Effects of chemotherapy upon fertility amongst women of reproductive age, using AMH as a marker of ovarian reserve

by

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TABLE OF CONTENTS

FIGURES

TABLES

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DECLARATION

'This thesis is submitted to the University of Warwick in support of my application for the degree of **Doctor of Medicine**. It has been composed by myself and has not been presented in any previous application for any degree, apart from the background material in introduction and methodology sections which was previously submitted for my upgrading from masters to doctoral study.

'The work presented (including data generated and data analysis) was carried out by the author except in the cases outlined below:

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Abstract

Nowadays, early detection of cancer and improving survival rates mean that more young women diagnosed with cancer have a normal life expectancy. For many of them the ability to conceive after successful treatment is one of their prime concerns. This dissertation evaluated the adverse effects upon fertility of some of the most common chemotherapeutic regimens used for cancers affecting women of reproductive age. Serial serum anti-Müllerian hormone (AMH) measurements were used to assess ovarian function in female patients with breast cancer or lymphoma before chemotherapy and compared longitudinally with same age healthy volunteers. Interestingly, women experiencing cancer during their reproductive years had a significantly lower ovarian reserve than healthy women, even before the start of chemotherapy. Overall 35% of reproductive age women newly diagnosed with cancer still expressed some desire to have children, while the same was true for nearly 50% of women aged ≤39 years. Simultaneously, only 9% of them had had a consultation with a fertility specialist regarding fertility preservation options or the impact of chemotherapy on ovarian function. Oncologists should consider women's wishes regarding fertility while counselling patients prior to cancer therapy. Overall in the cancer group, AMH declined significantly from the pre-chemotherapy level, remaining undetectable in 80% of patients within the first 12 months. It confirmed devastating effects of some of the chemotherapeutic agents on ovarian reserve. In the breast cancer group, only 15% of women had detectable AMH at 12 month follow-up. In contrast, amongst patients with Hodgkin lymphoma, more than 60% had a detectable degree of recovery of AMH concentrations at 12 months of follow-up, which was even higher if only ABVD regimen was used in women aged ≤ 32. Nearly 90% of those women reported the resumption of menstruation. My study offers reliable statistics as well as formulae calculating the probability of return of menses that can be used by oncology teams during consultations prior to chemotherapy. In contrast to other published results, I concluded that pre-treatment serum AMH concentrations are not correlated with amenorrhea or post-treatment AMH concentrations. However, AMH in combination with age could be used to calculate the risk of amenorrhea at 12 months using a prediction model constructed as a result of my work. In light of the recent debate about the reliability of AMH assays, my results confirmed the intra-subject reproducibility of the second generation AMH assay, providing evidence that is eagerly awaited by clinicians working in the field of Reproductive Medicine. My study supports the reliability of AMH measurements at unspecified times of the menstrual cycle. It provides guidance and reassurance to doctors using AMH assay in clinical practice.

CHAPTER 1

1 Introduction

1.1 Ovarian biology and ovarian ageing

It is widely believed that at birth, human ovaries contain a non-replaceable stock of oocytes, which has been estimated to comprise around 2 million oocytes (Wallace, 2010). Although this paradigm has been challenged by some evidence of oocyte renewal in mammals (Johnson *et al.*, 2005), it remains the most widely accepted theory of oogenesis (Wallace, 2010).

Oocytes are formed from primordial germ cells in the first trimester of pregnancy and by 16-21 weeks the first primordial follicles are present in foetal ovaries. The dialogue between somatic cells and oocytes plays a crucial role in their survival and further development (Telfer & McLaughlin, 2007). There is continuous recruitment of primordial follicles from the resting pool until the age of menopause when the number is around 1000 (Telfer & McLaughlin, 2007). It was found that the rate of follicular recruitment increases from birth until puberty when it reaches a plateau and then declines with age. Based on mathematical modelling, Wallace et al. suggested that the optimal recruitment rate of non-growing follicles is 880 follicles per month at age 14.2 in women with an average age of menopause (Wallace, 2010).

Adult ovaries contain follicles at different stages of development. The classes of follicles are categorised based on their number of granulosa cells and the time they take to transition between stages. *In vivo* these have been estimated as follows: primordial, primary (>150 days), secondary (120 days), pre-antral, antral (65 days), selected (10 days) maturing (10 days) and ovulatory follicles (Gougeon, 1986). The majority of oocytes are arrested in prophase of the first meiotic division and are surrounded by granulosa cells within primordial follicles. The recruitment of follicles into the growing pool is inhibited by anti-Müllerian hormone (AMH) (Strauss Iii & Williams, 2009).

The recruited primordial follicle undergoes mitosis within the granulosa, to form more layers of granulosa cells. When several layers have been laid down, the solid follicle forms an extracellular cavity within the granulosa, called the antrum. From an early antral stage, follicular development becomes dependent upon endocrine control, particularly FSH (follicle-stimulating hormone). At the beginning of each menstrual cycle, a group of small antral follicles is available for final selection, but normally only one is ovulated two weeks later. Only the ovulated oocyte has the potential to be fertilised and develop further. Remaining follicles become atretic and undergo apoptosis (Gougeon, 1996).

1.2 Ovarian ageing and fertility

1.2.1 Oocyte numbers and female age

Throughout a woman's life, the decline in reproductive capacity so called ovarian aging is related to the decreasing numbers of primordial follicles in the ovaries. The process is mainly age dependent and influenced by genetic factors, but lifestyle and medical or surgical interventions also play a role.

Some researchers have examined the age related decline in primordial follicles. One of the widely known models reports a bi-exponential relationship between follicle numbers and age, showing that loss of follicles accelerates from age 37.5, as shown in the graph below (Faddy *et al.*, 1992). Although, this model was questioned subsequently by its author and other research groups, as it seems unlikely that dramatic physiological changes in follicles dynamics would start at age of 37-38 when there is still a reasonable number of follicles remaining (around 25 000). Following reanalysis the differential equation model was proposed to be more reliable (Faddy & Gosden, 1996).

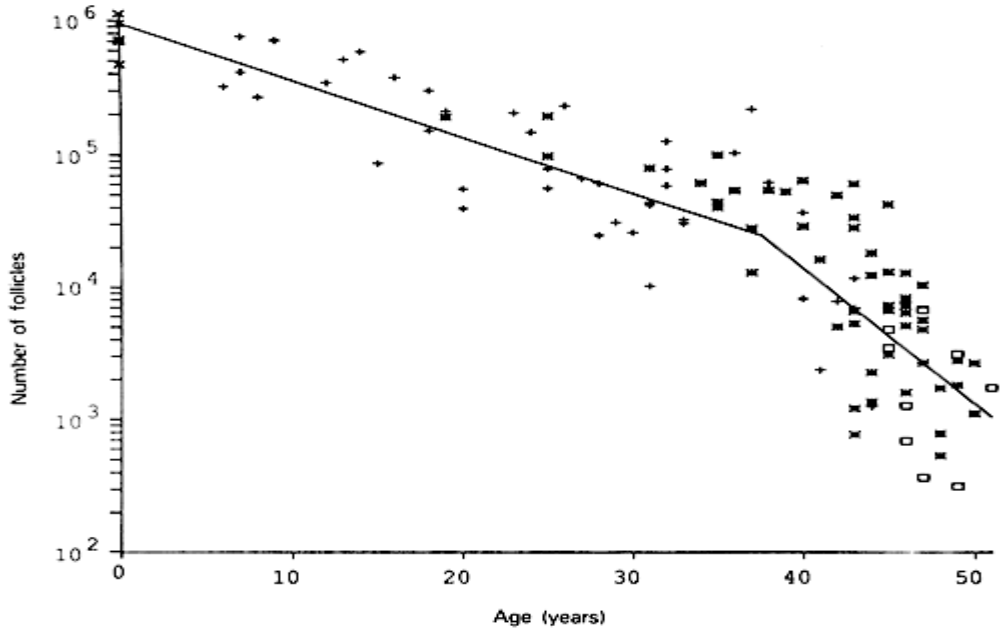


Figure 1 Bi-exponential fall in the number of follicles (from Faddy *et al.,* 1992).

This model is based on an assumption that the rate of decline is a function of the number of remaining follicles and results from its paracrine action (Faddy & Gosden, 1996). A recent comparison of statistical models of ovarian follicle loss favour the differential equation model alongside the power model suggested by Coxworth (Coxworth & Hawkes, 2010). In his model, he suggested that the rate of follicle loss due to atresia increases gradually with age rather than changing dramatically at a specific age. According to his model, only around 80% of the variation in the number of non-growing follicles could be explained by age alone. The author suggested that different variables (for example hormonal markers) could add more value to allow a more individualized assessment (Hansen *et al.*, 2008).

Wallace *et al.* published a different mathematical model of the decline in the numbers of non-growing follicles from conception until menopause. According to his calculations based on data retrieved from histological studies, the vast majority of women, by the time of their 30th birthdays, have already lost 88% of their pre-birth pool of follicles. A decade later only 3% of the stock remains (Wallace WH, 2010).

Many published studies describe genetic effects upon age at natural menopause. One large, prospective cohort study of the heritability of age at natural menopause, has shown that at least 50% of the individual factors accounting for age at menopause are genetic. There is a correlation between ages of menopause amongst daughters and mothers as well as twin sisters (Murabito *et al.*, 2005).

Other factors and its impact on menopausal age have been considered. A recently published meta-analysis of 11 studies has shown that smoking is a strong independent factor for the early age of natural menopause (Sun *et al.*, 2012, Nelson *et al.*, 2013). Overall, lifestyle factors have been estimated to have a relatively small contribution to variation in menopausal age. Apart from smoking, other factors: age at menarche, parity, regularity of menstrual cycles, use of contraception, breastfeeding, alcohol and coffee consumption, diet, body mass index, and socio-economic status are debatable in terms of their effects on menopausal age (Kok *et al*., 2005; van Noord *et al.,* 1997).

