH, FSH, inhibin B, estradiol, LH

Rapid and profound changes in biochemical markers of ovarian reserve have been demonstrated in the first year after chemotherapy treatment, with the majority of data relating to women receiving chemotherapy for newly diagnosed breast cancer [14-16, 120]. Different markers demonstrate distinct patterns of change. AMH shows a dramatic and profound reduction shortly after chemotherapy administration [14, 16, 120], which is sustained over a year after treatment (the maximum duration of these studies). Inhibin B also demonstrates a rapid decrease although not as dramatic as that seen with AMH [14]. FSH levels increase significantly but may show some recovery in the first year after treatment, unlike AMH which showed no recovery for the duration of these studies [14, 16, 120]. All of the studies discussed in this section are summarized in table 3.

Yu et al provide an elegant description of AMH, FSH and estradiol levels before and at 6, 12, 36 and 52 weeks after chemotherapy in a nested cohort of women diagnosed with breast cancer before age 40 years taking part in a larger clinical trial [16]. The impact of chemotherapy on AMH levels is particularly dramatic. Prior to chemotherapy there was no significant difference in AMH levels between patients and controls. However, AMH was profoundly reduced just 6 weeks after chemotherapy (from a median of 0.86ng/ml to 0.08ng/ml). Thereafter levels showed no recovery, remaining suppressed with a median level of 0.07ng/ml 12 months after chemotherapy. A similar effect was previously demonstrated by Anderson et al, who report mean AMH falling from 1.11ng/ml to approximately 0.13ng/ml three months after chemotherapy administration in 42 premenopausal women newly diagnosed with breast cancer [14]. Many women had AMH levels close to the lower limit of detection. Anders et al in a similar study found that AMH levels were reduced from 2.72 to 0.03 pg/ml three months after adjuvant chemotherapy in those aged < 35 years and from 0.47 to 0.03 pg/ml in those aged ≥ 35 years [120]. At 12 months after chemotherapy, AMH level remained very low in both groups, showing no recovering over the study period (0.07 pg/ml in those < 35 years and 0.03pg/ml in those >35 years). Anders et al also found that women who were amenorrheic at one year post-chemotherapy had lower AMH and inhibin B levels prior to commencing chemotherapy.
FSH levels increase significantly post chemotherapy, with significant differences seen as early as 6 weeks [14, 16, 120]. Yu et al found FSH levels increased from a baseline median value of 5.2 IU/L to a maximum of 55 IU/L at 12 weeks after chemotherapy [16]. While levels were significantly elevated in comparison with baseline at all time-points in the study, recovery did occur after 12 weeks with median values of 15.3 IU/L reached at 12 months. Anderson et al demonstrated a similar pattern with a four-fold increase in FSH in the first three months after chemotherapy (p < 0.05) [14]. Anders et al demonstrated a much greater increase in FSH in women aged >35 years in comparison with those <35 years of age with maximal differences seen at 6 months after chemotherapy [120].

Anderson et al also report significant reductions in inhibin B levels, which fell to approximately 50% of pre-treatment levels at the three-month mark (p<0.001) and remained suppressed at one year after chemotherapy [14]. Levels were undetectable in 20/42 (48%) subjects at six months. Lutchman-Singh et al found significantly reduced inhibin B levels in women treated with chemotherapy in comparison with controls (74.97±17.7 versus 19.25±4.56 pg/ml, p<0.05) [15]. The longitudinal component of this study included only three estimates of inhibin B, which were reduced after chemotherapy. Chatterjee et al tested pituitary-gonadal function in a small group of women undergoing bone marrow transplant and found that the anterior pituitary gland retained its trophic hormone reserve and secretory capacity while the ovary suffered an acute insult demonstrated by significant increases in FSH and LH [121].

Antral follicle count and ovarian volume

The immediate effect of chemotherapy is confirmed by biophysical tests with significant reductions in antral follicle count and ovarian volume when measured before and after adjuvant chemotherapy for breast cancer [14, 15]. Anderson et al demonstrated a decrease in mean antral follicle count from 5.8 ± 0.7 before chemotherapy to 2.1 ± 0.3 (p < 0.001) at 12 months after the start of chemotherapy [14]. The decrease occurred predominantly in the first half of the year with a mean follicle count of 3.1 seen at six months. Mean ovarian volume followed a similar pattern with a significant decrease from 4.3 ± 1.3 ml to 3.0 ± 0.2 ml (p = 0.047) in the 12 month period.
Lutchman-Singh et al provide a comparison between premenopausal women newly diagnosed with breast cancer and 24 healthy, age-matched controls, demonstrating no significant difference in total antral follicle count prior to chemotherapy treatment (16.77±1.63 versus 17.05±1.21) [15]. After chemotherapy, total antral follicle count was significantly reduced in comparison with controls at 7.8 ± 0.85 (p < 0.001). A comparison restricted to eight women who attended both before and after chemotherapy demonstrated a significant reduction in total antral follicle count from 15.02±2.7 to 5.1±0.7 (p<0.01) after chemotherapy. Ovarian volume was also reduced in the women treated with chemotherapy, but this difference did not reach statistical significance.

**Long-term effect of chemotherapy (more than 1 year from treatment)**

**Biochemical markers – AMH, FSH, inhibin B, estradiol, LH**

Seven of the ten available studies demonstrate altered hormonal profiles consistent with long-term depletion of ovarian reserve after a variety of treatments for breast cancer, haematological cancer and childhood cancer. Three studies demonstrate no significant difference between survivors and controls. One of these studies specifically recruited survivors with FSH levels within the normal range, therefore we might expect biochemical profiles to be comparable with the control group [124]. However, even in this study, significant reductions in antral follicle count and ovarian volume were found. The second of these studies demonstrated significant differences in subset analysis related to treatment received [122] but no difference overall when compared with a control group. The third measured only FSH and LH – samples were taken at a comparable time point in the menstrual cycle but not during the early follicular phase, a non-standard design that may increase the variability within the sample and reduce the probability of detecting a true difference [126]. All of the studies discussed in this section are summarized in table 4.

**Childhood Cancer Survivors**

Seven studies examine long-term ovarian reserve in childhood cancer survivors [62, 75, 78, 122, 124-126]. By far the largest study recruited 185 participants and evaluated a single random AMH level in 182 of those, eliminating three survivors who had surgical removal of one or both ovaries [122]. The study included female survivors of childhood cancer >18 years of age, treated at a single institution between 1958 and 2000. Those with a central nervous system malignancy
were excluded. Median follow up was 18.1 years. Survivors were recruited regardless of menopausal status and included 33 (18%) with regular menstruation, 28 (15%) with oligomenorrhea or amenorrhea, 98 (54%) taking an oral contraceptive, 5 (3%) taking hormonal replacement therapy and 4 (2%) who were pregnant. Treatment consisted of non-alkylating chemotherapy +/- radiotherapy (38%) or alkylating chemotherapy (55%). There was no significant difference in overall AMH levels between survivors and 42 healthy controls (1.7 versus 2.1 µg/L, p = 0.57), although 27% of survivors had AMH levels below the 10th percentile. Subset analysis showed significant reductions in AMH levels in those receiving chemotherapy and total body irradiation or radiation to a pelvic field (<0.1 versus 2.1 µg/L, p < 0.001) and in those receiving three or more cycles of MOPP chemotherapy (0.5 versus 2.1 µg/L, p = 0.004).

To my knowledge, this is the first publication reporting on the use of a single random AMH measurement as an estimate of ovarian reserve in cancer survivors. In fact, 21% of participants attended during the early follicular phase of the menstrual cycle. It is interesting to note that there was no significant difference in AMH levels in those tested at a random time point and those tested during the early follicular phase of the menstrual cycle [127].

Two other studies provide evidence of significant long-term reductions in AMH levels in childhood cancer survivors compared to controls [62, 78]. Van Beek et al found reduced AMH in 30 survivors of childhood Hodgkin’s disease at a median follow up of 11.6 yrs [78]. Eighteen women in this group were current users of the OCP and one was using HRT. Survivors had received ABVD / EBVD chemotherapy with or without MOPP chemotherapy. Some received radiotherapy but only to fields above the diaphragm. Interestingly, AMH levels were comparable with controls in those receiving ABVD / EBVD chemotherapy only. AMH was significantly reduced in those receiving 3 or more cycles of MOPP chemotherapy. Those treated with MOPP chemotherapy also had significantly reduced inhibin B and elevated FSH in comparison with controls. Inhibin B levels demonstrated a downward trend with increasing number of MOPP cycles received.

Bath et al specifically recruited childhood cancer survivors with regular menstrual cycles and no evidence of premature ovarian failure a median of 8 years after chemotherapy and/or radiotherapy treatment [62]. The group had a median age of 23 years and half were current users of the oral contraceptive pill. Even in this highly selected group of women, AMH was significantly reduced in survivors who were not taking the OCP when compared with controls.
(13±3 versus 21±3.4 pmol/l, p<0.05). FSH levels were significantly increased (7.5±1.4 versus 4.2±0.3, p=0.02). Estradiol and inhibin B levels were comparable between the two groups. An FSH stimulation test was also performed; as expected this had no effect on AMH levels.

Larsen et al have published two studies relating to their cohort of childhood cancer survivors [75, 124]. The group includes women treated between 1970 and 1996 with a non-CNS malignancy, with a median time to assessment of over 16.5 years. The larger study includes 100 women, subdivided into three groups according to increasing risk of ovarian toxicity; group 1 received non-alkylating chemotherapy, group 2 alkylating chemotherapy +/- radiotherapy, group 3 received alkylating chemotherapy and total body irradiation prior to bone marrow transplant. 70% of survivors had regular menstrual cycles at the time of inclusion in the study. Those with regular cycles had similar FSH and LH levels to controls, but demonstrated reduced inhibin B and increased estradiol levels.

**Adult cancer survivors**

Three studies provide data on long-term ovarian reserve after cancer treatment in adulthood, two relating to breast cancer and one to hematological cancer. In the largest study published to date, Su et al recruited 127 survivors of stage I – III breast cancer who were premenopausal at the time of diagnosis (defined as having had at least one period in the year prior to treatment) [18]. Women currently receiving endocrine therapy were included. 110 survivors with a median age 46.1 yrs were matched by age and race with a control group from the Penn Ovarian Ageing Study and assessed at two time points a median of 2.1 years and 5.3 years after chemotherapy. Ovarian reserve was assessed only at the first time-point. Chemotherapy-related amenorrhea was defined as ≥ 12 months of amenorrhea after chemotherapy and was present in 70 (55%) at first assessment and 62 (56%) at second assessment. In pair-wise comparisons, survivors had reduced AMH (53.1 versus 99.5 pg/mL, p = 0.004), increased FSH (35.6 versus 13.3 IU/L, p = <0.001) and reduced inhibin B (12.7 versus 38.5 pg/mL, p <0.001). These findings persisted in a regression model adjusting for potential confounders: gravidity, BMI, smoking and alcohol exposure. The use of dose dense chemotherapy or inclusion of a taxane in the chemotherapy regimen was not associated with chemotherapy related amenorrhea. 71% of the group were on tamoxifen at the time of ovarian reserve assessment. There was no significant difference in AMH, inhibin B and estradiol levels in those taking tamoxifen versus those not taking tamoxifen.
FSH levels were significantly reduced in those taking tamoxifen (35.5 IU/L versus 42.8 IU/L, p=0.04).

Partridge et al recruited 20 premenopausal breast cancer survivors free of recurrence at least one year from diagnosis [17]. Premenopausal status was defined as having had at least one period in the previous six months, although 80% of the women recruited reported regular monthly menstruation. Survivors had a mean age of 36.8 years. The majority had been treated with AC or dose dense AC chemotherapy and 45% had received a taxane. 50% were on tamoxifen at the time of the study. 12 (60%) survivors were more than three years from diagnosis. AMH was significantly reduced in survivors compared to healthy controls matched for age and gravidity (mean AMH 0.4 versus 1.4 ng/mL, p = 0.0004). FSH was significantly increased in survivors (9 IU/L versus 6.9 IU/L, p=0.02) and inhibin B was reduced (11.5 pg/ml versus 33.1 pg/ml, p=0.02). Multiple logistic regression demonstrated that AMH was the best predictor of antral follicle count (OR 7.63). Addition of the other biochemical tests did not improve the model. The survivors who were currently taking tamoxifen had lower AMH and inhibin B levels, increased estradiol and reduced antral follicle count. The small sample size precluded formal statistical comparison of these groups.