Possible underlying mechanism of ovarian aging has been widely described. Recent reports highlight an increasing incidence of DNA (Deoxyribonucleic acid) double-strand breaks in primordial follicles in mouse and human causing accelerated loss of the follicles related to age and presenting a marked increase from age of 36 (Couzin-Frankel,2013; Johnson & Keefe, 2013; Titus *et al.*, 2013). In parallel, declining expression of DNA repair genes such as BRCA1, MRE11, Rad51 and ATM was observed. Overall, the efficiency of mechanisms to repair damaged DNA was found to decrease with age that negatively affected ovarian reserve and the functionality of the remaining follicles.

In women with an impaired capacity to repair damaged DNA due to a faulty BRCA1 gene, the decline in ovarian reserve may become evident even at a younger age (Titus *et al.*, 2013).

1.2.2 Oocyte quality with female age

It is known that not only the quantity but also the quality of oocytes declines with age. Statistical analysis shows that women of older age are far more likely to have a miscarriage, which is believed to be related to the higher incidence of chromosomal abnormalities in embryos (Cowchock *et al.*, 1993).

Factors responsible for declining quality and the higher chances of chromosomal abnormalities in oocytes with age are still not fully understood One of the theories is that impaired mitochondrial function plays a significant role (Bentov *et al.*, 2011). Further evidence of impaired oocyte function in older women comes from clinical experience where older women achieve pregnancy and live birth rates similar to that of young women if they receive donated eggs from a young donor (Nelson, 2011; Nelson *et al.*, 2013).

1.2.3 Uterine function and female age

Although important, the quality and quantity of oocytes is not the only fertility issue in older women. There is a substantial body of evidence, mainly from animal studies or observational studies in humans that uterine decidua and placental development may become impaired with maternal age. In older women using oocytes donated by younger women, despite good pregnancy rates, the incidences of premature delivery and low birth weight in infants are higher (Nelson, 2011; Nelson & Lawlor, 2011). National data show that older women have a much higher frequency of Caesarean sections, even after adjusting for confounding factors such as gestational diabetes. Myometrium obtained at planned Caesarean section had lower contractility in older women, which may be one of the reasons for a higher rate of poor progress in labour and an increased need for surgical intervention in this age group

(I. Demeestere, 2012; Smith *et al.*, 2008).

The currently available evidence supports the idea that women are born with a fixed number of oocytes that declines throughout reproductive life. There is significant variation in the rate of follicle loss among individuals, which cannot be explained by lifestyle factors. None of the mathematical models can be reliably used for counselling of individuals about their fertility prospects based on age alone. Therefore, assessment of the ovarian reserve at a given time in a woman's life could offer a more accurate estimate of the remaining fertile years for those concerned about their present and future fertility (Coxworth & Hawkes, 2010).

1.3 Ovarian reserve

Ovarian reserve describes the estimated number of oocytes in a woman's ovaries. As described earlier, at any given time, the majority of oocytes are enclosed in primordial or so-called 'resting' follicles while some are in the process of growing. A small proportion of primordial follicles is recruited to grow each day. The majority of growing follicles eventually become atretic, some will continue to grow and become responsive to follicle-stimulating hormone (FSH). The pool of resting follicles, is impossible to measure directly without using invasive methods, however, it is linked to the pool of small, growing follicles, of which estimation can be made more readily (McGee & Hsueh, 2000; Wallace WH, 2010). Indirect measures of the ovarian reserve are used as 'ovarian reserve tests'.

Several such tests are available to assess ovarian reserve including measurement of hormones in blood, such as FSH, luteinizing hormone (LH), inhibin B, estradiol (E2), AMH, antral follicle count (AFC) using transvaginal ultrasound, and dynamic tests (Broekmans *et al.*, 2006) but the perfect marker has not yet been identified. Ideally, an ovarian reserve test (ORT) should be informative of not only the quantity but also the quality of oocytes. The menstrual cycle interplay of hormones used for assessment of ovarian reserve is described below.

1.3.1 Physiology of hypothalamo-pituitary-ovarian axis

Early stages of follicular development are independent of gonadotropins – FSH and LH – however gonadotropin sensitivity develops with growth. In natural menstrual cycles, each month, a small cohort of antral follicles becomes sensitive to FSH, from which one follicle (occasionally two) becomes more responsive to FSH and LH and continues to grow. The outer layer of the follicle (theca cells) has receptors for LH and produces enzymes necessary for synthesis of androgens, while the inner layer (granulosa cells) has receptors for FSH and produces aromatase needed for conversion of androgens to estrogens. Thus, working together, the two cell types produce estrogens from androgen, via the 'two cells, two gonadotropins' model of steroidogenesis.

The rise in blood levels of FSH during the early follicular phase is a response to increased hypothalamic gonadotropin-releasing hormone (GnRH) output resulting from lowering estrogen and progesterone levels. While the recruited antral follicle grows and secrete estradiol (E2), E2 exerts an adverse feedback on the pituitary gland and hypothalamus, reducing FSH production and preventing recruitment of more antral follicles. The selected follicle continues to develop until >20-25mm diameter, when an LH surge is triggered from the pituitary gland, which causes maturation of the oocyte from the 'immature' stage of female meiosis (Prophase I) to the 'mature' stage (Metaphase 2 arrest). The mechanism of triggering the LH surge is complex and involves increasing levels

of E2, associated with an increased pituitary responsiveness and sensitivity. After the release of the oocyte, progesterone produced from the corpus luteum suppresses secretion of FSH. If pregnancy occurs, human chorionic gonadotropin from the trophoblast continues to support progesterone production. If not, after 12-14 days, the progesterone level drops significantly resulting in menstruation.

Superimposed upon the gonadotropin cycling, additional actions of inhibin B and inhibin A are also important. Inhibin B plays a role in controlling FSH secretion while inhibin A may have an impact on the LH surge and maintenance of the corpus luteum. They are known for their paracrine regulation of theca cells and androgen production in the ovary, while granulosa cells are affected by the structurally related activins (Shaw *et al.*, 2003, Luesley & Baker, 2004).

1.4 Overview of ovarian reserve tests (ORTs)

1.4.1 Follicle stimulating hormone (FSH)

The basal level of FSH in serum, measured between day two and five of the menstrual cycle (where day 1 is the first day of menstruation), is the most widely used test of ovarian reserve in fertility clinics worldwide. The pituitary secretion of FSH varies substantially throughout the cycle and is under estradiol and inhibin feedback, so correct timing of the blood sample is important. FSH in the early menstrual cycle provides an indication of the tonic level of gonadotropin stimulation needed to drive the menstrual cycle. As a woman ages, and/or as her ovarian reserve declines, FSH increases early in the cycle because the pituitary gland has to produce more FSH in order to generate an estrogen response from the waning ovarian follicle population.

Among the profound limitations of serum basal FSH as an ovarian reserve test are intra- and inter-cycle variations and a lack of clear cut off points for meaningful clinical interpretation (Bancsi *et al.*, 2003; Johnson *et al.*, 2006). Concentrations and threshold values of FSH can vary significantly between laboratories, depending on assay methodology (Scott & Hofmann, 1995). In addition, the rise in basal FSH is a relatively late indicator of decreasing follicle numbers, so by the time that an increased FSH level is noted, the oocyte number is already in decline (de Vet *et al.*, 2002).

A meta-analysis on basal FSH as a prognostic marker of IVF (in vitro fertilisation) outcome was performed on 21 studies (Bancsi *et al.*, 2003). The predictive ability of basal serum FSH for inadequate response to ovarian stimulation was found to be moderate while for non-pregnancy even less useful. The clinical application of FSH testing prior to an IVF cycle was only proved for a minority of patients, who had very high basal FSH concentrations. The burdens of this analysis were a lack of clear, uniform criteria for poor response and cycle cancellation in the evaluated studies. Furthermore, in the conducted analysis, data on clinical and ongoing pregnancies were not considered separately.

A subsequent meta-analysis on FSH versus AFC (AFC is discussed further in section 1.4.5) in predicting IVF outcome included 32 studies on FSH. The ability of FSH measurements to predict response to ovarian stimulation was found to be inferior to AFC. Estimated summary ROC curves for the prediction of non-pregnancy shown a poor performance for both markers (Hendriks *et al.*, 2005).

A more recent systematic review of ovarian reserve tests identified 37 studies of basal FSH that were suitable for analysis. As previously, the authors stressed the wide range of methods used in the studies as well as lack of standard definitions. For basal FSH, accuracy for the prediction of inadequate response to ovarian stimulation and non-pregnancy was evident at relatively high FSH levels that would apply only to a small group of women with normal regular

cycles. Considering its high false positive rate, basal FSH would not be considered the best marker of ovarian reserve (Broekmans *et al.*, 2006).

In the general population, basal FSH was not related to pregnancy outcomes. For women aged >30, without a history of subfertility, day 3 FSH concentrations does not correlate with the risk of miscarriage (van Montfrans *et al.*, 2004).

1.4.2 Basal estradiol (E2)

Estradiol is a steroid hormone produced by granulosa cells by aromatization of androstenedione from theca cells and conversion from estrone to estradiol. Smaller amounts of precursors are also produced by the adrenal cortex and fat cells (Shaw *et al.*, 2003, Luesley & Baker, 2004). Estradiol is involved in the feedback between the growing follicle and the pituitary. High concentrations of estradiol early in the menstrual cycle reflect the advanced follicular development and early selection of the dominant follicle. As the ovary ages, day 2-5 estradiol is frequently seen to increase transiently before levels drop at the menopause. In a younger woman, raised estradiol in the early follicular phase can indicate the presence of a functional ovarian cyst.