Lie Fong et al compared AMH levels before and after chemotherapy in 25 survivors of haematological malignancy analyzed in two groups according to treatment received (Group A – alkylating agents, group B – stem cell transplant after conditioning regimen including total body irradiation) [128]. Interestingly, survivors in both groups had lower AMH levels than controls prior to the administration of chemotherapy (1 versus 2.1 µg/L, p < 0.001 in group A, 0.9 versus 2.1 µg/L, p < 0.05 in group B). This is unexpected given that the controls were slightly older than the survivors. The authors do not provide a explanation for this finding. Group A was reassessed at a median interval of 3.4 years at which time median AMH was reduced from 1.0µg/L to 0.3µg/L. Group B was reassessed at a media interval of 2.8 years, at which time median AMH levels were 0. Post-treatment FSH, inhibin B and estradiol levels also demonstrated significant depletion of ovarian reserve in both groups in comparison with controls. As might be expected as a result of treatment, the magnitude of ovarian reserve depletion was greater in group B, with median FSH of 64.4 IU/L (compared to 8.5 IU/L in group A) and undetectable levels of inhibin B. This was also evident in the menstrual history as none of the survivors in group B had spontaneous menstruation at the second assessment.
Lie Fong et al also calculated the remaining fraction of AMH. This entity represents the drop in AMH per year independent on the AMH level at the start of the study and is calculated by adjusting the difference between two AMH measurements for the time interval between them. The remaining fraction of AMH for survivors in Group A was not significantly different from the control group. The authors therefore postulate that the insult is the result of damage to the primordial follicle pool as opposed to an increased rate of follicle loss.

**Antral follicle count and ovarian volume**

Six of the ten studies discussed included ultrasound estimations of antral follicle count and / or ovarian volume in varying cohorts of cancer survivors. All but one [125] demonstrated reduced ovarian reserve in cancer survivors as evidenced by reduced follicle counts [17, 128], reduced ovarian volumes [62] or both [62, 75, 124]. In general, studies demonstrating reduced ovarian volume or antral follicle count also found alteration of biochemical indices consistent with reduced ovarian reserve. Some investigators estimate that cancer survivors had an ovarian age ten years old than their chronological age [75]. In a highly selected population of survivors, all of whom had FSH levels < 10 IU/L and regular menstrual cycles, reduced antral follicle count (8 versus 11, p=0.002) and reduced ovarian volume (4.9 versus 6.8, p=0.008) identified reduced ovarian reserve in the presence of unchanged biochemical levels [124].

Of further interest in this study, Larsen et al report antral follicle counts for follicles measuring <5mm in size and for those 5 – 10 mm in size. This subdivision demonstrates that the reduction in follicle number in the cancer survivors is confined to the smaller follicles < 5mm in size [75]. The authors suggest that chemotherapy has a disproportionate effect on the smallest follicles. Given that AMH is largely produced by these smaller follicles, it is perhaps to be expected that chemotherapy induces the profound depletion of AMH observed in the studies discussed earlier.

1.5.4 Occult depletion of ovarian reserve

It is clear from the available literature that significant depletion of ovarian reserve can exist in the presence of regular menstrual cycles. Two of the studies discussed in detail above specifically recruited cancer survivors who had regular menstrual periods after cancer treatment [17, 62]. In comparison with controls, 20 breast cancer survivors had significantly reduced AMH, inhibin B and antral follicle count [17]. Likewise, 20 survivors of childhood cancer were
found to have significantly reduced AMH, increased FSH and decreased ovarian volume when compared with controls [62].

A large subset of the study by Larsen et al (n=70) were survivors of childhood cancer who reported spontaneous menstruation [75]. In comparison with controls, this group had a significantly reduced mean antral follicle count (7.5 versus 11, p < 0.001) and mean ovarian volume (4.8cm$^3$ versus 6.8 cm$^3$, p = < 0.001). FSH and LH levels were similar but inhibin B levels were reduced and estradiol levels increased in the survivors in comparison with controls. As mentioned above, Larsen et al further sub-divided the same population of childhood cancer survivors in a second analysis comparing 21 survivors with an FSH level < 10 IU/ml and regular menstrual cycles with healthy controls. Even this restricted group of survivors demonstrated significant reductions in antral follicle count (8 versus 11, p = 0.002) and ovarian volume (4.9 cm$^3$ versus 6.8 cm$^3$, p = 0.008), underlining the inadequacy of menstrual histories in the detection of depleted ovarian reserve.

It is interesting to note that AMH testing demonstrates significant occult reduction in ovarian reserve in both studies testing for AMH, while the traditional ovarian reserve indices showed inconsistent results across the two studies. It would be fascinating to analyze AMH levels in Larsen et al’s selected group of survivors with regular periods and FSH level < 10 IU/ml, however AMH testing was not part of the study design.

1.5.5 Ovarian reserve and the prediction of the outcome of assisted reproduction techniques.

AMH has been investigated as a predictor of both quantitative and qualitative aspects of assisted reproductive therapy (ART). The European Society of Human Reproduction and Embryology point out the value of AMH in predicting both poor response and hyper-response to ovarian stimulation for IVF [19]. The currently available literature suggests that AMH and AFC are superior to other predictors including day 3 FSH, estradiol and Inhibin B in this regard [19]. A recent meta-analysis including 13 studies on AMH and 17 on AFC found that both were equally clinically useful predictors of poor response to ovarian stimulation [129]. For the prediction of pregnancy, AMH and AFC were found to be equally poor predictors. The use of AMH levels to individualize assisted conception strategies is currently under investigation. In a prospective cohort study recruiting 538 women the incorporation of AMH levels in treatment decisions was
associated with maintained pregnancy rates and reduced risk of harm due to ovarian hyper-
response [130]. AMH measurements were taken randomly during the menstrual cycle in this
study reinforcing this as the optimum method of AMH estimation in clinical practice.

1.6 Summary

It is clear that chemotherapy has an immediate detrimental effect on ovarian reserve. AMH
measurement provides the most dramatic example of this with profound reduction in AMH
levels evident as early as 6 weeks after commencing chemotherapy with no recovery up to a year
after treatment [16]. These effects are sustained over time, with depleted ovarian reserve seen in
childhood cancer survivors up to 18 years after treatment [75, 122].

The reduction of the primordial follicle pool induced by chemotherapy will shorten the
reproductive lifespan of the ovary. The limited data available on long-term menstrual patterns
clearly indicate that young women receiving chemotherapy are at significant risk of premature
menopause. However, the natural history of ovarian reserve prior to the manifestation of
amenorrhea remains poorly described.

Significant occult depletion of the ovarian reserve is evident in cancer survivors with regular
menstrual periods, underlining the need for accurate estimation of the ovarian reserve by
biochemical and/or biophysical means in order to appreciate the true extent of ovarian toxicity.
In fact, AMH may be the key indicator of ovarian toxicity secondary to chemotherapy; it is
largely produced by smaller follicles at an earlier stage of maturation, and it is these follicles
which seem to be disproportionately affected by chemotherapy [62, 75, 122]. A single AMH
measurement is an effective indicator of depleted ovarian reserve in cancer survivors.
Furthermore, a single random sample is sufficient; coordination of testing with the early
follicular phase of the menstrual cycle is not required. This makes AMH a far more acceptable
test for patients and a more practical one for clinical services [16, 18, 120, 122].

1.7 Limitations of the existing literature

There are five studies in the literature describing ovarian reserve in women treated for breast
cancer [14-18]. Three of these studies exclusively examine ovarian reserve in breast cancer
survivors in the first year after chemotherapy treatment and clearly demonstrate a marked and
sustained reduction in ovarian reserve [14-16]. There are two studies examining ovarian reserve
more than a year after chemotherapy, suggesting that the initial impact on ovarian reserve is evident after one year [17, 18]. The first included 20 breast cancer survivors and only four of these were more than five years out from completion of chemotherapy at the time of participation in the study [17]. The second study included 110 breast cancer survivors, but the median follow up was 2.1 years and none of the participants were more than five years out from chemotherapy treatment at the time of ovarian reserve estimation [18]. In addition, the survivors in this study had an average age of 46.1 years, 6 – 10 years older than the average in other published studies relating to young breast cancer survivors. This may be due to the inclusion criteria used – participants were required to have had one period in the year preceding the commencement of chemotherapy as opposed to a requirement for regular menstruation after chemotherapy treatment in other studies. It is concerning therefore that the ovarian reserve of this group of survivors is not truly representative of young women who regain regular menstrual function after chemotherapy.

In conclusion, there are few data describing the natural history of ovarian reserve in young women receiving adjuvant chemotherapy for breast cancer. In particular, there is an almost complete lack of data regarding ovarian reserve in long-term breast cancer survivors.
2 Study hypothesis & objectives

2.1.1 Hypothesis

It is clear from the literature review that adjuvant chemotherapy for breast cancer treatment causes an immediate depletion of ovarian reserve manifest as reduced ovarian reserve markers in the first two years after chemotherapy treatment. The hypothesis is that this reduction in ovarian reserve is permanent and will be evident in long term breast cancer survivors.

2.1.2 Objectives

1) To compare ovarian reserve in young breast cancer survivors and controls

2) To explore potential factors influencing AMH level in young breast cancer survivors treated with chemotherapy

3) To model the decline in ovarian reserve with increasing age in young breast cancer survivors and controls

2.1.3 Endpoints

The primary endpoint is AMH level in young breast cancer survivors and controls. Secondary endpoints include the following measures of ovarian reserve in young breast cancer survivors and controls: FSH, LH, estradiol, inhibin B, total antral follicle count and ovarian volume.
3 Material and Methods

3.1 Study design

This is a cross-sectional, comparative study. Ovarian reserve was estimated on one occasion in a cross-sectional sample of eligible breast cancer survivors drawn from a patient group within a single institution. Primary and secondary endpoints are measures of ovarian reserve and will be compared in breast cancer survivors and a control group.

3.2 Research Ethics Board Approval

Approval for this study was granted by the University Health Network Research Ethics Board and the Mount Sinai Hospital Research Ethics Board.

3.3 Study participants

3.3.1 Breast cancer survivors

Breast cancer survivors were recruited from the patient population within the Princess Margaret Hospital. Potential study participants were identified in two ways; by submitting a query to the institutional breast cancer registry and by screening relevant outpatient clinic lists on an ongoing basis. Inclusion and exclusion criteria are summarized in table 5. Since initial recruitment was poor, protocol amendments designed to improve accrual were implemented after the study commenced. These changes are highlighted in italics in table 5 and discussed in more detail below.
Table 5 Breast Cancer Survivors - Inclusion and Exclusion Criteria

<table>
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<tr>
<th>Inclusion criteria</th>
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<tr>
<td>1. Histological confirmation of breast cancer</td>
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<td>2. Diagnosed after January 1996</td>
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<td>3. Received chemotherapy treatment for breast cancer before 40 years of age</td>
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<td>4. Currently premenopausal – defined as having at least one menstrual period within</td>
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<td>the preceding six months. <em>Women who were amenorrheic but within 18 months of</em></td>
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<td><em>chemotherapy were included after January 2009</em></td>
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<td>5. Age ≤ 50</td>
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<table>
<thead>
<tr>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Breast cancer recurrence</td>
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<tr>
<td>2. Oophorectomy</td>
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<tr>
<td>3. <em>Currently on hormonal therapy i.e. tamoxifen or aromatase inhibitors</em> – women</td>
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<tr>
<td><em>on hormonal therapy were included in the study after December 2008</em></td>
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<tr>
<td>4. Currently on ovarian suppressive therapy i.e. gonadotrophin releasing hormone</td>
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<td>analogues</td>
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<td>5. Currently on the oral contraceptive pill (can be recruited if discontinued for</td>
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<td>12 weeks)</td>
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<td>6. Currently pregnant</td>
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<tr>
<td>7. Other cancer, excluding non-melanoma skin cancer</td>
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### 3.3.2 Controls

The control group were recruited from two sources; women treated for breast cancer who had not received chemotherapy and healthy volunteers. The inclusion and exclusion criteria used for these two groups are illustrated in tables 6 and 7. All control participants were required to have regular menstrual cycles, defined as cycle length of 21 – 25 days. Those currently taking endocrine therapy or using the oral contraceptive pill were excluded. Eligible healthy volunteers gave no previous history of cancer or chemotherapy treatment for any other reason. It was important to recruit a control group with a similar age profile to group of survivors treated with chemotherapy. While the control group was not formally matched with the test group, controls were required to be between 30 and 50 years of age.
Table 6 Controls (Survivors) - Inclusion and Exclusion Criteria

| Inclusion Criteria | 1. Histological confirmation of breast cancer  
|                   | 2. Currently premenopausal – defined as having regular menstrual cycles (21 – 35 days)  
|                   | 3. Current age 30 – 50 years  
| Exclusion Criteria | 1. Any chemotherapy treatment  
|                   | 2. Breast cancer recurrence  
|                   | 3. Oophorectomy  
|                   | 4. Currently on hormonal therapy i.e. tamoxifen or aromatase inhibitors  
|                   | 5. Currently on ovarian suppressive therapy i.e. gonadotrophin releasing hormone analogues  
|                   | 6. Currently on the oral contraceptive pill (can be recruited if discontinued for 12 weeks)  
|                   | 7. Currently pregnant  
|                   | 8. Other cancer, excluding non-melanoma skin cancer  

Table 7 Controls (Healthy Volunteers) - Inclusion and Exclusion Criteria

| Inclusion Criteria | 1. Age >30 years, < 50 years  
|                   | 2. Regular menstrual periods (defined as cycle length 21 – 35 days)  
| Exclusion Criteria | 1. History of treatment / investigation for infertility  
|                   | 2. Any diagnosis of cancer  
|                   | 3. Any chemotherapy treatment  
|                   | 4. Currently on hormonal medications i.e. oral contraceptive pill  
|                   | 5. Currently pregnant  
|                   | 6. Oophorectomy  

3.4 Protocol Amendments

3.4.1.1 Random AMH testing

It became apparent in the initial eight months of the study that the number of survivors and controls likely to be recruited and tested was significantly lower than expected. One of the reasons for this was a higher than anticipated rate of refusal. It was clear from my experience of discussing the study with potential participants that the requirement to attend for ovarian reserve estimation during the early follicular phase of the menstrual cycle (day 2 – 5 inclusive) presented
an absolute barrier to participation for many women who were otherwise quite interested in taking part in the study. The unpredictability of menstrual cycles and the necessity to arrange time off work or alternative childcare at very short notice with the arrival of their menstrual period made participation extremely difficult. In several cases, women submitted signed consent forms in good faith, but then found it impossible to attend for testing in spite of their best efforts. It was clear from discussion with this cohort that the ability to attend at a convenient time for random AMH testing would facilitate their participation greatly.

As discussed in the introduction, there is a significant amount of data demonstrating the consistency of AMH results during a single menstrual cycle and across consecutive cycles. Reported fluctuations in AMH levels during menstrual cycle are of small amplitude and therefore considered of minor significance when interpreting data for clinical purposes according to the European Society of Reproduction and Embryology [19].

During our study, Lie Fong et al published one of the largest studies in the literature reporting AMH levels in cancer survivors [122]. Personal communication with the chief investigator indicated that 21% of the 182 participants were tested during the early follicular phase of the menstrual cycle. There was no statistically significant difference in AMH levels for survivors tested during the early follicular phase of the cycle when compared with those tested at a random time-point in the cycle. It had been their experience that random AMH testing was far more acceptable to survivors and resulted in significantly increased participation rates.

Based on these accumulated data, I felt it was reasonable to request a protocol amendment allowing random AMH testing in an effort to facilitate recruitment of both survivors and controls. This amendment was submitted to the Research Ethics Boards and approved in early 2009. Those women attending for random AMH testing were not offered transvaginal ultrasound and would not have FSH, LH, estradiol and inhibin B levels included in the study analysis as interpretation of these tests requires that they are performed during the early follicular phase of the menstrual cycle.
3.4.1.2 Ovarian reserve estimation while amenorrheic post chemotherapy

While screening potential survivor participants, a small number of women were identified who were amenorrheic and still within 18 months of completion of chemotherapy. These women fulfilled all other criteria and were excluded on the basis of amenorrhea only. As discussed in the introduction, the majority of women receiving adjuvant chemotherapy for breast cancer before the age of 40 years regain menstrual function, although it may take up to 18 months for periods to return. We considered that the risk of recruiting a woman who will not subsequently regain menstrual function, thus creating a heterogeneous sample group was small. A protocol amendment was submitted to the Research Ethics Boards allowing the recruitment of these women. This amendment was approved in early 2009.

3.4.1.3 Recruitment of healthy volunteers as controls

The original protocol stated that breast cancer survivors who had not received chemotherapy would be recruited as control subjects. However, it was immediately apparent that the rate of recruitment was extremely low. The majority of survivors identified in the institutional breast cancer registry were treated with chemotherapy. A protocol amendment was designed allowing the recruitment of healthy volunteers who would answer a four-item questionnaire and undergo biochemical estimation of ovarian reserve only. Healthy volunteers were not offered transvaginal ultrasound. This protocol amendment was approved in early 2009.

3.4.1.4 Recruitment of breast cancer survivors currently receiving endocrine therapy

The original protocol excluded those breast cancer survivors who were currently receiving hormonal therapy on the basis that tamoxifen is associated with significantly reduced FSH levels [131]. Currently there is no evidence to suggest that tamoxifen has a significant impact on the primary endpoint, AMH. In practice, this single criterion excluded significant numbers of survivors. Other authors have included survivors currently taking tamoxifen in studies of ovarian reserve [17, 18] Given that our primary analysis was unlikely to be affected, we decided to
modify our criteria to facilitate recruitment. An amendment to the protocol allowing recruitment of these women was approved by the Research Ethics Board and implemented May 2010.

### 3.5 Recruitment procedures

#### 3.5.1 Database queries

Potentially eligible women were identified by extracting a list of candidates from two electronic sources. The first was the Princess Margaret Hospital breast cancer registry, an institutional database, which extends over the required time period. The second was Impac, an electronic record system used within the Radiation Medicine Program, which covered the latter part of the study period. This second query was designed to identify women receiving radiotherapy for ductal carcinoma in situ (DCIS) who were potentially eligible as controls and might not be included in the breast cancer registry.

The two patient lists were cross-referenced to exclude duplicates. A spreadsheet was designed including all patients identified in these two database searches. For each patient, the electronic record (EPR) was carefully reviewed according to the inclusion and exclusion criteria described above. If a clear reason for exclusion was documented in the EPR, these details were noted in the spreadsheet and the case considered ineligible.

In the case of a potentially eligible case, all entries in the EPR were reviewed in detail. The data required to complete the case record form was extracted. All available contact details were transferred to the spreadsheet. At this point, potentially eligible survivors were contacted by sending an information package to their current address as listed in the EPR. This information package included an introductory letter, a patient information leaflet and the consent form. The introductory letter summarized the study and provided a dedicated telephone line set up for the purposes of study recruitment. Women were advised that they could return a signed consent form or call the number to discuss the study further with one of the research team. A woman who did not wish to participate was advised to contact the dedicated phone line to opt out. The letter also stated we would follow up with a phone call offering participation in the study if we received no reply after two weeks.
Women who returned a signed consent form were contacted to review their history and ensure eligibility for the study. In the case of no reply, the potential participant was contacted by phone using the current contact details in the EPR. If the woman was interested in participating eligibility was confirmed and the study was explained in detail. The consent form was then signed and returned by mail at which point the woman was included in the study.

Several attempts were made to contact potential participants. When at least three attempts failed or phone numbers were found to be no longer in use, alternative addresses as listed in the EPR were also tried. If contact failed after at least three attempts using all available contact details, the person was recorded in the trial database as uncontactable.

3.5.2 Healthy volunteers

Healthy volunteers were recruited from Princess Margaret Hospital staff by means of flyers distributed throughout the building inviting participation and providing the dedicated phone line for the study. Women attending surgical clinics with benign breast disease were also offered participation by direct approach in the clinic and by distribution of flyers within the clinic waiting areas. Those interested in participation answered a brief questionnaire to ensure compliance with inclusion and exclusion criteria detailed above. Eligible women were provided with a consent form. Once written consent was obtained, women were included in the study.

3.6 Data Collection

3.6.1 Case specific data

In the case of breast cancer survivors, all data regarding diagnosis and treatment were gathered in a retrospective manner. In brief details regarding age at diagnosis, stage of disease, surgical procedures and obstetric history before and after diagnosis were recorded. Details of surgery and radiotherapy treatments were recorded. All available information regarding chemotherapy regimens including drugs used, dosage and number of cycles was recorded. Menstrual patterns before and after chemotherapy treatment were recorded. In each case, detailed review of the Princess Margaret Hospital EPR was performed and the information crosschecked and supplemented on discussion with study participants during the study visit.

For healthy volunteers age, height, weight and date of first day of menstrual period were recorded during their study visit.
3.6.2 Ovarian reserve estimation

The early follicular phase of the menstrual cycle was defined as day 2 – 5 inclusive. On day 1 of their menstrual cycle, participants contacted the dedicated study phone line and made arrangements to attend for their study visit. Those having a transvaginal ultrasound performed had all tests done at the Center for Fertility and Reproductive Health in Mount Sinai Hospital. Those attending for blood tests alone were facilitated in Princess Margaret Hospital. Participants attending for random AMH testing made an appointment at their own convenience. During the study visit, required information was documented in person with study participants.

Biochemical assays

AMH and inhibin B assays were run in batches on stored serum samples. Prior to the initiation of this study, AMH was not available within Mount Sinai Hospital or Princess Margaret Hospital. A commercially available kit was obtained and subjected to in-house laboratory quality assurance procedures prior to use. The commercially available kit was a two-step sandwich type enzyme immunoassay kit (Immunotech, Beckman Coulter, France) with a lower limit of detection of 0.14 ng/mL. The intra- and inter-assay coefficients of variation (CV) were ≤ 12.3% and ≤14% respectively or 1pM. Inhibin B was estimated using a sandwich type enzyme immunoassay (Diagnostic Systems Laboratories, Inc., Beckman Coulter, USA) with a minimum detection limit of 7 pg/mL. Intra-assay CV was 3.5 – 5.6% in three samples, each tested 23 times. Inter-assay CV is 6.2 – 7.6% in three samples, each tested 14 times. The FSH, LH and estradiol levels were determined by electrochemiluminescence assays run on an automated immunoanalyzer (Roche Modular Analytics E170, Roche Diagnostics, IN, USA). For FSH, the lower limit of detection was 0.1 mIU/mL and intra- and inter-assay CV were 2.5 – 2.8% and 3.6 – 4.5% respectively. For LH, the lower limit of detection was 0.1 mIU/mL and intra- and inter-assay CV were 0.7 – 1.2% and 1.6 – 2.2% respectively. For estradiol, the lower limit of detection was 18.4 pmol/L and the intra- and inter-assay CV were 1.4 – 3.3% and 2.2 – 4.9% respectively.

Imputed values

Some cases returned a value below the level of detection for the inhibin B and AMH tests. For inhibin B, these values were reported as < 7pg/ml. A value of 3.5 pg/ml was entered for the purposes of analysis. For AMH, these values were reported as <1 pmol/L. A value of 0.5 pmol/L
was entered for the purposes of analysis. The proportion of cases in each group for whom values were imputed are summarized in table 8.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inhibin B</strong></td>
<td>8 of 29 (28%)</td>
<td>12 of 24 (50%)</td>
</tr>
<tr>
<td>(tested day 2 – 5 of menstrual cycle)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>AMH</strong></td>
<td>1 of 54 (2%)</td>
<td>10 of 52 (19%)</td>
</tr>
</tbody>
</table>

**Biophysical tests**

All transvaginal ultrasounds were performed by experienced fertility specialists at the Center for Fertility and Reproductive Health in Mount Sinai Hospital. For practical reasons it was not possible to have all ultrasound scans performed by one person. Follicles were measured in the largest diameter and those measuring 2 – 10 mm in size were counted as antral follicles. Ovarian volume was calculated using the formula for a prolate ellipsoid (0.5236 * D1 *D2 *D3, where D1, D2 and D3 represent the maximum measurements in the x, y and z dimensions). The ultrasound report was provided on a standardized form designed for use in the study.

**3.6.3 Communicating results to participants**

Fertility is a potentially high emotive issue. In order to avoid any unnecessary stress for participants, a standard process was adopted as regards discussing results with participants. All participants were asked if they wished to receive the results of ovarian reserve estimates. Those who wished to know their results were contacted by me initially and advised as to whether results of FSH, LH, estradiol and ultrasound were within the normal range. All women were offered the option of consultation with a fertility expert at the Centre for Fertility and Reproductive Health in Mount Sinai Hospital. All participants who had questions regarding potential future fertility were advised that I could not provide that opinion and they were offered
referral to a fertility expert. In addition a fertility expert reviewed report forms on ultrasounds conducted in Mount Sinai Hospital as part of the study.

When a woman was referred to the Centre for Fertility and Reproductive Health in Mount Sinai Hospital, the family physician was informed in writing and a copy of the tests performed as part of the study included with the letter. All further management was at the discretion of the relevant fertility expert and the individual and did not form any part of the study data.

3.7 Statistical analysis

3.7.1 Objective 1 – Comparison of primary and secondary endpoints in controls and survivors

All endpoints were tested for normality by visual review of the histogram, identification of outliers on box plots and by using the Kolmogorow-Smirnov test for normality. The primary endpoint AMH was not normally distributed; data demonstrated a positive skew, contained several outliers (as shown in figure 6) and the Kolmogorow-Smirnow test gave a highly significant result (p < 0.001) indicating non-normality. The option of log transformation was considered; this procedure provided a normal distribution for the control group but not for the survivors. Therefore the non-parametric Mann-Whitney test was used to compare medians between groups.

Sample size calculations were performed using mean, standard deviations and effect sizes seen in hormone levels in breast cancer survivors published in the literature. With \( \alpha = 0.05 \) and power = 0.80, a sample size of 20 – 40 subjects in each arm is required depending on the source data chosen. The study is therefore powered for the primary comparison of AMH levels. Additional comparisons are reported, as detailed in the results section. The p values for these comparisons should be interpreted with caution as the study is not powered for each of these additional comparisons.
3.7.2 Objective 2 – Evaluation of predictors of AMH level in breast cancer survivors

Potential correlations between variables and AMH level were explored using Spearman’s rank correlation coefficient. Variables tested included attained age, age at the time of treatment, time since treatment, endocrine therapy and cyclophosphamide dose. Regression analysis was also used to estimate the magnitude of the impact of associated variables. Given the presence of outliers and a positive skew in AMH data this technique was used with caution. The validity of the analysis produced was evaluated by visual assessment of the normal probability-plot of the regression standardized residuals – in all analyses reported here this plot indicated no great deviation from normality. The potential undue influence of individual cases was assessed by reviewing the Mahalanobis and Cook’s distances produced by the regression model. Analyses were also repeated with outliers omitted; given that the results were similar, the data is presented with all cases included.