Several publications have shown a correlation between high basal estradiol concentrations and cycle cancellation or a poor response to ovarian stimulation (Licciardi *et al.*, 1995, Evers *et al.*, 1998;). In these studies, despite normal basal FSH, high basal estradiol predicted a high cancellation rate and a low oocyte

yield. Patients fared best if they had both normal basal FSH and low estradiol concentrations on cycle day three. The authors concluded that serum measurements of day three estradiol could be useful in patients with normal FSH level. Another research group confirmed this finding but only for women above age 35 (Vázquez *et al.*, 1998). In a study by Frattarelli et al. (2000) not only very high, but also very low (<20 pg/ml) concentrations of basal estradiol were predictive of cycle cancellation (Frattarelli *et al.*, 2000), however, others did not find similar results (Phophong *et al.*, 2000). Those studies in favour of basal estradiol as a predictor of the ovarian response to stimulation, even so, found no evidence of a relationship between pregnancy rate and basal estradiol concentrations (Frattarelli *et al.*, 2000).

The clinical usefulness of basal estradiol in predicting the ovarian reserve and IVF outcome was not supported in a systematic review (Broekmans *et al.*, 2006). Basal estradiol concentration was found to be a poor predictor of ovarian response due to very low predictive accuracy and lack of clear threshold levels. The same shortcomings were noted for prediction of non-pregnancy (Broekmans *et al.*, 2006). Therefore, based on current evidence, early follicular phase estradiol cannot be used as a reliable predictor of response to IVF stimulation.

1.4.3 Inhibins

Inhibins are dimeric glycoproteins, consisting of α-βA (inhibin A) and α-βB (inhibin B) subunits, known for their ability to suppress FSH secretion (Lockwood, 2004). Inhibin A is mostly produced by the corpus luteum with the highest serum levels detectable in the luteal phase of the menstrual cycle. Inhibin B is secreted by small, pre-ovulatory follicles. Its concentrations start rising in the early follicular phase. Inhibin B peak is corresponding with FSH's decline. It then falls progressively during the rest of the follicular phase and rises again after the mid-cycle LH surge, remaining low for the remainder of the luteal phase (Groome *et al.*, 1996). The observed changes in the serum concentration throughout the cycle and initial studies on patients with polycystic ovarian syndrome showing elevated inhibin B levels (Anderson *et al.*, 1998) indicated that inhibin B might be a suitable marker of ovarian function and follicular reserve for a subfertile population having IVF treatment. Moreover, some studies suggested that serum inhibin B concentrations correspond with the number of small antral follicles on ultrasound scan, possibly allowing an indirect measurement of the size of follicle pool (Tinkanen *et al.*, 2001).

In a prospective comparison study on ovarian reserve tests including AFC, basal FSH and inhibin B, the authors found inhibin B to have a lower predictive value than ultrasound assessment of the number of antral follicles. However, it

was suggested that inhibin B measurements could potentially have some role as an additional test (Bancsi *et al.*, 2002), alongside ultrasound assessment.

Potential applications of measuring inhibin in peri-menopausal patients with normal FSH were identified in a few studies. These observed that women with diminished ovarian reserve have low inhibin B levels before any rise in FSH suggested the menopausal transition. Seifer et al. (1999) in a case control study, assessed 109 women with a non-ovarian cause of subfertility and 47 women with known low ovarian reserve. Despite normal basal FSH, patients with low serum concentrations of inhibin B, had a smaller number of oocytes retrieved in IVF cycle (Seifer *et al.*, 1999). A systematic review of ovarian reserve tests revealed the poor predictive performance of inhibin B in relation to response to ovarian stimulation and chances for pregnancy (Broekmans *et al.*, 2006).

Most authors of the analysed studies discussed the fact that the test for inhibin B is technically challenging and not readily available. A lack of international standardisation has resulted in contradictory data on inhibin B. A further disadvantage of the inhibin B test is its strict cycle-dependency, leading to a lack of time-flexibility that is challenging for any diagnostic test.

1.4.4 Anti-Müllerian Hormone (AMH)

Recently, AMH measurement in blood, as a novel method of assessing the ovarian reserve, has rapidly become popular amongst fertility specialists. AMH, also known as Müllerian-inhibiting substance (MIS), is a dimeric glycoprotein, similar in structure to inhibin and activin, from the transforming growth factor-β (TGF-β) family (Pepinsky *et al.*, 1988). In humans, the gene for AMH is on chromosome 19p13 (Cohenhaguenauer *et al.*, 1987) while that for its receptor is on chromosome 12 (Josso *et al.*, 2003).

The ovaries are the only source of AMH in women (La Marca *et al.*, 2005a). The AMH concentration in serum correlates with the number of antral follicles on ultrasound scan (Mendez Lozano *et al.*, 2006) and the woman's age (de Vet *et al.*, 2002). The first, direct confirmation of the association between serum Anti-Müllerian hormone concentrations and the number of remaining primordial follicles was demonstrated in mice (Kevenaar *et al.*, 2006). The results clearly indicated that the serum concentration reflected the size of the follicle pool, confirming the value of AMH as a test of ovarian reserve in mice. Recently, one study confirmed the same relationship between AMH and the size of follicle pool in women. Serum AMH concentrations, prior to oophorectomy for benign causes, were correlated with the histological findings after surgery (Hansen *et al.*, 2011).

Anti-Müllerian hormone is produced by granulosa cells of the ovarian follicles with expression initiated in small growing primary follicles and declining in the antral stages as follicles gain FSH dependence or become atretic (Baarends *et al.*, 1995; Shaw *et al.*, 2003, Durlinger *et al.*, 2002; Weenen *et al.*, 2004). Using immunohistochemistry, AMH can be detected most strongly in pre-antral and small antral follicles, while some weak signals have been observed in primary follicles while none was detected in primordial follicles (Weenen *et al.*, 2004).

AMH plays a role in both folliculogenesis and steroidogenesis. It is believed to act as a feedback mechanism that inhibits the transition from resting primordial follicles into growing follicles (Durlinger *et al.*, 1999). MIS plays a role in the recruitment of FSH-sensitive follicles in the antral stage and reducing follicle sensitivity to FSH (Durlinger *et al.*, 2001; Hirobe *et al.*, 1994; Ueno *et al.*, 1989). Nevertheless, in human ovaries, the auto- and paracrine actions of AMH are still not fully understood and further studies are needed to determine its mechanism of action.

The AMH signalling pathway is typical for the TGF family. In females, AMH receptors are present in ovaries, endometrium and breast tissue (Josso *et al.*, 2003). In general, AMH action has a repressing effect on the targeted genes. AMHR-II (AMH receptor) binds with AMH ligand before forming a complex with type I receptor (ALK 2, 3 and 6). The phosphorylated and activated complex binds to cytoplasmic effectors, (Smad 1, 5, 8). Cofactors, coactivators and

corepressors for AMH transduction and its relationship with other hormonal pathways still needs further study in humans (Josso *et al.*, 2005).

In granulosa cells of human antral follicles, gene expression of AMH was found to be positively correlated with expression of FSH and androgen receptors. AMH's impact on follicular estrogen production has been suggested by a number of authors (Andersen & Byskov, 2006; Dewailly *et al.*, 2014). Additionally a statistically significant association was confirmed between AMH gene expression and levels of testosterone and progesterone in surrounding follicular fluid (Jeppesen *et al.*, 2013). AMH concentrations were low or undetectable in follicles undergoing atresia (Weenen *et al.*, 2004). This is suggestive of multilevel actions of AMH on follicular selection and development, but more studies are needed to explain fully the impact of AMH on follicular growth and recruitment in humans.

In clinical setting, the serum concentration of AMH seems relatively independent of the menstrual cycle (Hehenkamp *et al.*, 2006; La Marca *et al.*, 2006b; Tsepelidis *et al.*, 2007). Although some studies have reported inter- and intra-cycle fluctuations in MIS concentrations (Cook *et al.*, 2000; Streuli *et al.*, 2009). A recently published small study on 12 women aged between 29 and 43 referred to an IVF unit due to primary or secondary infertility, presented repeated measurements of their endocrine profiles throughout the menstrual cycle (Hadlow *et al.*, 2013). AMH levels were found to be statistically higher in the

mid-follicular and pre-ovulatory phase and declined in the luteal phase, which was similar to previous reports in 2008-2010. The observed changes in AMH concentrations were more prominent in younger women, while only minimal changes were observed in the older population, which the authors explained by changes related to ovarian aging (Sowers *et al.*, 2010; Wunder *et al.*, 2008a). At present, the general consensus is that cyclic fluctuations are too small to affect AMH-guided clinical decisions in the IVF setting (La Marca *et al.*, 2009) and AMH measurement at unspecified times in the menstrual cycle is clinically acceptable (Anderson *et al.*, 2012). This statement is supported by results from other studies which demonstrated the predictive value of AMH, measured on any day of the follicular or luteal phase, in terms of the response to ovarian stimulation (La Marca *et al.*, 2007; Nelson *et al.*, 2009).

Interestingly, AMH was previously found to be an accurate measure of ovarian function even amongst women with irregular menstrual cycles (La Marca *et al.*, 2006a), taking oral contraception (Somunkiran *et al.*, 2007) or undergoing pituitary down-regulation using a GnRH super-agonist for up to 8 weeks (Mohamed *et al.*, 2006), which suggested that serum AMH levels may be unaffected by gonadotrophic status. However, studies published more recently offer opposing evidence. A significant reduction in AMH concentration has been noted in breast cancer patients using GnRH super-agonists (Anderson *et al.*, 2006b). A group of researchers investigating early maturing girls during GnRH agonist therapy presented similar findings. After 3 months of treatment, they

found that levels of AMH were around 50% lower than before commencing therapy (Hagen *et al.*, 2012a). One possible interpretation is that this reflected the declining number of growing follicles during pituitary suppression.