3.7.3 Objective 3 – to model the decline in ovarian reserve with increasing age in survivors and controls

A scatterplot of AMH results for both survivors and controls will be plotted against the attained age of both groups. Least square linear and quadratic functions will be plotted against the data to assess the direction and type of relationship between the two variables. Multivariate linear regression was performed bearing in mind the caveats associated with the use of this technique outlined in the section above. In addition, the results of multivariate regression must be considered with caution as the study was not specifically powered for this analysis. Variables found to be correlated with AMH level were included in multivariate analysis. Where a very strong correlation between variables was found (>0.7), one of those variables was omitted from the final analysis. Any addition to the model was assessed by reviewing the overall model significance, $R^2$ value, the significance of the individual variable and the impact on the standardized coefficient for the group variable.
4 Recruitment

4.1 Survivors

A total of 52 survivors took part in the study. 33 of these women were identified through the Princess Margaret Hospital Breast Cancer Registry. The remainder was identified by scanning relevant clinic lists on an ongoing basis.

A query submitted to the Princess Margaret Hospital Breast Cancer Registry resulted in a list of 1037 potentially eligible women treated for breast cancer between 1996 and 2009 inclusive. The flow diagram in figure 4 illustrates the outcome of the recruitment process for all 1037 women. 740 (71%) were deemed ineligible based on screening of the Princess Margaret Hospital EPR. Metastatic disease, attained age >50 years and current use of hormonal therapy were the commonest exclusions accounting for 12 – 14% of the total in each case. Reasons for exclusion classified as “other” included factors such as documented postmenopausal status, other cancer (most commonly thyroid cancer) or clear documentation of a move to another country. There was insufficient data available on which to judge eligibility in 64 cases (6%). The majority of these cases were women referred to PMH for a second opinion who had not actually received treatment in the hospital.

This initial screen of the EPR identified 231 (22%) potentially eligible breast cancer survivors treated with chemotherapy. 100 of these (43%) were ultimately deemed uncontactable. 131 (57%) were successfully contacted. At this stage, a further 42 (18%) were found to be ineligible. The commonest exclusion criteria included postmenopausal status, breast cancer recurrence or current use of hormonal medications. This information was not in the EPR as they were not attending for regular follow up.

The recruitment process therefore identified 89 survivors who were confirmed as eligible to take part in the study and who were contactable. 45 (51%) of these women declined to participate in the study. The remaining 44 provided informed consent and were included in the study. 33 of these women attended for ovarian reserve tests. The remaining 11 women did not attend due to logistical challenges to attending during the early follicular phase of the menstrual cycle. This finding is discussed further below.
Figure 4. A flow diagram illustrating the recruitment of breast cancer survivors and controls based on an initial search of the Cancer Centre Registry.

Cancer Centre Registry
Women treated for breast cancer 1996 – 2009, receiving adjuvant chemotherapy before age 40 years
n= 1037

Screening of medical record

Potentially eligible survivors
n = 231

Potentially eligible controls
n = 66

Contact by mail and phone

Exclusion Criteria

<table>
<thead>
<tr>
<th>Exclusion Criteria</th>
<th>No. (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastatic disease</td>
<td>144 (14%)</td>
</tr>
<tr>
<td>Attained age &gt; 50 years</td>
<td>143 (14%)</td>
</tr>
<tr>
<td>On hormonal therapy</td>
<td>128 (12%)</td>
</tr>
<tr>
<td>Other</td>
<td>128 (12%)</td>
</tr>
<tr>
<td>Oophorectomy</td>
<td>68 (7%)</td>
</tr>
<tr>
<td>Recurrence / deceased</td>
<td>65 (6%)</td>
</tr>
<tr>
<td>Insufficient data</td>
<td>64 (6%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>740 (71%)</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Survivors</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>Not eligible</td>
<td>42 (18%)</td>
</tr>
<tr>
<td>Declined participation</td>
<td>45 (19%)</td>
</tr>
<tr>
<td>Uncontactable</td>
<td>100 (43%)</td>
</tr>
<tr>
<td><strong>Consented</strong></td>
<td><strong>44 (19%)</strong></td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td><strong>231</strong></td>
</tr>
</tbody>
</table>

**Tested** | 33 (14%) | 3 (5%) |
4.1.1 Protocol Amendments

Random AMH Testing

This amendment was implemented in July 2009 and greatly facilitated study participation for both survivors and controls. Ultimately 29 (56%) survivors and 25 (46%) controls attended for random AMH testing. The following is an example of the impact of this amendment on recruitment of survivors. Ultimately 29 (56%) survivors and 25 (46%) controls attended for random AMH testing.

Almost all of the 44 survivors identified through the breast cancer registry consented early in the course of the study. It was clear by January 2009 that a substantial proportion of participants were having difficulty attending for ovarian reserve testing in the early follicular phase of the menstrual cycle. By June 2009, only 21 had completed study visits. Many of the women not tested at this point had contacted study staff on day 1 of several menstrual periods but had not been able to the hospital within the required 2 – 5 day time line due to work and/or family commitments. In some cases, participants were on regular follow up in the hospital but could not be tested on days when they attended for other appointments. This caused significant frustration particularly for women who did not live within easy access of the hospital with full time jobs and / or young children.

When random AMH testing was implemented in July 2009, all women were again contacted, this time offering a convenient appointment for AMH testing alone. Twelve women agreed to this and were tested in the following nine weeks. Random AMH testing therefore provided a substantial and immediate increase in the number of women able to complete their participation in the study. Eleven women declined to attend for AMH testing. Several of these women had been particularly frustrated by their difficulties in attending prior to that point and were not motivated to take part in the study in spite of a more convenient arrangement being available.

Testing While Amenorrheic post Chemotherapy

An amendment allowing the recruitment of breast cancer survivors who were amenorrheic in the first year after chemotherapy but otherwise eligible to participate was implemented in January 2009. Thirteen (25%) survivors recruited were in this group.
4.2 Controls

54 women were recruited as a control group. 83% of controls were healthy volunteers recruited from PMH staff or from clinic attendees. These women were recruited after implementation of the relevant amendment to the protocol in January 2009. The impact of this amendment is obvious; indeed prior to its implementation, no control participants had been recruited.

Seven women treated for DCIS were identified from database queries. Only two participants were breast cancer survivors who had not received chemotherapy treatment. A summary of recruitment of controls is provided in table 9. None of the controls had been treated with tamoxifen.

4.3 Attendance for ultrasound

All breast cancer survivors were offered the option of attending for transvaginal ultrasound during the early follicular phase of the menstrual cycle. Women taking part as controls were also offered the ultrasound test if they had been treated for DCIS or breast cancer. Healthy volunteers were not offered transvaginal ultrasound.

In total 31 women attended for ultrasound in the early follicular phase of the menstrual cycle (9 controls and 22 survivors). For two survivors, there was incomplete data as one or other ovary could not be visualized. Similar proportions of participants in both groups accepted the offer of an ultrasound test. 78% (7 of 9 eligible) of controls and 79% of survivors agreed to take part in this element of the study.

4.4 Summary of Recruitment

Table 9 provides a summary of recruitment of survivors and controls. Table 10 documents the numbers available for analysis for each of the ovarian reserve markers.
### Table 9 Summary of Recruitment for Survivors and Controls

<table>
<thead>
<tr>
<th></th>
<th>Survivors</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Institutional Breast Cancer Database</td>
<td>33 (63%)</td>
<td>45 (83%)</td>
</tr>
<tr>
<td>Weekly review of clinic lists</td>
<td>19 (37%)</td>
<td>7 (13%)</td>
</tr>
<tr>
<td>DCIS patients</td>
<td></td>
<td>2 (4%)</td>
</tr>
<tr>
<td>Cancer Survivors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>52 (100%)</td>
<td>54 (100%)</td>
</tr>
</tbody>
</table>

### Table 10 Summary of population analyzed for each ovarian reserve marker

<table>
<thead>
<tr>
<th></th>
<th>Survivors n (%)</th>
<th>Controls n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMH (measured at random or early follicular phase of cycle)</td>
<td>52 (100%)</td>
<td>54 (100%)</td>
</tr>
<tr>
<td>FSH, LH, estradiol, inhibin B (tested during early follicular phase of menstrual cycle)</td>
<td>28 (54%)</td>
<td>32 (62%)</td>
</tr>
<tr>
<td>Antral follicle count and ovarian volume (tested during early follicular phase of menstrual cycle)</td>
<td>20 (38%)</td>
<td>9 (17%)</td>
</tr>
</tbody>
</table>
5 Results

5.1 Breast cancer survivors – population characteristics

Age

Breast cancer survivors treated with chemotherapy had a median age of 39.2 years (28.5 – 50 yrs) at the time of participation in the study. As per study protocol all participants had been treated before the age of 40 years. Mean age at the time of treatment was 34.9 years (24.7 – 40 yrs). 24 (46.2%) of the group were < 35 years of age at the time of treatment.

Time Since Treatment

Mean time since treatment was 4.5 yrs, median 3.4 years (range 0.47 – 13.92 yrs). For 20 (38.5%) survivors, more than five years had elapsed since diagnosis. Four (7.7%) survivors were more than 10 years out from diagnosis. Figure 5 illustrates the spread of survivorship among the treated group.

Figure 5 Time since treatment for Breast Cancer Survivors

![Figure 5. Histogram showing distribution of time since treatment for breast cancer survivors.](image-url)
Menstrual Patterns

At the time of participation in the study, 10 (19%) survivors were amenorrheic – these women were recruited in the first 18 months after chemotherapy according to the protocol amendment discussed earlier. All other participants had had at least one period in the preceding six months. In fact, 29 (56%) of those recruited had regular monthly periods with an additional 2 reporting regular periods with a shortened interval. Eleven (21%) reported irregular periods with a frequency less than monthly.

Chemotherapy induced amenorrhea (CIA) occurred in 47 (90%) participants. The median duration of CIA was 6.9 months (1 – 77 months). Four women experienced CIA lasting > 12 months. Two of these women received tamoxifen therapy after chemotherapy and remained amenorrheic until after five years of tamoxifen therapy had been completed at which point menstruation returned.

Pregnancy Outcomes

Prior to chemotherapy treatment, 29 (48%) survivors had experienced at least one pregnancy, with 60 pregnancies recorded in the group overall. A total of 46 live births occurred in 25 women; eleven (21%) had one child, seven (13%) had two children and seven (13%) had three children. Nine women had a total of ten miscarriages and, five women had a total of six terminations of pregnancy and one had an ectopic pregnancy. After chemotherapy treatment, three (6%) became pregnant. One conceived on two occasions with one live birth, one had twins and one had a healthy singleton.

One woman reported undergoing cryopreservation of oocytes prior to chemotherapy. Two women had received gonadotrophin releasing hormone analogue (GnRH) during chemotherapy.

Disease Characteristics

Disease characteristics for breast cancer survivors are summarized in table 11. The majority of participants had stage II breast cancer and high grade primary lesions. There were almost equal numbers of hormone receptor positive and negative tumors. 21.2% of the group had Her2-neu
positive tumors. The Her2-neu receptor status was unknown in 15.4% of cases; these cases were treated in the 1990’s at a time when routine testing for the receptor had not commenced. One participant was known to be a carrier of the BRCA gene.

Table 11 Breast Cancer Survivors - Disease Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stage</strong></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>12 (23.1%)</td>
</tr>
<tr>
<td>II</td>
<td>28 (53.8%)</td>
</tr>
<tr>
<td>III</td>
<td>12 (23.1%)</td>
</tr>
<tr>
<td><strong>ER / PR status</strong></td>
<td></td>
</tr>
<tr>
<td>ER ± PR positive</td>
<td>25 (48%)</td>
</tr>
<tr>
<td>ER / PR negative</td>
<td>27 (52%)</td>
</tr>
<tr>
<td><strong>Her 2 status</strong></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>11 (21.2%)</td>
</tr>
<tr>
<td>Negative</td>
<td>33 (63.5%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>8 (15.4%)</td>
</tr>
<tr>
<td><strong>Pathology</strong></td>
<td></td>
</tr>
<tr>
<td>Ductal</td>
<td>47 (90%)</td>
</tr>
<tr>
<td>Lobular</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>Medullary</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>Other</td>
<td>3 (5%)</td>
</tr>
<tr>
<td><strong>Pathological grade</strong></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>-</td>
</tr>
<tr>
<td>Grade 2</td>
<td>13 (25%)</td>
</tr>
<tr>
<td>Grade 3</td>
<td>35 (67.3%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>4 (7.7%)</td>
</tr>
</tbody>
</table>
5.2 Breast cancer survivors – treatment characteristics

Chemotherapy

The chemotherapy regimen prescribed and the number of cycles given was clearly documented in the electronic medical record in all cases. The majority received adjuvant chemotherapy, given after definitive surgery. All but two women completed the planned chemotherapy course. In these two cases, chemotherapy was stopped before the planned number of cycles had been administered due to unacceptable levels of toxicity.

Women receiving chemotherapy were treated with a wide variety of chemotherapy regimens as illustrated in table 12. The commonest regimens used were ACT (Paclitaxel) and FEC-D. Overall, 34 (65.4%) women were treated with a regimen containing one of the taxanes (docetaxel or paclitaxel). The drug combinations and doses in each of these regimens are listed in table 1.

Table 12 Chemotherapy Regimens received

<table>
<thead>
<tr>
<th>Chemotherapy</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT (Paclitaxel)</td>
<td>14 (27%)</td>
</tr>
<tr>
<td>FEC-D</td>
<td>13 (25%)</td>
</tr>
<tr>
<td>CEF</td>
<td>7 (14%)</td>
</tr>
<tr>
<td>AC</td>
<td>3 (6%)</td>
</tr>
<tr>
<td>ACD (Docetaxel)</td>
<td>3 (6%)</td>
</tr>
<tr>
<td>CMF</td>
<td>3 (6%)</td>
</tr>
<tr>
<td>FEC</td>
<td>3 (6%)</td>
</tr>
<tr>
<td>Other</td>
<td>6 (12%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>52 (100%)</strong></td>
</tr>
</tbody>
</table>

Endocrine therapy

Nineteen (37%) survivors were treated with endocrine therapy as summarized in table 13 below. Nine (17%) were currently receiving hormonal therapy at the time of participation in the study. All of these women were taking tamoxifen tablets only. In all other cases tamoxifen therapy had been completed prior to participation.

Table 13 Endocrine Therapy Received

<table>
<thead>
<tr>
<th>Endocrine Therapy</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamoxifen</td>
<td>16 (84%)</td>
</tr>
<tr>
<td>Tamoxifen and goserelin</td>
<td>2 (11%)</td>
</tr>
<tr>
<td>Arimidex and goserelin</td>
<td>1 (6%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>19 (100%)</strong></td>
</tr>
</tbody>
</table>

Surgery

All participants had curative surgery. Thirty-one (60%) had breast conserving surgery. The rest of the group had a mastectomy with one woman also opting for a prophylactic mastectomy on the contra-lateral side. The majority of participants (79%) had an axillary lymph node dissection. The median number of nodes removed at axillary dissection was 14 (range 4 – 26). Sixteen (39%) of those having axillary dissection were node negative. Sixteen (39%) had 1 – 3 axillary nodes positive and nine had ≥ 4 nodes positive. Ten (19%) survivors had a sentinel lymph node procedure only. The median number of nodes removed at sentinel lymph node dissection was 2.5 (range 1 – 9). All of these women were node negative. All women with a positive sentinel lymph node procedure went on to have full axillary dissection performed in a second surgical procedure. Details of axillary surgery were unavailable in one case.

Radiotherapy

Radiotherapy was administered in 40 (77%) of cases. Eighteen women received radiotherapy to the conserved breast or chest wall only delivered via standard tangent fields. All of these women had node negative disease. Twelve women received radiotherapy to regional lymph nodes in
addition to treatment of the conserved breast or chest wall, delivered via tangent fields matched
with anterior and posterior fields treating the supraclavicular and/or axillary lymph node regions. Women treated with neo-adjuvant chemotherapy followed by surgery received radiotherapy after surgery. Women treated with adjuvant chemotherapy commenced radiotherapy after chemotherapy as per institutional protocol.

5.3 Controls - Characteristics

Mean age at the time of participation in the study was 40.7 years (range 28 - 50 years). In accordance with inclusion criteria, all women in the control group had regular periods and gave no prior history of a cancer diagnosis or chemotherapy treatment for any other reason.

5.4 Biochemical measures of ovarian reserve

A total of 52 breast cancer survivors were recruited and attended for AMH testing. A random AMH test was obtained in 29 (56%) survivors. The remainder was tested during the early follicular phase of the menstrual cycle (days 2 – 5 inclusive). All other ovarian reserve tests were performed during the early follicular phase of the menstrual cycle only. FSH, LH and estradiol results were available for 28 (54%) survivors. Inhibin B results were available for 24 (46%) survivors. Antral follicle count and ovarian volume were obtained in 20 (38%) survivors.

Biochemical results according to normal range

AMH results are tabulated according to a normal range of 14.3 – 48.6 pmol/l in table 14. This range is provided by the kit manufacturer (Immunotech, Beckman Coulter, France). Breast cancer survivors were significantly more likely to have AMH levels below the normal range when compared with controls (87% of survivors versus 57% of controls, p = 0.002, Chi square analysis with Yate’s continuity correction).

FSH, LH and estradiol results for both groups are tabulated according to normal values for the early follicular phase of the menstrual cycle (days 2 – 5 inclusive) in tables 15 – 17. These are the standard normal ranges used by the institutional laboratory. FSH was in the expected range
for 88% of controls and 61% of survivors. A Chi square test comparing these proportions did not demonstrate a significant difference (p = 0.61). LH was in the normal range in 94% of controls and 82% of survivors. Estradiol was in the normal range for 63% of controls and 71% of survivors. Chi square testing demonstrated no significant differences in these figures.

There was complete agreement between FSH and AMH tests in identifying breast cancer survivors with estimates outside the normal range consistent with reduced ovarian reserve – all survivors with a raised FSH level had a low AMH level. There was significant discordance between the tests when classifying breast cancer survivors as being within the normal range. Nineteen survivors had an FSH level < 14 IU/L consistent with the expected range during the early follicular phase of the menstrual cycle. Only five of these survivors had an AMH level in the normal range. Forty-two (81%) of survivors have an AMH level <10.96 pmol/l, the median value in the control group.

### Table 14 AMH results according to normal range

<table>
<thead>
<tr>
<th>AMH level*</th>
<th>Controls n (%)</th>
<th>Survivors n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 14.3 pmol/l</td>
<td>31 (57%)</td>
<td>45 (87%)</td>
</tr>
<tr>
<td>14.3 – 48.6 pmol/l</td>
<td>19 (35%)</td>
<td>7 (14%)</td>
</tr>
<tr>
<td>&gt; 48.6 pmol/l</td>
<td>4 (7%)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>54 (100%)</td>
<td>52 (100%)</td>
</tr>
</tbody>
</table>

* Based on a normal range of 14.3 – 48.6 pmol/l provided by the commercial ELISA kit manufacturer (Immunotech, Beckman Coulter, France)

### Table 15 FSH results according to normal range

<table>
<thead>
<tr>
<th>FSH level*</th>
<th>Controls n (%)</th>
<th>Survivors n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;3 IU / L</td>
<td>1 (3%)</td>
<td>2 (7%)</td>
</tr>
<tr>
<td>3 – 14 IU / L</td>
<td>28 (88%)</td>
<td>17 (61%)</td>
</tr>
<tr>
<td>&gt;14 IU / L</td>
<td>3 (9%)</td>
<td>9 (32%)</td>
</tr>
<tr>
<td></td>
<td>32 (100%)</td>
<td>28 (100%)</td>
</tr>
</tbody>
</table>

* Based on normal early follicular phase range of 3 – 14 IU/L
Table 16 LH results according to normal range

<table>
<thead>
<tr>
<th>LH level*</th>
<th>Controls n (%)</th>
<th>Survivors n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – 12 IU / L</td>
<td>30 (94%)</td>
<td>23 (82%)</td>
</tr>
<tr>
<td>&gt;12 IU / L</td>
<td>2 (6%)</td>
<td>5 (18%)</td>
</tr>
<tr>
<td></td>
<td>32 (100%)</td>
<td>28 (100%)</td>
</tr>
</tbody>
</table>

* Based on normal early follicular phase range of 1 – 12 IU / L

Table 17 Estradiol results according to normal range

<table>
<thead>
<tr>
<th>Estradiol level*</th>
<th>Controls n (%)</th>
<th>Survivors n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;90 pmol / L</td>
<td>10 (31%)</td>
<td>5 (18%)</td>
</tr>
<tr>
<td>90 - 716 pmol / L</td>
<td>20 (63%)</td>
<td>20 (71%)</td>
</tr>
<tr>
<td>&gt;716 pmol / L</td>
<td>2 (6%)</td>
<td>3 (11%)</td>
</tr>
<tr>
<td></td>
<td>32 (100%)</td>
<td>28 (100%)</td>
</tr>
</tbody>
</table>

* Based on normal early follicular phase range of 90 – 716 pmol / L

**Distribution of biochemical results – box plots**

The distribution of results for each biochemical test is illustrated in individual box plots in figures 6 - 10 inclusive. As discussed in the methods section, AMH, the primary endpoint is not normally distributed in either controls or survivors. The box plot of AMH data in 6 illustrates the presence of outliers with high values in both groups. Data regarding outliers was reviewed to ensure compliance with inclusion criteria. The most extreme outlier is a control with a value of 134.9 pmol/l. The same individual had an LH level above normal with the other biochemical tests falling within the normal range. All other cases classified as outliers (both controls and survivors) were found to have FSH, LH and estradiol levels within the normal ranges. The box plot suggests that AMH levels are lower in survivors, with the median value clearly below that of the control group. This is confirmed on statistical comparison discussed in the analysis of objective 1 below.
The box plot of FSH data illustrated in figure 7 also illustrates the presence of outliers with two high values occurring in each group. Two controls recorded values of 32 and 45 IU/L. Both women met the inclusion criteria for control participants and were aged 44 and 48 years respectively. Both had low AMH levels. Box plots for FSH and LH (figures 7 & 8) suggest increased values in breast cancer survivors. These differences were confirmed on statistical comparison, discussed in the analysis of objective 1 below. As shown in figure 9, there are no outliers in the inhibin B data. However, there is a wide variability in the data in both groups as evidenced by the size of the boxes. Although the median values appear quite different, this difference did not reach statistical significance as discussed below. Estradiol datapoints are plotted in figure 10. Again, there are several outliers in both groups with higher values.

**Figure 6 AMH Results - Boxplot**

![AMH Results Boxplot](image)

Figure 6. Boxplot of AMH values in breast cancer survivors and controls. AMH is not normally distributed: both groups include outliers with higher AMH values. Median AMH is lower in survivors.

**Figure 7 FSH Results - Boxplot**

![FSH Results Boxplot](image)

Figure 7. Boxplot of FSH values in breast cancer survivors and controls. There are two outliers in each group with higher FSH values. Median FSH is higher in survivors.
Figure 8 LH Results - Boxplot

Figure 8. Boxplot of LH values in breast cancer survivors and controls. There are outliers in each group with higher LH values. Median LH is higher in survivors.

Figure 9 Inhibin B results - Boxplot

Figure 9. Boxplot of Inhibin B values in breast cancer survivors and controls.

Figure 10 Estradiol Results - Boxplot

Figure 10. Boxplot of estradiol values in breast cancer survivors and controls. Both groups contain outliers with elevated estradiol levels.
5.5 Objective 1 – Comparison of ovarian reserve in survivors and controls

5.5.1 Biochemical measures

Table 18 illustrates mean and median values for all biochemical measures in the two groups. The Mann-Whitney test was used to compare groups. Survivors had significantly lower AMH values than the control group \( p < 0.001 \). Absolute mean and median values for survivors were one third of those seen in the control group. FSH and LH values were significantly higher in survivors. Survivors had lower inhibin B values consistent with reduced ovarian reserve but the difference did not reach statistical significance.

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>Mean</th>
<th>Median</th>
<th>Standard deviation</th>
<th>Mann-Whitney p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AMH pmol/L</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivors</td>
<td>52</td>
<td>6.65</td>
<td>3.42</td>
<td>8.14</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>54</td>
<td>17.43</td>
<td>10.96</td>
<td>22.07</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>FSH IU/L</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivors</td>
<td>28</td>
<td>15.53</td>
<td>9.55</td>
<td>15.18</td>
<td>0.029</td>
</tr>
<tr>
<td>Controls</td>
<td>32</td>
<td>9.72</td>
<td>7.55</td>
<td>8.21</td>
<td></td>
</tr>
<tr>
<td><strong>LH IU/L</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivors</td>
<td>28</td>
<td>9.16</td>
<td>7.05</td>
<td>6.87</td>
<td>0.005</td>
</tr>
<tr>
<td>Controls</td>
<td>32</td>
<td>5.55</td>
<td>4.25</td>
<td>3.25</td>
<td></td>
</tr>
<tr>
<td><strong>Estradiol (pmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivors</td>
<td>28</td>
<td>333.57</td>
<td>165</td>
<td>488.65</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>32</td>
<td>217.91</td>
<td>134</td>
<td>315.73</td>
<td>0.155</td>
</tr>
<tr>
<td><strong>Inhibin B (pg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivors</td>
<td>24</td>
<td>25.88</td>
<td>5.5</td>
<td>26.96</td>
<td>0.295</td>
</tr>
<tr>
<td>Controls</td>
<td>29</td>
<td>32.16</td>
<td>29.4</td>
<td>25.59</td>
<td></td>
</tr>
</tbody>
</table>
5.5.2 Biophysical measures

Summary statistics for total AFC and mean ovarian volume in both study groups are provided in table 19. Absolute values for mean and median total AFC and mean ovarian volume are lower in breast cancer survivors consistent with reduced ovarian reserve in survivors. However, these differences did not reach statistical significance.