A large Danish study of 228 users and 504 non-users of oral combined hormonal contraception showed that, after adjusting for age, AMH was 29.8% lower in users compared to non-users. It was noted that for every year of use of hormonal contraception there had been a decline in AMH level of 2.3% (Bentzen *et al.*, 2012). The recovery of ovarian function and a possible subsequent increase in AMH levels after discontinuing combined contraception has not yet been evaluated in large prospective studies. However, a small study which included 25 women using combined oral contraception reported an increase in AMH concentrations in two following cycles in women who stopped taking hormonal contraception (van den Berg *et al.*, 2010).

As mentioned earlier, AMH is produced mainly by small pre-antral and antral follicles - the latter are sensitive to gonadotropins and their number is reduced in women who have suppressed pituitary function. Pituitary suppression may affect the overall quantity of AMH produced by this population of follicles and, therefore, the serum levels of AMH. However, it remains unclear which size of follicles contributes most to the serum AMH levels. Intra-follicular levels of AMH, the population of follicles (e.g. a large group of small pre-antral follicles versus a small group of follicles selected for dominance), the quantity of granulosa cells

in a given size of follicles or its volume, it all affect serum AMH concentrations. One of the recently published studies, based on in vivo modelling, suggested that the follicles contributing the most to serum concentrations are those of size 5-8 mm (60% of overall serum AMH levels). This was concluded from measurements of intra-follicular levels of AMH and the overall quantity of granulosa cells in a range of sizes of follicles as well as overall volume, after examining ovarian tissue of 157 women aged 1 to 39, who underwent oophorectomy for fertility preservation prior to gonadotoxic treatment. Despite more intense AMH gene expression in smaller follicles <5 mm and higher intra-follicular concentrations of AMH, larger follicles (5-8 mm) were believed to have a higher overall content of AMH due to more granulosa cells and a greater volume. The smaller follicles accounted for only 25% of the serum AMH concentration (Jeppesen *et al.*, 2013).

To conclude, the impact of gonadotropins on serum AMH levels is possible. Serum AMH measurements obtained under pituitary suppression would reflect the size of the pre-antral follicle pool which is considered to be a more accurate reflection of the true ovarian reserve. However, calculation of reference ranges of AMH was performed without any additional hormonal suppression. AMH as a marker of the ovarian reserve would be still useful if obtained on hormonal contraception or while taking GnRH analogues, but the results require interpretation in that context.

Measurements of FSH and inhibin B are strictly cycle dependent and need to be done on a specific day of the menstrual cycle, usually between day 2 and 5. The ability to assess ovarian reserve at any time of the menstrual cycle and in non-menstruating women makes AMH testing more convenient compared with other ovarian reserve tests. This particular characteristic could have clinical value in women having irregular cycles due to gonadotoxic treatment or severe illness (Anderson *et al.*, 2012).

1.4.4.1 Serum Anti-Müllerian hormone (AMH) and its accuracy in predicting responses to fertility treatment

One valuable clinical application of ovarian reserve testing is its ability to predict ovarian response to stimulation with gonadotropic drugs.

Seifer et al. (2002) published one of the first studies that demonstrated the relationship between serum AMH concentrations and response to ovarian stimulation (Seifer *et al.*, 2002). A number of authors have shown that AMH has greater association with the number of collected oocytes than day three FSH, Inhibin B or estradiol in all age groups (Shaw *et al.*, 2003, Fiçiciog *et al.*, 2006; Riggs *et al.*, 2008). A statistically significant difference between serum AMH levels in ‘poor’ compared to ‘good’ responders was found by others (Tremellen *et al.*, 2005) confirming AMH’s value as a quantitative marker of ovarian function.

AMH measured at non-selected time of the menstrual cycle was found to be predictive of the ovarian response, numbers of oocytes retrieved and dose of FSH required for ovarian stimulation (La Marca *et al.*, 2007, Elgindy *et al.*, 2008). Similarly, Nelson et al (2009), in a prospective cohort study of 538 patients, measured AMH on an unspecified day of the menstrual cycle, showing the relationship between serum AMH concentrations and response to ovarian stimulation. This study confirmed the clinical usefulness of AMH in IVF settings (Nelson *et al.*, 2009).

1.4.4.2 AMH as a predictor of hyperstimulation, poor response and cycle cancellation

AMH measurements were also found to be useful in predicting the extremes of ovarian response to gonadotropin stimulation. McIlveen *et al.,* (2007) found that low serum AMH concentrations were associated with poor response to ovarian stimulation and a higher risk of cycle cancellation. (McIlveen *et al.*, 2007).

In another study, AMH was superior to FSH in predicting poor response to ovarian stimulation (Nardo *et al.*, 2009). Other researchers suggested that AMH levels $\leq$1.26 ng/ml were highly predictive of poor ovarian response, but confirmation of findings by AFC improved the reliability of ovarian reserve testing (McIlveen *et al.*, 2007, Gnoth *et al.*, 2008).

AMH measurements may be helpful in predicting hyperstimulation syndrome which is a potentially life-threatening condition resulting from use of exogenous gonadotropins (Tremellen *et al.*, 2005; Nakhuda *et al.*, 2007; Lee *et al.*, 2008, Salmassi *et al.,* 2015).

In one of the studies, serum AMH concentrations were as effective as estradiol concentration on the day of human chorionic gonadotropin (HCG) administration in identifying women who would develop ovarian hyperstimulation syndrome (Lee *et al.*, 2008).

Later, a meta-analysis including nine studies on AMH and five studies on AFC showed that both tests had good predictive value for excessive response to stimulation in IVF cycles. AMH was shown to have 82% sensitivity and 76% specificity and shared the same clinical value as AFC. The authors were able to demonstrate that at a certain cut off points, the probability of an excessive response was 70% (Broer *et al.*, 2011a).

The following meta-analysis tested the prediction of excessive response to ovarian stimulation if individual patient data, such as age, duration of infertility, or body mass index (BMI) were included. It showed that the predictive performance of AMH was not altered by patients' age, BMI or duration of infertility. The accuracy of AMH remained good in all subgroups. Moreover, the authors concluded that a combination of AFC and AMH had high predictive accuracy, even without taking age into consideration (Broer *et al.*, 2013).

Although, this analysis was performed on old data, acquired before 2009 when two different assays have been in clinical use. Different threshold levels of AMH have been reported by several authors, resulting in conflicting evidence. Problems identified have included differences in the assays applied and methods used for identifying thresholds, as well as different units of measurement. All this has led to the application of many different cut off points for AMH predictive testing (Nelson & La Marca, 2011). Newer studies have confirmed that AMH is a good predictor of hyper-response in normo-ovulatory women undergoing stimulation with clomiphene citrate and gonadotropin, but not in women with PCOS (polycystic ovary syndrome) (Kim *et al.*, 2013).

1.4.4.3 AMH as a predictor of pregnancy and non-pregnancy

A number of studies have suggested a predictive value of serum AMH concentrations for occurrence of pregnancy (Hazout *et al.*, 2004; Nelson *et al.*, 2007; Elgindy *et al.*, 2008; Wunder *et al.*, 2008b, Iliodromiti *et al.,* 2014) and live birth (Nelson *et al.*, 2007) while others have found contrasting results (Fiçiciog *et al.*, 2006; McIlveen *et al.*, 2007, Gnoth *et al.*, 2008).

A meta-analysis of 13 studies demonstrated the poor predictive ability of serum AMH measurements in predicting the chances of pregnancy in IVF cycle (Broer *et al.*, 2009). It is likely that the positive association found by some researchers

is a consequence of the cycle performance and oocyte yield per se rather than a direct relationship with oocyte or embryo quality.

A research group from Sweden recently explored this point in a prospective longitudinal study including 892 patients undergoing intracytoplasmic sperm injection (ICSI). They found that pre-treatment AMH levels were associated with embryo scores, pregnancy and live birth rates, even after adjusting for age and oocyte yield (Brodin *et al.*, 2013). However, another group published opposing results on the predictive ability of AMH measurements in assessing the chances of pregnancy in the young, healthy population (Hagen *et al.*, 2012b). This research group prospectively assessed 186 couples (women aged 19-35) and followed them up until pregnancy or till 6 months. It was found that AMH levels were not predictive of achieving natural pregnancy in young women with no previous history of infertility (Hagen *et al.*, 2012b).

Another group led by Olemdo et al. (2013) investigated AMH levels in 145 women undergoing ICSI. After adjusting for confounding factors, they found that AMH < 3 pmol/L was associated with chances for pregnancy of around 31%, while AMH > 15 pmol/L was associated with chances of up to 35% (Brugo Olmedo *et al.*, 2013). Overall, patients with lower AMH concentrations had fewer follicles, lower fertilization rates and generated fewer embryos (Lekamge *et al.*, 2007).

In summary, serum AMH measurement appears to indicate the size of the ovarian follicle pool and as such is a useful marker of ovarian reserve. Its ability to predict pregnancy seems to be a reflection of the quantitative measurement of oocyte numbers and its predictive accuracy is poor (Iliodromiti *et al.,* 2014). It remains debatable whether the quality of oocytes or live birth could be predicted by a single serum AMH measurement because the achievement of pregnancy and live birth is always multi-factorial.

1.4.4.4 Anti-Müllerian Hormone (AMH) with age

Unlike other markers, AMH was found to be reliable in the assessment of ovarian aging. A considerable literature has been published on the relationship between AMH and ovarian ageing (Lee *et al.*, 1996; van Rooij *et al.*, 2004, Broer *et al.*, 2011; Hagen *et al.*, 2010, Nelson & La Marca, 2011, Freeman *et al.*, 2012, Kelsey *et al.*, 2012,Tehrani *et al.*, 2013, Nair *et al.,* 2015, Dolleman *et al.,* 2015). The results indicated that serum AMH levels decline throughout reproductive life, even in young women (age 20-35) with normo-ovulatory cycles. This is in contrast to FSH, E2 or inhibin B, which remain relatively static in young women (de Vet *et al.*, 2002).

Initially, small studies showed a linear decline of AMH with age (Sowers *et al.*, 2008), however, a non-linear decline was demonstrated by others (de Vet *et al.*, 2002). More recently validated mathematical models have provided a

normogram of AMH with age (Nelson & La Marca, 2011). Nelson et al., have suggested that the best model to describe AMH's decline with age is a quadratic equation. Their dataset was derived from around 4500 subfertile women (Nelson & La Marca, 2011).

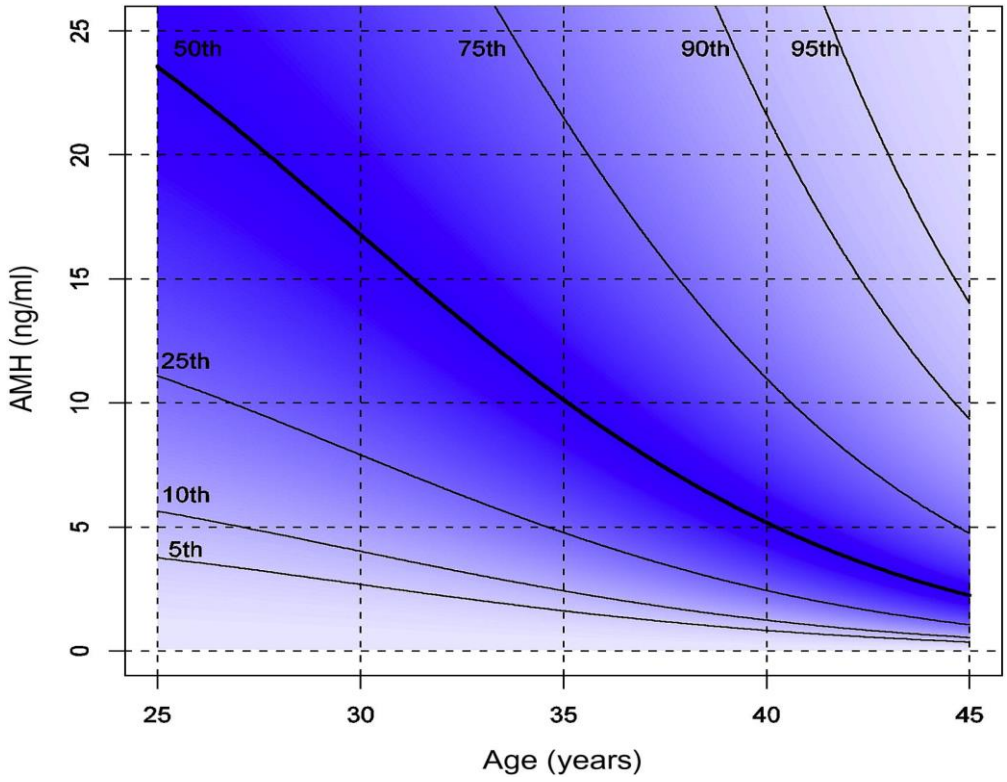


Figure 2 Normogram of AMH with age (from Nelson & La Marca*.*, 2011).

A large cross sectional study of 926 healthy volunteers between the ages of 0 and 69 determined age-related changes in AMH levels. The peak of serum AMH concentration was in the early 20s. From the age of 25, the decline in AMH was

observed with levels of AMH being undetectable at an age of 46.7 (Hagen *et al.*, 2010).

The mathematical model from combined histological data examined the relationship between serum AMH concentrations and changes in the primordial follicle pool. The falling concentration of AMH in adult women was consistent with the progressive loss of primordial follicles (Kelsey *et al.*, 2012).

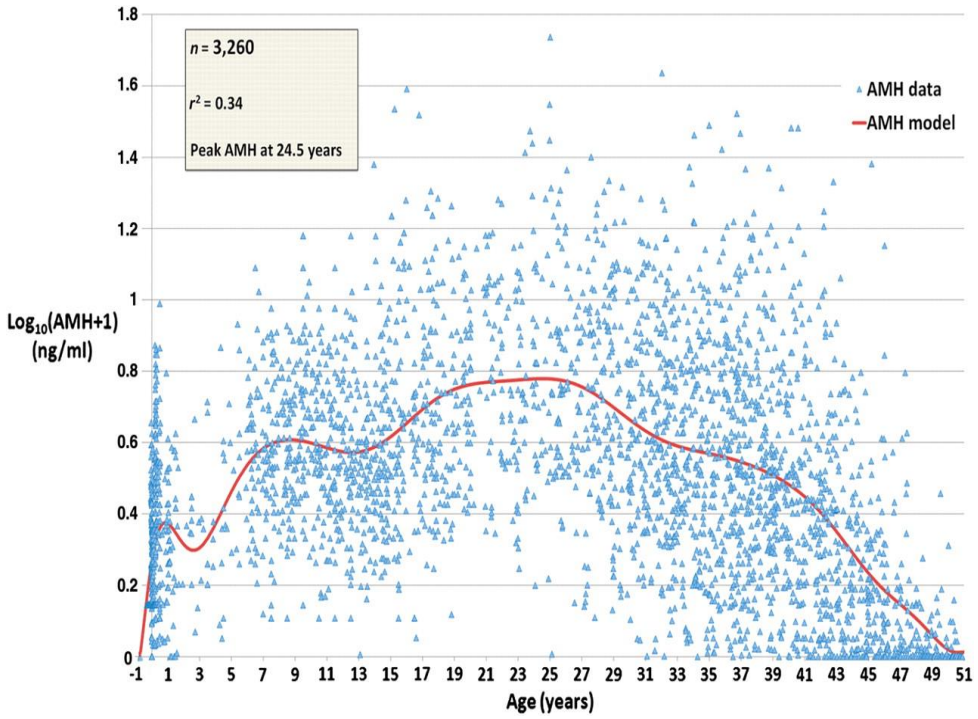


Figure 3 Concentrations of serum AMH in women at different ages (from Kesley *et al.,* 2012).

AMH levels start increasing from birth, with a quite distinctive rise in the first few months of life, then continue to increase until reaching a peak in the mid-20s. Following that, there is a steady decline in AMH concentrations until becoming undetectable around five years before menopause (Hagen *et al.*, 2010). A slight decrease in AMH concentration, observed around puberty, coincides with the time of maximal follicular recruitment (Nelson *et al.*, 2013).

Relative to other endocrine markers, a decrease in serum AMH concentration is observed much earlier on the approach to menopause (Lee *et al.*, 1996; van Rooij *et al.*, 2004, Broer *et al.*, 2011;). This is helpful in clinical applications since other markers remain relatively static until close to the menopause.

A growing body of evidence suggests the usefulness of AMH as a predictor of menopause. A prospective longitudinal study of 257 women aged 21-46, followed up for 11 years, and has shown that AMH is highly predictive of age at menopause. This study was able to provide an age-specific AMH normogram for prediction of age at menopause. In contrast, in another study, the use of AFC and FSH failed to provide a better correlation than age alone (Broer *et al.*, 2011). Another prospective longitudinal study followed up 401 women in their late reproductive years for 14 years. It confirmed that AMH was a good predictor of time to menopause if measured in women at an age of around 40 (Freeman *et al.*, 2012). Another large study on 1015 women with follow-up for at most 10

years presented a prediction model for age at menopause that was accurate in 92% of women (Tehrani *et al.*, 2013).

1.4.4.5 AMH testing in clinical practice

The first documented development of an AMH assay was by Hudson et al.(1990) followed by Long et al. (2000) and Al-Qahtani et al. (2005a) until the currently used monoclonal antibodies 12H and 7A were patented by Groome et al. (2006).

The first assays showed variability in AMH levels depending on the storage and freeze-thaw characteristics of the tested samples. The DSL Active AMH assay and generation II assay were greatly improved versions mainly because they used a pair of monoclonal antibodies directed to epitopes in the mature region of the AMH molecule. The 12H antibody recognizes the mature region of the AMH molecule directly while the 7A antibody initially binds to a pro-region, but after a longer period of observation, then binds to the mature region. The mature region of the AMH molecule is more stable against proteolysis than the pro-region. Previous antibodies were directed to epitopes in the pro-region, which may have explained the discrepancy in AMH results related to AMH proteolysis in blood samples exposed to prolonged transport and different storage conditions (Kumar *et al.*, 2010).

Before the second generation assay, the two available assays (DSL and Immunotech) used different antibodies and different standards, creating uncertainty about cut off points and reference ranges for AMH results. Several authors tried to find correlations between the two assays, suggesting initially 4.6-fold lower results with DSL (Fréour *et al.*, 2007). More recently others confirmed the reliability of both assays and similar reference values (Streuli *et al.*, 2009, Lee *et al.*, 2011;). Additionally different reporting of results either in ng/ml or pmol/L has added to the lack of uniformity in reference ranges for these commercially available assays.

The second generation assay available from 2010 is standardised against the Immunotech calibration material, traceable to an independent reference material in Paris. Calibrators are prepared using a bovine serum (Kumar *et al.*, 2010). An international standard is yet to be established by the National Institute for Biological Standards and Control (Nelson & La Marca, 2011). Second generation assay calibrators and controls are provided separately from the AMH ELISA plate and should be stored at -20 °C (as opposed to 2-8 °C for DSL Active AMH ELISA) (Kumar *et al.*, 2010).

The methodology of the first DSL assay carried a risk of interference by heterophile antibodies in the serum samples, which was corrected by the use of filters in second generation assay (Kumar *et al.*, 2010). This has practical importance in patients with cancers treated with monoclonal antibodies (such

as Herceptin and Avastin) or using animal serum-based medications where AMH results could have been potentially affected by an 'HAMA response' (allergic reaction to mouse antibodies). In such situations, AMH results should be read with caution.