Table 19 AFC and ovarian volume - comparison of groups

<table>
<thead>
<tr>
<th>Variables</th>
<th>n</th>
<th>Mean</th>
<th>Median</th>
<th>Standard deviation</th>
<th>Mann-Whitney p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Ovarian volume (cm³)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivors</td>
<td>20</td>
<td>5.34</td>
<td>4.04</td>
<td>5.39</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>9</td>
<td>8.09</td>
<td>7.09</td>
<td>6.90</td>
<td>0.258</td>
</tr>
<tr>
<td>Total AFC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivors</td>
<td>20</td>
<td>6.8</td>
<td>5.00</td>
<td>5.268</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>9</td>
<td>9.56</td>
<td>9.00</td>
<td>6.37</td>
<td>0.287</td>
</tr>
</tbody>
</table>
5.6 Objective 2: Predictors of ovarian reserve

Attained age

Age is a known predictor of ovarian reserve. Although the two groups were not formally age-matched, the inclusion and exclusion criteria were designed to try and ensure a similar age distribution between the survivors and the controls. Figure 11 is a histogram illustrating a broadly similar age distribution in the two groups. A two-tailed t-test shows no significant difference in mean age between controls and survivors (mean age 40.7 versus 39.2 yrs, p = 0.169).

Within the control group, there was a highly significant correlation between attained age and AMH level (p < 0.001, Spearman’s correlation coefficient). Linear regression demonstrated that AMH decreased by 0.547 for every year increase in age (p < 0.001, R² = 0.30). This relationship is illustrated in figure 12. There was also a significant correlation between attained age and inhibin B (p = 0.047). FSH, LH and estradiol did not show a significant correlation with attained age in the control group.
For breast cancer survivors, there was a significant correlation between attained age and AMH level (p = 0.044). Linear regression demonstrated that AMH decreased by 0.304 for every year increase in age (p = 0.029, R² = 0.09). This relationship is illustrated in figure 13. There was also a significant correlation between attained age and inhibin B (p = 0.021). As in the control group, there was no significant correlation between age and FSH, LH or estradiol.

**Figure 12 AMH and attained age in controls**

![Figure 12 AMH and attained age in controls](image)

Figure 12. Scatterplot of AMH level against attained age for controls with superimposed regression line. AMH level declines with increasing age (p < 0.001).

**Figure 13 AMH and attained age in survivors**

![Figure 13 AMH and attained age in survivors](image)

Figure 13. Scatterplot of AMH level against attained age for breast cancer survivors with superimposed regression line. AMH level declines with increasing age (p = 0.029).
Time since treatment

There is no statistically significant correlation between AMH and time since chemotherapy treatment \((p = 0.988)\). Survivors were divided into two groups either more or less than five years since treatment. Twenty survivors were more than five years from treatment and had a median AMH of 4.86 pmol/L compared with 32 survivors less than five years from treatment who had a median AMH of 3.36 pmol/L. There was no significant difference between the two groups \((p = 0.755)\). Figure 14 illustrates the relationship between AMH level and time since chemotherapy treatment. These data show no recovery in AMH with increasing time since treatment.

Age at treatment

Mean age at the time of treatment was 34.7 years \((\text{range} \ 34.7 \sim 39.9 \text{ years})\). There is a significant correlation between AMH and age at the time of diagnosis \((p = 0.046)\). Linear regression suggest that AMH is decreased by 0.284 for each year increase in age at the time of treatment \((p = 0.41, R^2 = 0.08)\). This relationship is illustrated in figure 15.
Figure 15 AMH and age at treatment in survivors

![Figure 15](image_url)

**Timing of AMH testing**

AMH results were compared for survivors tested at a random time-point versus those tested in the early follicular phase of the menstrual cycle. The same comparison was performed for the controls. The absolute figures for mean and median values are higher in both controls and survivors tested at random time-points during the menstrual cycle but these differences did not reach statistical significance as shown in table 20. Thirteen (25%) of survivors were tested while less than 18 months out from chemotherapy treatment in accordance with the protocol amendment discussed previously. Median AMH level was lower in these survivors (2.53 pmol/L versus 4.93 pmol/L in those tested later), although this difference did not reach statistical significance (p = 0.152).

**Table 20 Random versus early follicular phase AMH testing**

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean pmol/L</th>
<th>Median pmol/L</th>
<th>Standard Deviation</th>
<th>Mann-Whitney p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Survivors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Random</td>
<td>29</td>
<td>7.27</td>
<td>3.42</td>
<td>8.63</td>
<td></td>
</tr>
<tr>
<td>Early follicular phase</td>
<td>23</td>
<td>5.86</td>
<td>3.3</td>
<td>7.60</td>
<td>0.705</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Random</td>
<td>25</td>
<td>22.96</td>
<td>14.06</td>
<td>28.83</td>
<td></td>
</tr>
<tr>
<td>Early follicular phase</td>
<td>29</td>
<td>12.65</td>
<td>8.60</td>
<td>12.57</td>
<td>0.129</td>
</tr>
</tbody>
</table>

Figure 15. Scatterplot of AMH against age at the time of treatment in breast cancer survivors with superimposed regression line. AMH level decreased with increasing age at time of treatment (p = 0.041).
**Tamoxifen therapy**

Nine survivors were currently receiving hormonal therapy, while ten had completed hormonal therapy prior to taking part in the study. Median AMH values are similar in these two groups – 4.93 pmol/L versus 3.59 pmol/L respectively. Our study was not powered to detect a potential difference and sample size was too small to allow statistical comparison. Median AMH was 3.27 pmol/L for all nineteen survivors treated with tamoxifen versus 4.62 pmol/L for those 33 survivors who had never received tamoxifen (p = 0.871). FSH, LH and inhibin B were not statistically different in tamoxifen users versus non-users.

**Chemotherapy**

The distribution of AMH values according to cyclophosphamide dose is illustrated in figure 16. There is no statistically significant association between AMH level and cyclophosphamide dose although absolute AMH level tends to decrease with increasing cyclophosphamide dose (p = 0.275). Mean and median AMH levels according to alkylator score generated from cyclophosphamide doses received are given in table 21. The two groups with highest and lowest scores were compared. There was no significant difference in median values (p = 0.364). In addition, survivors who had received a cumulative cyclophosphamide dose of >6,000mg/m^2 were compared with those who received a lower dose. For the latter comparison, the relationship approached significance with p = 0.057.

Chemotherapy was also grouped according to whether regimens contained a taxane or not. Thirty-four (65.4%) survivors were treated with a regimen containing a taxane. Eighteen (34.6%) received a chemotherapy regimen which did not include a taxane. Median AMH levels were 3.42 pmol/L versus 3.81 pmol/L respectively. A Mann-Whitney test showed no significant difference in the two groups (p = 0.418).
Figure 16 AMH levels and cumulative cyclophosphamide dose

![AMH levels and cyclophosphamide dose](image)

Figure 16. Scatterplot of AMH level against cyclophosphamide dose. AMH tends to decrease with increasing dose of cyclophosphamide received but this relationship is not statistically significant (p = 0.275).

Table 21 AMH values and alkylator score

<table>
<thead>
<tr>
<th>Alkylator score</th>
<th>n</th>
<th>Mean</th>
<th>Median</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pmol/L</td>
<td>pmol/L</td>
<td></td>
</tr>
<tr>
<td>1 (lowest cyclophosphamide dose)</td>
<td>36</td>
<td>7.47</td>
<td>3.54</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>8.16</td>
<td>2.00</td>
<td></td>
</tr>
<tr>
<td>3 (highest cyclophosphamide dose)</td>
<td>13</td>
<td>4.01</td>
<td>3.42</td>
<td>0.364*</td>
</tr>
</tbody>
</table>

* comparison of lowest and highest dose groups, p value based on Mann-Whitney test.

BMI

Mean BMI was similar in controls and survivors (24.2 and 24.9 respectively) and comparison using a t-test confirmed a lack of difference (p = 0.468).
Multivariate analysis

Multiple linear regression analysis was performed including variables found to be significant on univariate analysis. Attained age and group both remained significant on multivariate analysis. Age at the time of treatment was also added to the model but was not significant. The model parameters are summarized in table 22. Given findings of borderline significance for cyclophosphamide dose on univariate tests, two cyclophosphamide variables were evaluated in a multivariate model including attained age. Neither the alkylator score based on tertile cyclophosphamide doses, nor a cyclophosphamide dose >6,000 mg/m$^2$ were significant predictors of AMH level.

Table 22 Multivariate model

\[
R^2 = 0.285, \text{ Model significance } < 0.001
\]

<table>
<thead>
<tr>
<th>Standardised coefficients</th>
<th>p</th>
<th>upper</th>
<th>lower</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>-0.368</td>
<td>&lt;0.001</td>
<td>-9.332</td>
</tr>
<tr>
<td>Attained age</td>
<td>-0.439</td>
<td>&lt;0.001</td>
<td>-1.932</td>
</tr>
</tbody>
</table>

\[
R^2 = 0.285, \text{ Model significance } < 0.001
\]

<table>
<thead>
<tr>
<th>Standardised coefficients</th>
<th>p</th>
<th>upper</th>
<th>lower</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>-0.373</td>
<td>0.003</td>
<td>-10.72</td>
</tr>
<tr>
<td>Attained age</td>
<td>-0.406</td>
<td>0.023</td>
<td>-2.404</td>
</tr>
<tr>
<td>Age at treatment</td>
<td>-0.46</td>
<td>0.797</td>
<td>-1.706</td>
</tr>
</tbody>
</table>
5.7 Objective 3 – To model the decline in ovarian reserve with increasing age in survivors and controls

Figure 17 below is based on the regression analyses detailed above. While both regression lines tend towards a very low AMH level at approximately fifty years of age, the control group records much higher levels in the third decade. Based on the relationship demonstrated in this graph, a 35 year old breast cancer survivors treated with chemotherapy has an AMH level equal to that of a healthy 45 year old woman.

Figure 17 Decline in AMH with age in survivors and controls

![Graph showing decline in AMH with age in survivors and controls](image-url)
### 5.8 Correlation between biochemical markers of ovarian reserve

A correlation matrix for all ovarian reserve markers for the entire study population combined is given in table 23. AMH had a highly significant positive correlation with total antral follicle count (AFC) and inhibin B and a highly significant negative correlation with FSH and LH. There was no significant correlation between AMH level and ovarian volume or estradiol. The strongest correlation was seen between AMH and total AFC with a correlation coefficient of 0.755. There was also a significant correlation between total antral follicle count and both inhibin B and estradiol. Inhibin B showed a strong positive correlation with AFC with a coefficient of 0.652. Neither FSH nor LH was correlated with antral follicle count. Not unexpectedly FSH and LH were significantly correlated with each other. There was a strong positive correlation with a coefficient of 0.660.

**Table 23 Correlation Matrix - markers of ovarian reserve**

<table>
<thead>
<tr>
<th>Variable</th>
<th>AMH</th>
<th>Total AFC</th>
<th>Mean OV</th>
<th>FSH</th>
<th>LH</th>
<th>Inhibin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correlation coefficient</td>
<td>0.755</td>
<td>&lt;0.001</td>
<td>0.096</td>
<td>0.642</td>
<td>-0.339</td>
<td>-0.180</td>
</tr>
<tr>
<td>Significance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.096</td>
<td>0.174</td>
<td>0.619</td>
<td>0.008</td>
<td>-0.392</td>
<td>-0.159</td>
</tr>
<tr>
<td>Significance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>-0.424</td>
<td>-0.288</td>
<td>-0.165</td>
<td>0.001</td>
<td>0.660</td>
<td>0.003</td>
</tr>
<tr>
<td>Significance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>-0.339</td>
<td>-0.180</td>
<td>0.003</td>
<td>0.660</td>
<td>0.008</td>
<td>0.001</td>
</tr>
<tr>
<td>Significance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.642</td>
<td>0.652</td>
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6 Discussion

Our study demonstrates significant long-term, irreversible depletion of ovarian reserve in breast cancer survivors. These data provide the largest sample thus far described for breast cancer survivors more than five years from diagnosis. We successfully recruited a group of survivors likely to be representative of women for whom fertility after a breast cancer diagnosis is important: women treated with modern adjuvant chemotherapy regimens before 40 years of age who remain premenopausal after treatment. We have also provided additional data supporting the use of random AMH testing as a measure of ovarian reserve in breast cancer survivors.

6.1 Long-term ovarian reserve after chemotherapy for breast cancer

There are only two other studies providing data on ovarian function in breast cancer survivors beyond the immediate post-chemotherapy phase. Our results compare well with these two papers although the number of long-term survivors in these studies is very small.