The lack of manufacturer's recommendations for transferring and handling samples from distant laboratories prompted me to perform a pilot study prior to deciding on the final protocol. Based on those results, I instructed research nurses and the laboratory team on sample handling and the need for strict adherence to the study protocol. Any samples received in MFS Laboratory more than 48 hours from venepuncture have been not included in the analysis and repeat blood samples were obtained where possible.

A recently published pioneer study by Fleming et al*.,* (2013) indicated that prolonged storage of whole blood at room temperature may increase the AMH measurement due to a direct effect of blood cells. His results indicated that storage of whole blood at room temperature up to 44 hours from venepuncture may lead to an increase in measured AMH levels by 12% which is within the normal variation of the assay and is considered to be acceptable. However, the authors strongly recommended the separation of serum from whole blood within 24 hours of venepuncture (Fleming *et al.*, 2013a). Despite this, there remains today no universally agreed protocol for sample handling between the patient and the AMH assay laboratory. Given the challenges of the assay itself, it is

generally run commercially in a few specialised laboratories and transport of samples is, therefore, a routine necessity.

Interestingly, a recently published study reporting results from 10 laboratories in an external assurance scheme using the second generation II assay showed good reproducibility for each laboratory but the significant variability between laboratories. A large coefficient of reproducibility of 38.8% between Laboratories was reported. Individual laboratories had different average bias values relative to the consensus value ranging from -24.0% to +22.7%. The interpretation of these results is difficult, but it emphasises the fact that the technical aspects of performing each assay as well as the handling and storage of the kit and blood samples should be clearly documented in publications. It has been recommended that the same laboratory should be used to manage patients in an individual clinic in order to avoid confusion, especially with repeated results (Wallace *et al.*, 2011). It is evident that the AMH assay is still under development.

1.4.5 Ultrasound scan (USS) tests of ovarian reserve

The ovaries of women of reproductive age contain populations of follicles at different stages of development. These are: resting reserve of primordial follicles, growing pre-antral follicles, small antral follicles (1-5 mm) which are 'selectable' and larger antral follicles (5-12 mm), which are FSH dependent. The number of antral follicles, known as the antral follicle count (AFC), is believed to

reflect the pool of resting follicles in each ovary. This association has been confirmed by Gougeon et al. (1984) and repeated by others (Gougeon, 1984; Pellicer *et al.*, 1998).

Hansen et al. (2011) tested the ovarian reserve using three-dimensional ultrasonography prior to oophorectomy to ascertain the population of follicles ready to be recruited during the early follicular phase of the menstrual cycle and confirmed the same on histological slices post-surgery (Hansen *et al.*, 2011).

1.4.6 Antral follicle count

The method by which antral follicles are counted in the early follicular phase varies among studies. Some measure the number of follicles of diameter 2-10 mm (Chang *et al.*, 1998; Scheffer *et al.*, 2003), others 2-5 mm (Jarvela *et al.*, 2003) or 2-6 mm (Haadsma *et al.*, 2007) using two- (Scheffer *et al.*, 2003) or three-dimensional ultrasound (Jayaprakasan *et al.*, 2007). The diversity of methods used, inter-cycle and inter-observer differences might have an impact on the performance of AFC as a diagnostic test.

So far the use of 2D or 3D ultrasound does not seem to have a significant impact on AFC when used in predicting IVF outcomes (Jayaprakasan *et al.*, 2007). Another method called Sono-Automatic Volume Calculation (Sono AVC) provides automated measurements and has given promising initial results (Sherbahn & Deutch, 2009).

There have been attempts to detect early signs of ovarian aging using basal FSH, inhibin B, estradiol and ovarian volume compared with AFC. In the study by Scheffer et al. (2003) the number of 2–10 mm antral follicles appeared to have better correlation with age than other markers of ovarian reserve (Scheffer *et al.*, 2003).

The performance of AFC as an ovarian reserve test has been assessed in a number of meta-analyses. In one meta-analysis comparing AFC and FSH, the superiority of the counting of antral follicles over basal gonadotropin measurements was clearly demonstrated (Hendriks *et al.*, 2005). A further comparative meta-analysis of AFC and ovarian volume also showed superior performance of AFC in predicting poor response compared to ovarian volume. However, for the prediction of cases of non-pregnancy, both tests were inadequate (Hendriks *et al.*, 2007).

A systematic review of ovarian reserve tests classified AFC as a reliable predictor of response to ovarian stimulation (at low threshold levels), while its prediction of non-pregnancy was very poor (Broekmans *et al.*, 2006). AFC was shown to be an equally good predictor of hyper-response to ovarian stimulation as AMH (Broer *et al.*, 2011a).

In the systematic review of ultrasound tests of ovarian reserve, it was concluded that AFC, using a cut-off point of fewer than four, had high specificity in predicting non-pregnancy but low sensitivity. It would lead to a high rate of false

negative results if used routinely in clinical practice. In the same study, a meta-analysis of AFC revealed that women with AFC of four or more had only a 2.5% risk of cycle cancellation. The authors concluded that it might be a good test to identify those patients with a low risk of inadequate response (Gibreel *et al.*, 2009).

1.4.6.1 Ovarian volume

Two of the first systematic reviews on ovarian reserve tests, found that ovarian volume had poor clinical value as a routine marker of ovarian reserve for both poor response to stimulation and non-pregnancy in an IVF cycle (Broekmans *et al.*, 2006; Hendriks *et al.*, 2007).

However, a more recent analysis of ovarian volume (assessed using 2D USS) showed high specificity in predicting non-pregnancy and cycle cancelation at a very low volume of $3ml^3$ (Maheshwari *et al.*, 2009). The authors discussed issues of reproducibility of the test, including inter-observer and inter-cycle variability, which seemed to be a greater problem than for AFC (Jayaprakasan *et al.*, 2008). There was insufficient data available to assess outcomes such as pregnancy and live birth.

1.4.6.2 Ovarian stromal blood flow

In a meta-analysis of measurement of ovarian stromal blood flow using transvaginal Doppler ultrasound, six relevant studies were identified. The analysis of three selected studies with same cut off values of 3 cm^3 indicated promising results with a relatively high specificity for the cancellation of the treatment cycle and non-pregnancy in the prediction of IVF outcome. However, this analysis needed a larger number of patients and live birth rate as an outcome measure in order to have clinical meaning (Maheshwari *et al.*, 2009).

1.5 Cancer in young women- selection of patients

1.5.1 Introduction

The treatment of cancer is well known to cause a range of serious side effects. Like some other tissues, ovaries, seem to be particularly sensitive and readily damaged by certain treatment regimens, causing loss of ovarian reserve. Persistent amenorrhoea is an obvious indicator of an effect upon ovarian function. However, the recovery of menses does not imply that the ovary has recovered completely (Partridge *et al.*, 2010b). Occult loss of oocytes as a result of chemotherapy means that ovarian reserve testing in reproductive age women with cancer would be particularly valuable.

The extent of chemotherapy-induced follicle loss would at least partially depend on how large the ovarian reserve is before treatment begins. Ideally, testing prior to chemotherapy could give women the opportunity to discuss fertility preservation options, in light of knowledge about their own ovarian reserve (Anderson & Cameron, 2011). Secondly, depending on the type of chemotherapy and dosage of each agent, the estimated degree of damage could be discussed individually. With increasing numbers of women living longer after cancer therapy, the remaining fertility potential and risk of early menopause become more important. In the next section, I provide a brief overview of risk

factors, incidence rates and the most commonly used chemotherapy protocols in breast cancer and lymphoma sufferers.

1.5.2 Breast cancer

The most common cancer in women, breast cancer, accounts for around 30% of all cancers. The Office for National Statistics reported that 41,259 new cases were diagnosed in England in 2010, and an increase of 1.8% from 2009 was noted (ONS, 2012a). Overall survival rates have been improving with screening and more aggressive treatment. The current death rate is 24 in 100,000 and has fallen significantly over the last 40 years. For women diagnosed with breast cancer, the five-year survival estimate is around 85% (ONS, 2012a). There is an increasing number of new cases in younger women, with overall 18% of breast cancer cases being diagnosed in women aged 49 and below (NCIN, 2011). Those statistics provide evidence for the growing number of young women, who as a result of their diagnosis, will be experiencing chemotherapy-related health issues. In the USA, it is estimated that around 15% of women diagnosed with breast cancer are of reproductive age (Oktay, 2005). Assuming that a similar percentage would be applicable in the UK, around six thousand young women each year face the risk of early menopause and infertility due to breast cancer therapy.

The risk of developing breast cancer in women is age-related. According to statistics provided by NHS Cancer screening programme, the estimated risk of

developing breast cancer is ‘1 in 2,000 at age 29, 1 in 215 at age 39, 1 in 50 at age 49 and 1 in 13 at age 69 (PHE, 2013).

Early menarche, late menopause and full term pregnancy at an older age increase the risk of breast cancer, while high parity and an extended duration of breastfeeding up to 24 months have a protective effect (Cancer, 2002; Ewertz *et al.*, 1990). The use of exogenous estrogen in the form of oral contraceptives or hormone replacement therapy (HRT) is also a well-known risk for developing breast cancer. Women taking HRT (current users) have a 66% higher risk of developing breast cancer versus non-users. If HRT is stopped, within 5 years, the risk reduces to that of non-users (Beral, 2003).

Genetic and family breast cancer susceptibility have been widely investigated. It is known that carrying the gene mutation BRCA1 and BRCA2 gives a 45-65% chance of developing breast cancer, and overall 10-fold increased risk compared with normal controls (Antoniou *et al.*, 2003). However, overall the BRCA mutation accounts for only around 2% of all breast cancer cases. Other known gene mutations include TP53, PTEN, STK11/LKB1, ATM) (Turnbull & Rahman, 2008, Cancer, 2001).

Additional risk factors include high BMI, daily alcohol intake (10g of alcohol daily increases the risk by 15-20%), exposure to ionising radiation, and night shift work (most likely due to reduced levels of melatonin which has an anti-

carcinogenic effect) (Megdal *et al.*, 2005; Parkin, 2011; Parkin & Boyd, 2011; Parkin & Darby, 2011).

The most common type of breast cancer is ductal breast carcinoma (70-80% of all breast cancer cases). Invasive lobular breast cancer is diagnosed in 10-15% of all cases. Rare types of breast cancer include inflammatory breast cancer 4%, Paget's disease (1-2%). Other less common types include medullary (5%), mucinous (2%), tubular breast cancer (1%) and metaplastic breast cancer (Vogel, 2008). Important biological characteristics affecting clinical decisions are the presence of estrogen and progesterone receptors, and low or high proliferation measured by Ki-67 and HER-2. Based on those characteristics breast cancer can be then divided into:

- Triple negative (around 15% of all) with negative estrogen, progesterone, and HER-2 receptors, more frequent in the younger population.
- HER-2 positive (around 10% of all) - strongly positive HER-2 by immunochemistry/immunofluorescence testing, estrogen receptor negative, more common in the younger population.
- Luminal A (around 15% of all) - strongly positive estrogen and progesterone receptors, low proliferation, negative Her-2.
- Luminal B (around 60%) low level estrogen and progesterone receptors but high Ki-67 and HER-2 positive (Kim *et al.*, 2011).

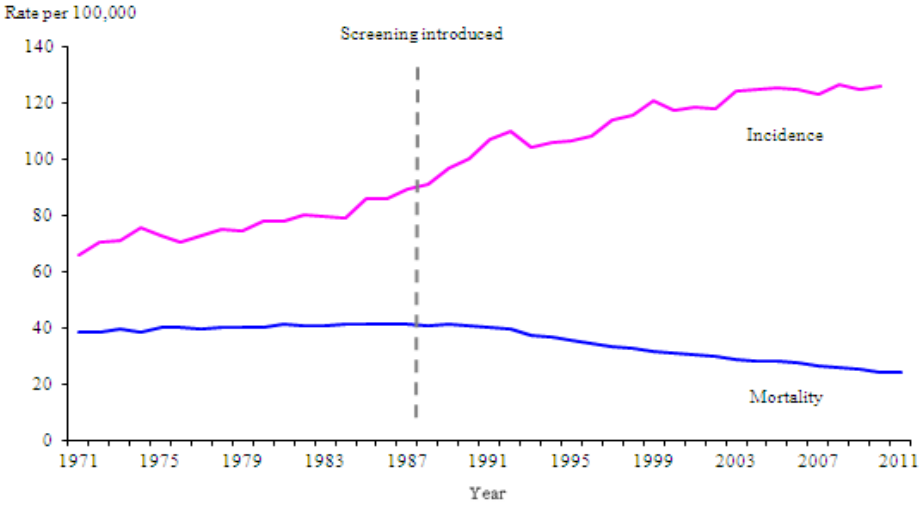


Figure 4 Incidence and mortality rates for breast cancer. UK (source Office for National Statistics)

Breast cancer treatment usually includes adjuvant therapy after primary surgery for early and locally advanced breast cancer (stage I, II and III). In case of advanced metastatic cancer endocrine therapy; single agent chemotherapy and biological therapy are most commonly chosen (NICE, 2009a). The overall decision about treatment is based on the stage of cancer its biological characteristics, hormone receptor status of tumour, patient's age, menopausal status as well as patient's choice (NICE, 2009b).

The aim of surgery is to remove the tumour from breast tissue and lymph nodes. It additionally helps to obtain more information on the tumour's biological characteristics. Adjuvant treatment includes radiotherapy, chemotherapy, endocrine therapy or therapy with monoclonal antibodies (Trastuzumab). Hormonal therapy includes treatment with aromatase inhibitors or Tamoxifen (NICE, 2006). There is emerging evidence from RCTs and meta-analysis showing that aromatase inhibitors improve clinical outcomes as compared with Tamoxifen, mainly assessed as the disease-free interval in postmenopausal breast cancer survivors (Burstein *et al.*, 2010; NICE, 2006; Xu *et al.*, 2011).

Aromatase inhibitors act by inhibiting the conversion of androgen to estrogen which reduces the levels of circulating estradiol and its negative feedback on the hypothalamic-pituitary axis. In pre-menopausal women, this can increase FSH/LH production leading to follicular growth and ovulation. Most women above the age of 40 would develop amenorrhea following chemotherapy. However, it is uncertain whether the suppression of ovaries is permanent. Some observational studies have reported an increased risk of return of ovarian function in peri-menopausal women with breast cancer taking aromatase inhibitors compared with non-users (Burstein *et al.*, 2006; Smith *et al.*, 2006). This subsequently carries risks related to increased estrogen levels and the possibility of pregnancy. Therefore, a marker which could reliably confirm the permanent suppression of ovarian function and assure the safety of aromatase inhibitors in such patients is sought after by oncologists (Henry *et al.*, 2014).

Chemotherapy regimens used for breast cancer include CMF (cyclophosphamide, methotrexate, fluorouracil); Epirubicin followed by CMF; AC (anthracycline and cyclophosphamide), which can be used for women with lymph node negative disease. Other regimens used are FEC (fluorouracil, epirubicin and cyclophosphamide) with docetaxel in case of node positive cancer and FAC (fluorouracil, adriamycin=*doxorubicin* and cyclophosphamide). The decision about chemotherapy regimen used is based mainly on the biological profile of the tumour, its spread to lymph nodes, the risk of recurrence and patients underlying medical conditions. NICE recommends multi-agent therapy with the usage of antracyclines (epirubicin or doxorubicin) (NICE, 2013b).

Cyclophosphamide is effective, and one of the most widely used agent against breast cancer but its use is associated with a high risk of ovarian damage. Depending on the combination of drugs included in the regimen and the number of cycles of chemotherapy administered, it may have a variable damaging effect on ovarian tissue. The risk of amenorrhoea after 6 cycles of CMF or four cycles with AC is estimated at 33%, while six cycles of FEC or FAC increases the risk to 50-60% (Kim *et al.*, 2012). Some new drugs used in HER-2 positive patients such as trastuzumab (humanised monoclonal antibody) have not yet been assessed in longitudinal studies and, therefore, the long term effects on ovaries are uncertain.

1.5.3 Lymphoma

Hodgkin lymphoma is the third most common cancer in people aged 15-29. In women, the peak of disease is around age 20-24 and later in life at age 70-74. Overall, the incidence in females is 2.4 per 100,000 and has remained stable from the mid-1970s. Although there has been a significant fall in the number of deaths from Hodgkin lymphoma, from 1.1 to 0.3 per 100,000 in the UK according to Office for National Statistics and The National Cancer Intelligence Network (NCIN, 2009; ONS, 2011; ONS, 2012). Based on histology, the types of lymphoma are divided into several subtypes with Classical Lymphoma being the most common and represented by nodular sclerosis type (around 50% of all cases), mixed cellularity, lymphocyte rich and lymphocyte depleted types (Adami, 2008).

Patients with early stages of Hodgkin lymphoma receive four courses of ABVD (adriamycin, bleomycin, vinblastine, dacarbazine) which is estimated give <10% chance of premature ovarian insufficiency. For more advanced stages of the disease, further courses of ABVD or BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine and prednisolone) can be used. BEACOPP's risk of inducing early menopause is 50% in women exposed before the age of 30 (Kim *et al.*, 2012). ChlvPP (chlorambucil, vinblastine, procarbazine and prednisolone). the combination is used in elderly patients not suitable for ABVD (Weekes *et al.*, 2002). Another protocol, Stanford

V (mustine, doxorubicine, vincristine, vinblastine, bleomycin, etoposide and steroids) was found to be inferior to ABVD in terms of survival in a 10 year follow-up clinical trial (Chisesi *et al.*, 2011)

Non-Hodgkin Lymphoma is the fifth most common cancer in UK. Although its incidence is higher in older people, increasing from the age of 50-54. Around 3.8% are diagnosed at age 20-34 and 6.8% between 35 and 44. In women, the incidence in the last 10-15 years has increased by 11% (NCIN, 2009; ONS, 2012b). The survival rate for women under the age of 49 is estimated at 80-85% (Kim *et al.*, 2012).

The regimen commonly used in this group is CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisolone) which has been associated with a 5% risk of premature ovarian insufficiency (Kim *et al.*, 2012).

1.6 Effect of cancer and chemotherapy on ovarian function: possible mechanisms

The impact of cancer and its treatment on ovarian biology and function is not fully understood in humans, and some of the studies give opposing theories. Here, I present an overview of the literature on the mechanism of chemotherapy-induced follicle damage, the impacts of different types and dosages of chemotherapy, as well as the range of effects in different age groups.

1.6.1 Ovarian reserve before chemotherapy

In order to understand the impact of cancer therapy upon female reproductive function, it is important to assess whether there is any association between the occurrence of a disease, or an effect of severe illness (e.g. cancer) on ovarian reserve prior to any treatment being commenced. There are several publications comparing AMH in cancer patients and the infertile population, however the latter may not be a suitable control group because infertility may be associated with a reduced follicle pool (Knopman *et al.*, 2009; Quintero *et al.*, 2010). Even if cases of male factor infertility were analysed, it couldnot be excluded that, on average, levels of AMH may be lower in the infertile population.

Others have tried to assess the ovarian reserve by checking the regularity of menstrual cycles, a method which is known to be unreliable (Bath *et al*., 2003; Larsen *et al*., 2003).