In 2009, Partridge et al reported a well designed study with features similar to ours although it had a smaller sample size and included only 4 women with more than 5 years follow up [17]. They recruited a total of 20 premenopausal breast cancer survivors with a mean age of 36.6 years who were free from disease recurrence at least one year after chemotherapy treatment. As in our study, premenopausal status was defined as having had at least one menstrual period in the preceding 6 months; 75% of those included were menstruating monthly. Half of the cohort was currently taking tamoxifen therapy. Ovarian reserve was estimated using the same markers as in our study on a single visit during the early follicular phase of the menstrual cycle. Results were compared with a control group matched on age and gravidity.

AFC was the primary outcome measure and was significantly lower in survivors (6 versus 10 in controls, p = 0.042). By comparison we did not demonstrate significant differences in AFC between survivors and controls. However, our sample size was quite small for this comparison. As in our study AFC was highly correlated with AMH, which was 0.4 ng/ml in survivors versus 1.4 ng/ml in controls (p < 0.004). This three-fold difference in AMH levels is consistent with the magnitude of difference seen in our study. Differences in FSH and inhibin B suggested lower ovarian reserve in survivors but were not statistically significant. Although the small sample size
precludes formal analysis, those survivors on tamoxifen were found to have slightly lower AMH and AFC than those not taking tamoxifen. We compared 33 survivors who had not received hormonal therapy with 19 who had. AMH level was slightly lower in those treated with hormonal therapy (median 3.27 versus 4.61 pmol/L respectively) but there was no statistically significant difference (p = 0.871).

Su et al report on a group of 110 women who had ovarian reserve testing performed within 1 – 4 years of commencing chemotherapy for breast cancer [18]. Participants were required only to be premenopausal before chemotherapy, defined as having had at least one period in the year preceding the start of chemotherapy. There were no restrictions regarding age or menopausal status after chemotherapy. Survivors were older than those in our study with a median age of 46.1 years and had ovarian reserve testing performed a median of 2.1 years after commencing chemotherapy. 55% of the group was amenorrheic for at least 12 months at the time of ovarian reserve assessment. 71% of those tested were currently taking tamoxifen. AMH, FSH, inhibin B and estradiol levels were estimated on one occasion at a random time-point during the menstrual cycle.

As in our study, survivors were found to have significantly reduced AMH and increased FSH levels. In addition, they had significantly reduced inhibin B and increased estradiol levels in comparison with age-matched controls. AFC, ovarian volume and LH tests were not performed. A comparison of 87 women taking tamoxifen with 23 not treated with tamoxifen showed no significant difference in AMH levels, inhibin B or estradiol levels. FSH was significantly lower in those taking tamoxifen (35.5 versus 42.8 IU/L, p = 0.04). Three separate logistic regression analyses were used to compare AMH, inhibin B and FSH levels in survivors and controls while controlling for potential confounders including smoking, alcohol use, BMI and gravidity. Ovarian reserve markers remained significantly different between the two groups.

The consistent finding in these two studies and ours is a significant reduction in AMH level in breast cancer survivors when compared with controls. While differences in other indices consistently suggest reduced ovarian reserve in survivors, statistical significance for FSH, LH and inhibin B comparisons is variably demonstrated. Uniquely in our dataset, more than one third of survivors were > 5 years from diagnosis (n = 20) compared with only 4 in the Partridge study. For our group median survival at the time of ovarian reserve testing was 3.4 years (range
0.47 – 13.92 years) compared with 2.1 years (range 1 – 4.9 years) for Su’s dataset. None of the participants in Su’s study had ovarian reserve testing performed more than 5 years after chemotherapy treatment. Our study therefore provides initial data regarding the long-term natural history of ovarian reserve in breast cancer survivors. We have demonstrated that the dramatic early changes in AMH level previously reported in the first year after chemotherapy are sustained in long-term survivors.

6.2 Predictors of ovarian reserve after chemotherapy for breast cancer

**Patient related factors**

As expected AMH level showed a significant relationship with attained age in both survivors and controls. As discussed in the introduction, publications analyzing increasingly large datasets in the normal population clearly demonstrate the close relationship between decreasing AMH level and increasing age in women of child-bearing age [11]. These data suggest that 30% of the variation in AMH level is due to age alone, a similar proportion to that seen in our control group. Anders has previously demonstrated the ability of AMH to discriminate between women receiving treatment for breast cancer aged more or less than 35 years [120].

Age at the time of treatment was also associated with AMH level in breast cancer survivors. This has not previously been reported, but it to be expected given the correlation between age at the time of treatment and attained age. Time since treatment was not significantly associated with AMH level.

**Treatment related factors**

This study was not specifically designed to evaluate the impact of individual chemotherapy or endocrine drugs on AMH level. There were no inclusion or exclusion criteria related to type of adjuvant treatment received. As expected, participants were treated with a variety of chemotherapy regimens as summarized in table 12. The majority of women received cyclophosphamide as a component of a multi-drug regimen. As shown in figure 16, AMH level decreases with increasing cyclophosphamide dose. Non-parametric tests demonstrated a
relationship between AMH level and cyclophosphamide dose approaching significance (both alkylator score and cyclophosphamide dose were analyzed). Given the potential relationship demonstrated in figure 17, a t-test was also employed as a parametric analysis may be more likely to detect an actual relationship. In fact, a t-test suggests that AMH levels are significantly lower in women receiving a cumulative cyclophosphamide dose of >6,000mg/m$^2$ ($p = 0.031$). However, a linear regression model controlling for the influence of attained age on AMH level, demonstrates that cyclophosphamide dose is not a significant predictor of AMH level ($p = 0.90$). A potential relationship between cyclophosphamide dose and AMH level would compare well with clinical data demonstrating higher rates of amenorrhea after CMF type chemotherapy when compared with more modern chemotherapy regimens. As shown in table 1, a complete course of CMF contains a much higher cumulative dose of cyclophosphamide than a more modern regimen such as FEC-D (8.4gm/m$^2$ cyclophosphamide for CMF versus 1.5gm/m$^2$ for FEC-D). It may be the case that a larger sample size is required to detect the effect of lower cumulative cyclophosphamide doses. The administration of a taxane was not significantly associated with AMH level. This analysis is compromised by the fact that taxanes were administered with a variety of other drugs and the varying impact of these combinations cannot be accounted for in our analysis.

Nineteen survivors received endocrine therapy, consisting of tamoxifen in the vast majority of cases. There was no significant difference in AMH levels in those treated with endocrine therapy. The potential impact of tamoxifen therapy on ovarian reserve has not been elucidated. Su provides the only other statistical comparison regarding the impact of tamoxifen on AMH level in breast cancer survivors and also found no significant difference [18]. Of note, Su also found significantly decreased FSH level in tamoxifen users versus non-users. We found a non-significant reduction in FSH level in tamoxifen users.

### 6.3 Pattern of ovarian reserve after chemotherapy for breast cancer

This dataset allowed us to explore for the first time the pattern of changing AMH levels in a reasonable sample of long-term breast cancer survivors. Figure 17 clearly illustrates the marked difference in the pattern of AMH decline across the span of reproductive life in survivors and controls. While both regression lines tend towards the same low point at the end of reproductive
life, breast cancer survivors have markedly lower AMH levels throughout resulting in a relatively flat slope in the associated regression line. These data indicate that a 35 year old breast cancer survivor has an AMH level similar to a 45 year old control, suggesting permanent depletion of ovarian reserve.

We found no correlation between AMH level and time since treatment \((p = 0.988)\). This finding may support the theory that damage induced by chemotherapy results in immediate follicle loss, but does not result in an increased rate of follicle loss in subsequent years. This pattern has previously been postulated by Anderson et al [14]. Lie Fong provides some supportive clinical data, demonstrating that the remaining fraction of AMH is similar in 25 female survivors of hematological and controls [128]. AMH was measured at two time intervals and the remaining fraction calculated as the ratio of the two measurements adjusted for the time interval in each case. We were not able to demonstrate any recovery in AMH level after chemotherapy treatment for breast cancer. The major driver of decreasing AMH level after treatment was increasing age.

There are no published data that can be easily compared with the findings illustrated in figure 17. In the studies discussed previously, Partridge and Su provide only a straightforward comparison of means [17, 18]. Three studies focusing on hormonal patterns in the first year after chemotherapy have demonstrated significant increases in FSH, LH and decreases in AMH, inhibin B and antral follicle count [14-16]. AMH levels in particular demonstrate an immediate and dramatic reduction in response to chemotherapy administration with no recovery seen up to one year later. Our data suggest that this decrease is sustained with AMH ultimately declining from a post-chemotherapy low towards postmenopausal levels.

### 6.4 Relevance of our study population

We successfully recruited a cohort of women diagnosed with breast cancer before the age of 40 years, requiring adjuvant chemotherapy, who are long-term survivors and continue to menstruate. This is the specific group for whom fertility after breast cancer is of greatest concern.

Unlike some studies in the literature, we recruited only women treated before that age of 40 years. 45% of the participants in our study had actually received chemotherapy before the age of 35 years. It has been shown that women diagnosed with breast cancer before the age of 35 or 40
years are more likely to have treatment related fertility concerns at the time of diagnosis and furthermore, that they may modify treatment decisions based on these concerns [25]. The detrimental effect of impaired fertility in this group persists, resulting in long-term adverse effects on quality of life [29, 30].

In order to recruit a group receiving drug combinations typical of modern oncology practice, we limited our patient population to women receiving chemotherapy treatment within the last ten years. The adjuvant chemotherapy regimens used to treat breast cancer have changed significantly over the past 10 – 15 years. CMF, one of the first regimens to demonstrate efficacy is now infrequently used in the adjuvant setting. Young women in particular are more likely to receive intensive chemotherapy regimens including an anthracycline and a taxane drug. While it is not possible to elucidate the effect of a single drug, data relating to rates of amenorrhea after treatment suggest that the pattern of ovarian toxicity is quite different after older regimens such as CMF when compared with anthracycline containing regimens such as AC.

We recruited women who remained premenopausal after chemotherapy treatment, as defined by having had at least one period in the preceding 6 months. In fact, 80% of survivors included in the study reported ongoing menstruation.

6.5 Recruitment

As discussed earlier, it became clear quite early in the study that the rate of recruitment was poor in both the survivor and the control group. There were three main barriers to the recruitment of breast cancer survivors; 43% of potentially eligible women identified in the Breast Cancer Registry could not be contacted as their contact details in the EPR were no longer valid, 51% of those potentially eligible and contactable declined to take part in the study and the requirement for an additional hospital visit during the early follicular phase of the menstrual cycle proved a logistical obstacle for many would-be participants. Ultimately three protocol amendments were submitted which facilitated successful recruitment.

Survivors

Our initial search of the Breast Cancer Registry from 1996 to 2009 inclusive identified 1,037 women who had been diagnosed with breast cancer before the age of 40 years. Careful screening of the EPR resulted in 221 (22%) potentially eligible breast cancer survivors treated with
chemotherapy. Of those potentially eligible 43% could not be contacted, because they were no longer on follow in the hospital and their contact details were out of date. Ultimately 89 (8.5%) women were both eligible and contactable. Perhaps most disappointing at this stage was that 45 (51%) of these women declined to participate in the study. Prior experience within our research group had indicated a significant interest in fertility outcome after chemotherapy and a high degree of willingness to take part in research studies relating to this topic. A previous study conducted in the same institution examining fertility outcomes in survivors of Hodgkin’s disease treated with ABVD combination chemotherapy, found that 94% of eligible lymphoma survivors agreed to take part [85]. Hodgkin’s disease survivors constitute a different population, being younger on average at the time of diagnosis and having a better prognosis overall. In addition, this study was based on an extensive questionnaire, which could be completed with participants over the phone and did not require an additional attendance at the hospital or any additional testing.

Those who gave a reason for non-participation in our study most commonly cited busy schedules (full time jobs, small children) as the main obstacle to them taking part. Only two women were unwilling to revisit the hospital or spend time discussing their treatment details as they found the subject distressing. Prior to the implementation of the protocol amendment allowing random AMH testing, the logistical requirement to attend during the early follicular phase of the menstrual cycle remained a significant obstacle to study completion for many potential participants. The majority of participants were strongly motivated by the potential future benefits of the study for other young women diagnosed with breast cancer and they found this element of the study design quite frustrating. In particular, those who attended the hospital regularly for follow up expressed dissatisfaction at the necessity of an additional hospital visit timed with the early follicular phase of the menstrual cycle.

Some published articles on ovarian reserve in breast cancer survivors do not adequately describe study recruitment procedures. In many cases it is not possible to define the proportion of potentially eligible survivors who ultimately took part. Reading the available data, one comes to the unsurprising conclusion that studies requiring a significant time commitment on the part of participants are more difficult to complete. The largest published study recruited 182 survivors of childhood cancer to a study requiring a single blood draw for random AMH assessment [122]. Each participant nominated a convenient time for assessment, most commonly during a routine
hospital visit. 97% of eligible women agreed to take part in this study. By contrast, Larsen et al designed a study requiring attendance for ovarian reserve estimation in the early follicular phase of the menstrual cycle on three or four occasions and found that 39% of eligible women agreed to participate [124].

The use of random AMH testing based on a single sample taken at a time convenient for participants was the single most significant factor in facilitating recruitment to our study. Furthermore, it is likely that the women who consented to the study but subsequently failed to attend for testing during the early menstrual phase of the cycle would have been successful participants had they had the option of a single random AMH test from the start of the study. Unfortunately, enthusiasm for the study had waned in this small group of women when they were later offered random AMH testing after implementation of the associated protocol amendment and only a proportion availed of the test at that stage.

Significant barriers to survivor participation have been encountered in various research settings. Germino et al specifically highlight the difficulty in recruiting busy young breast cancer survivors who have many responsibilities and demands on their time [132]. In attempting to recruit African American breast cancer survivors in particular, they successfully overcame recruitment challenges by employing a multifaceted strategy aimed not only at improving accessibility and relevance of study information to the target population but also at understanding and engaging with relevant cultural, ethnic and religious aspects of potential participant’s lives.

Some have approached survivorship research by recruiting former participants in large clinical trials. This provides the advantage of detailed knowledge of the original disease and treatment and a defined cohort of potential participants. However, this approach is also associated with challenges as described by Ganz et al in a study recruiting colorectal cancer survivors 5 – 20 years out from treatment who were participants in five phase III randomized controlled NSABP clinical trials [133]. Sixty of sixty-five treating institutions agreed to take part. However, registration forms were received on only 41% of potentially eligible patients. 95% of the participants who expressed an interest ultimately completed interviews; however, this represented only 29% of the overall potential sample. Barriers to recruitment included difficulty locating patients, a lack of institutional commitment and a lack of patient interest.
One of the more effective approaches to date in terms of data collection, is the multicentre cooperative approach represented by the Childhood Cancer Survivors Study. The coordinating group in St. Jude Children’s Research Hospital, have published data on the feasibility of recruiting survivors to studies examining late effects [134]. Eligible survivors were offered participation in this particular study on three levels; 1) comprehensive medical evaluation requiring attendance at the hospital; 2) limited medical evaluation performed at home or; 3) completion of health surveys only. 91.8% of those eligible and contactable agreed to take part in the study. Strikingly 88.6% consented to attend for comprehensive medical evaluation, 11% opted for the survey only and 0.4% elected to undergo home evaluation. Those who declined to participate were likely to be male (p=0.001). In our study, potentially eligible breast cancer survivors were allowed to opt out of transvaginal ultrasound testing. Ultimately 42% of survivors taking part also attending for transvaginal ultrasound.

We did not offer an incentive to participation in our study, although participants were offered a refund for the cost of their parking if applicable. Ashing-Giwa et al have reported that payment of incentives upfront did not dramatically affect participation rates for long term breast cancer survivors [135]. In a study using a mailed quality-of-life questionnaire half of the participants were given payment in advance and half were offered payment on completion of the study. There were equal response rates in both groups, leading the authors to conclude that payment on completion was the more cost-effective option.

**Controls**

The study was initially designed to recruit control participants from among breast cancer survivors who had not received chemotherapy. Very few controls were identified based on these criteria. The recruitment of healthy volunteers as controls provided a straightforward and successful solution. Healthy volunteers are commonly employed in published studies and arguably, the study should have been designed this way from the outset.

**6.6 AMH as the test of choice**

**AMH is user-friendly**

Our experience suggests that random AMH testing is the most practical way to assess ovarian reserve in breast cancer survivors. The practical difficulties associated with ovarian reserve
testing based on traditional biochemical markers and transvaginal ultrasound during the early follicular phase of the menstrual cycle are allayed by the introduction of random AMH testing. The test is user-friendly, acceptable to survivors and can be easily combined with routine follow up care.

Currently available data support the theory that a single, random AMH test is a useful estimate of ovarian reserve. It has been established that AMH levels in healthy women do not fluctuate significantly during the menstrual cycle in contrast to FSH and LH levels which undergo large, cyclical variations [66-68]. The first study employing random AMH testing in cancer survivors was published while our study was ongoing and is the largest published to date, including 182 childhood cancer survivors [122]. Subset analysis in this study indicates no difference in AMH results obtained during the early follicular phase of the cycle compared to those obtained at a random time point [127]. We also found no significant difference between 23 AMH results obtained in the early follicular phase and 29 AMH results obtained at a random time point in breast cancer survivors. A similar comparison in the control group showed no significant difference.

Antral follicle count is traditionally estimated by counting follicles visualized during transvaginal ultrasound. This test has the obvious disadvantage of being invasive, but also requires specialist on-site staff and attendance by the patient during the early follicular phase of the menstrual cycle. Partridge et al found that AMH was the strongest predictor of AFC in breast cancer survivors [17]. Furthermore the predictive ability of this model was not improved by the addition of FSH, LH or inhibin B. We found that AMH was more strongly correlated with AFC than with any other marker of ovarian reserve (Spearman correlation coefficient 0.755, p < 0.001).

**AMH as an accurate marker of chemotherapy-induced ovarian toxicity**

All of the published data on breast cancer survivors have demonstrated significantly reduced AMH levels in those treated with chemotherapy when compared with healthy controls [15-18]. Findings based on other ovarian reserve markers are less consistent. For instance, FSH levels are increased in survivors in most but not all studies [17]. In women tested before and immediately after chemotherapy, AMH is the marker that demonstrates the most profound changes, with
dramatic reductions to barely detectable levels seen as early as six weeks after commencing chemotherapy. Our data suggests that this depletion is permanent.

These findings are consistent with the suggestion that AMH is an accurate indicator of the true ovarian toxicity of chemotherapy. It has been suggested that chemotherapy disproportionately affects the smaller follicles, which are at an early stage of development. Larsen et al have demonstrated greater depletion of smaller follicles in survivors treated with chemotherapy [124]. It is these smaller follicles that are largely responsible for the production of AMH. One would therefore predict that AMH levels would fall disproportionately with significant cell kill among these follicles. Follicles at this early stage of development are not under the cyclical influence of the gonadotrophins – while still an indirect measure of the remaining follicle pool, AMH may therefore provide a more faithful estimation of chemotherapy induced toxicity.

**AMH as an accurate marker of ovarian age in young women**

Young women with breast cancer are generally defined as those diagnosed before 35 – 40 years of age. In women at this stage of reproductive life, gonadotrophin levels do not discriminate age differences, as increasing FSH levels are a late event in the menopausal transition. Here again, AMH provides an advantage. In women diagnosed with breast cancer, AMH can distinguish between those aged less than 35 years and those age 35 – 40 years old [120]. In the same comparison, FSH showed no difference. In our study AMH is significantly correlated with attained age in both survivors and controls while FSH and LH are not. Survivors were significantly more likely than controls to have an AMH level below the normal range. While the proportion with a raised FSH level was higher among survivors, this comparison with controls did not reach statistical significance. All survivors with a high FSH value also had a low AMH value. However, 14 survivors with normal FSH values had an AMH value less than normal.

In the age group of interest therefore, AMH not only provides an accurate estimate of chemotherapy-induced ovarian toxicity, but can also distinguish the natural decline in ovarian reserve with ageing.

**Standardization of AMH testing**

An internationally accepted means of testing for AMH does not yet exist. Two distinct commercially available kits based on an enzyme-linked immunosorbent assay (ELISA) have
used for AMH testing, largely in the research setting (produced by Beckman-Coulter (IBC) and Diagnostic Systems Laboratories (DSL). In addition, some authors utilized in-house assays and adjusted their results for comparison with controls tested using commercial kits [122, 128]. It has been shown that the two commercially available tests produce significantly different results [136].

Furthermore, Beckman Coulter has now introduced the GEN II assay which utilizes a more stable antibody than their previous assay. It is of interest to note that 46 of our controls had both Beckman Coulter tests performed. Mean values with the two tests differed by 30% and a paired t-test showed a significant difference between the results (p < 0.001). Clearly, standardization is required if results are to be comparable between research groups. To this end, it is interesting to note that the same company now produces both commercial kits.

Very large studies have confirmed the close correlation between decreasing AMH level and increasing age. The largest report to date included 15,234 tests performed using a single assay in a reference laboratory servicing fertility clinics in the United States [73]. Kelsey et al report on a dataset including over 3,000 AMH estimates in healthy women throughout reproductive life [11]. The standardization of AMH testing will also facilitate the development of a clinical tool based on data such as these, allowing easy comparison of AMH results with the expected levels in healthy women of a similar age.

### 6.7 Weaknesses of study design

This is a cross-sectional study based in a single institution, which may therefore be subject to selection bias. Disease and treatment details were captured in a retrospective fashion. Our overall sample size and our inclusion of women treated with any type of chemotherapy during the study period precludes an analysis of the impact of various chemotherapy regimens on long-term ovarian reserve. We have attempted to analyze various factors, which may influence long-term ovarian function such as age at treatment and time since treatment. Although we did find some significant factors as discussed above, a larger sample size would be required to exclude significant effects with confidence.
6.8 Future directions

Ideally, young women newly diagnosed with breast cancer, for whom future fertility is a significant concern would be able to make decisions regarding adjuvant chemotherapy in the context of a full appreciation of the long-term fertility outcomes associated with specific treatment regimens. Those women with a lower estimated risk of disease recurrence might be strongly motivated to choose a less gonadotoxic regimen or to avoid chemotherapy altogether in order to preserve fertility. However, the information required in the decision making process remains uneven. There is extensive clinical trial data available describing the magnitude of improvement in recurrence and mortality outcomes with specific chemotherapy regimens. In recent years, the OncotypeDX multigene assay (® Genomic Health, California, USA) has been incorporated into several international breast cancer treatment guidelines, allowing physicians to further individualize adjuvant therapy recommendations, based on patient specific prediction of recurrence scores. On the other side of the equation, much remains to be elucidated regarding the fertility outcomes associated with adjuvant therapies.

The individualization of treatment recommendations will require an understanding of the impact of specific drugs and drug combinations on ovarian reserve as measured by indices such as a random AMH test. This will require a prospective, longitudinal study which includes only women treated with certain chemotherapy regimens or which has a very large sample size. Alkylating agents such as cyclophosphamide have long been recognized as mediators of ovarian toxicity. Our study suggests that a cumulative dose of cyclophosphamide in excess of 6,000 mg/m² is associated with lower AMH levels in young breast cancer survivors. The cumulative cyclophosphamide dose in modern adjuvant chemotherapy regimens, although lower than that in older regimens such as CMF varies quite significantly (1,500 mg/m² for FEC-D, 2,400 mg/m² for AC and TC and 3,000 mg/m² for FEC, full details of each regimen given in table 1). If a linear relationship exists between cyclophosphamide dose and AMH level, is it likely that a large sample size will be required to demonstrate potential differences between a dose in the range of 1,500 mg/m² and 3,000 mg/m². Given the challenges that we have faced in recruiting young breast cancer survivors, a large sample size will only be achieved by multi-institutional initiatives. The most accurate means of evaluating specific chemotherapy regimens is in the setting of a clinical trial, given the variability of prescription seen in clinical practice. A random AMH test could easily be incorporated into a trial design requiring regular follow up to evaluate.
five or ten year recurrence and/or survival outcomes, albeit with an additional cost implication. The longitudinal aspect of such a study would include pre-chemotherapy estimation of ovarian reserve and fertility outcomes allowing an analysis of whether pre-chemotherapy values are predictive of post-treatment outcomes.

As discussed in the introduction, successful pregnancy is a function not only of quantitative ovarian reserve but also the quality of the remaining oocytes. As the ovary ages and the quantity of oocytes decreases, so too does the quality of the remaining oocytes. A normal ovarian reserve estimate therefore does not guarantee successful pregnancy. In the infertility setting, a large meta-analysis has shown that antral follicle count and AMH are equally poor predictors of successful pregnancy [129]. The authors of a recent paper employing large datasets to examine the relationship between AMH and age in the general population point out that the next step is an examination of the relationship between AMH level, age and individual pregnancy outcomes in large population based datasets [11]. This work is ongoing. Ultimately, it may be possible to design a clinically useful tool, predicting pregnancy rates based on age and ovarian reserve level. Similar data in young breast cancer survivors may allow not only a prediction of long-term ovarian reserve after a particular chemotherapy treatment but also the fertility outcomes associated with remaining ovarian reserve after chemotherapy treatment.

6.9 Conclusion

We have demonstrated significant long-term occult ovarian toxicity associated with adjuvant chemotherapy treatment for breast cancer in young women. Our data suggest that the immediate reduction in ovarian reserve induced by chemotherapy is irreversible. Regression analysis indicates that a 35 year old breast cancer survivor has an AMH level similar to that of a 45 year old control. These data have significant implications for young women newly diagnosed with breast cancer who wish to have children after treatment.
References


22. *Cancer Research UK.*


Copyright Acknowledgements

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The third graph is from Kelsey et al, 2011 A Validated Model of Serum Anti-Mullerian Hormone from Conception to Menopause. PLoS ONE 6(7):e22024.
doi:10.1371/journal.pone.0022024.