Two recently published studies compare AMH in cancer patients and healthy volunteers. A study of 108 breast cancer patients, pre-chemotherapy, versus 99 volunteers, reported no statistical difference in AMH concentrations between the groups. However, the groups were not age matched (the breast cancer group was significantly older) and volunteers with a previous history of polycystic ovarian syndrome (PCOS, known to be associated with raised AMH) or infertility were not excluded. Moreover, information about the type of contraception used was not taken into consideration (Su *et al.*, 2013).

Another recently published study from the FERTIPROTEKT Network in Hodgkin and non-Hodgkin lymphoma patients reported significantly lower levels of AMH in blood when compared with age-matched healthy volunteers, having a normal BMI and regular menstrual cycles. However, nearly 60% of volunteers were using oral contraception which could perhaps have affected the results (Lawrenz *et al.*, 2012). Interestingly, when the same authors compared the number of retrieved oocytes for breast cancer and lymphoma patient groups (even those with normal results of ovarian reserve tests) following ovarian stimulation for fertility preservation, the lymphoma patients had significantly lower results. The authors hypothesised that reduced ovarian reserve may have

been related to high cytokine levels which are believed to be the reason for poorer quality sperm in male patients with lymphoma (Agarwal & Said, 2004).

1.6.2 Mechanism of chemotherapy-induced ovarian damage

The gonadotoxic influence of chemotherapy on ovaries includes effects on both somatic and germ cells as well as vascular damage. The degree of follicular apoptosis and cortical fibrosis may vary with different types and dosages of chemotherapy. Also, effects upon the pool of primordial follicles may not be the same as on other classes of follicles e.g. maturing follicles which contain dividing granulosa cells. The latter would be the prime target of chemotherapeutic agents as they directly affect actively dividing cells (Meirow *et al.*, 2010).

Overall, the prevailing view is that the number of germ cells is negatively affected by chemotherapy. As the destroyed germ cells cannot be regenerated, the damage is irreversible (Demeestere *et al.*, 2012). A counter view was proposed in 2005 by Johnson et al. (2005) supporting the theory of neo-oogenesis. While controversial, there have been some recent publications in support. However, neo-oogenesis is considered unlikely to impact significantly upon chemotherapy-induced damage (Johnson *et al.*, 2005).

Among the first publications studying the chemotherapy-induced ovarian damage, Ataya & Moghissi (1989) examined cyclophosphamide effects in rats

and concluded that the main action of chemotherapy is through direct effects on rapidly dividing cells with mitotic activity. The main effects observed were in the structure and function of proliferating granulosa cells, oocyte fertilization, and cleavage. They believed that surviving primordial follicles and oocytes had escaped the damaging effects and may be functioning normally after treatment is finished (Ataya & Moghissi, 1989).

In the 1990s, an Italian group performed studies on the human ovarian tissue. They recruited 12 reproductive age women with Hodgkin disease and performed ovarian biopsies before and after chemotherapy with alkylating agents. Using electron microscopy they observed a reduction in the overall number of primordial follicles in all biopsied samples, as well as cellular features suggestive of early atresia in some of the surviving oocytes and follicle cells. This was interpreted to mean that chemotherapy not only immediately affected quantity but may also influence the quality of surviving follicles by starting early atretic changes which could potentially have a long term effect (Familiari *et al.*, 1993).

However, Meirow et al. (2007) examined the changes in cryopreserved ovarian tissue in young women exposed to non-sterilising doses of chemotherapy and presented another idea. They provided evidence of disturbed blood supply to ovaries caused by obliteration of small blood vessels resulting in focal fibrosis of the ovarian cortex and loss of primordial follicles. They hypothesised that the

endothelial injury might cause long term effects resulting from fibrosis and follicle disappearance with subsequent abnormal neovascularisation (Meirow *et al.*, 2007).

A more recent study from a different group supported Meirow's work on the vascular damage (Ben-Aharon *et al.,* 2012, Ben-Aharon *et al.,* 2015). Following animal studies, Ben-Aharon et al*.* (2012) recruited 20 premenopausal women at a mean age of 34, undergoing adjuvant or neoadjuvant treatment with anthracycline or taxane for non-metastatic breast cancer. Shortly following chemotherapy, Doppler-flow velocity index and size of the ovaries were measured. The results revealed significantly reduced blood flow and a decrease in size of ovaries in those patients. A more pronounced decrease was observed with sequential chemotherapy. Serum AMH measured at the same time revealed a drastic reduction, suggesting rapid changes in granulosa cells (Ben-Aharon *et al.*, 2012, Ben-Aharon *et al.,* 2015).

Proliferating granulosa cells may be a target for most chemotherapeutic agents, and loss of support from granulosa cells may lead to the death of oocytes (Morgan *et al.*, 2012). Thus, if the activated, growing follicles are destroyed, the inhibiting effect of hormones such as AMH, produced by growing follicles and feeding back on follicular recruitment, is reduced. Therefore, a higher number of primordial follicles becomes recruited and then atretic at an increased rate which accelerates the loss of the ovarian reserve (Durlinger *et al.*, 2002). This

is called the follicular burn-out hypothesis and states that ovaries undergo premature ‘burn-out’ due to an increased rate of recruitment and atresia of primordial follicles. If this were correct, the ratio between the number of primary/secondary follicles and primordial follicles would be increased. It has been observed in animal studies. However, there is a lack of human studies to confirm the same mechanism (Meirow *et al.*, 2010).

A number of other studies, in both humans and animals, have been reported, but none has a definitive answer to describe the mechanism of chemotherapy-induced ovarian damage fully. It is possible that several mechanisms, which could be drug-specific or regimen-specific, take place at the time of exposure and may continue thereafter. Some of the damage may only become evident later. A constant dialogue between somatic cells and germ cells, which is necessary to regulate the growth and maturation of both, may be interrupted, and its re-establishment after profound changes due to chemotherapy exposure may be defective.

It has been hypothesised that, based on mechanism of action and current studies, some agents such as doxorubicin and cyclophosphamide act more on dividing granulosa cells while others such as Cisplatin or Topoisomerase enzymes target the oocyte directly (Morgan *et al.*, 2012).

Table 1 Summary of literature that examines the direct action of chemotherapy on the ovary (from Morgan *et al.,* 2012).

Chemotherapeutic agent	Author	Species	Affected cell type	Affected follicle class
Doxorubicin	Perez *et al.* (1997)	Mouse	Oocyte	
	Jurisicova *et al.* (2006)	Mouse	Oocyte	
	Ben-Aharon *et al. (*2010)	Mouse	Granulosa cells	Secondary
	Bar-Joseph *et al.* (2010)	Mouse	Oocytes	
	Soleimani *et al*. (2011)	Human, Mouse	Oocyte, granulosa cells, stroma, blood vessels	Primordial, pre-antral
Cisplatin	Gonfloni *et al*. (2009)	Mouse	Oocyte	Primordial, primary
Cisplatin/paclitaxel	Yucebilgin *et al.* (2004)	Rat		Primordial
Irinotecan	Utsunomiya *et al*. (2008)	Mouse	Granulosa cells	Pre-antral, antral, pre-ovulatory
Cyclophosphamide	Zhao *et al*. (2010)	Rat	Granulosa cells	
	Desmeules and Devine (2006)	Mouse	Oocyte and granulosa cells	Primordial, primary
	Petrillo *et al.* (2011)	Mouse, rat	Mainly Oocyte	Primordial, primary
	Oktem and Oktay (2007a)	Human	Oocyte	Primordial
	Raz *et al*. (2002)	Human	Granulosa cells	

1.6.3 Types of chemotherapy and effects on ovarian function

The most commonly used types of chemotherapeutic drugs affect cell divisions and DNA synthesis. These types include: alkylating antineoplastic agents (mechlorethamine, cyclophosphamide, chlorambucil, ifosfamide); platinum derivatives (cisplatin, carboplatin, oxaliplatin), anti-metabolites (azathioprine and mercaptopurine); vinca alkaloids (vincristine, vinblastine); taxanes (paclitaxel, docetaxel); topoisomerase (irinotecan, topotecan, teniposide and etoposide); anthracycline (doxorubicin, epirubicin). Some of these agents and certain regimens seem to have a greater impact upon ovarian reserve than others. Most damage to the ovary is believed to be caused by alkylating agents. Cisplatin (and other platinum agents) is classified as a drug with a high impact but less than cyclophosphamide. Intermediate risk is associated with taxanes. The risk decreases further with vinca alkaloids, and the lowest risk of ovarian toxicity is associated with antimetabolites (Meirow *et al.*, 2010, Demeestere *et al.*, 2012) .The anthracycline group has been previously classified as ‘low risk’, but recently that has been questioned (Letourneau *et al.*, 2012) as it seems to have a higher impact on ovarian reserve than initially suggested. Although, the dosages, types, and regimens of chemotherapy continue to change.

1.6.4 Dosage of chemotherapy and its effect on ovarian function

The effect of chemotherapy upon the ovary has been proven to be dose dependent. Meirow et al. (1999) in experiments on mice, showed that single increased doses of cyclophosphamide caused the loss of increasing numbers of primordial follicles in each subject. Follicular destruction was found to be proportional to the dose of cyclophospamide and was evident in all cases even at the lowest dose of 20 mg/kg. A dose of 75 mg/kg caused loss of 54% of primordial follicles (in humans the average dose of cyclophosphamide would be 40-50 mg/kg given in divided doses). The relationship between the dose of chemotherapy and the reduction in the number of primordial follicles best fits an exponential curve. The authors highlighted the fact that even if the ovulation rate and pregnancy rate were not affected by chemotherapy in young animals in the short-term, damage to primordial follicles was still observed to increase the risk of early ovarian failure and a narrowed period of time to conceive (Meirow *et al.*, 1999).

Those findings are consistent with some observational studies in humans, reporting a high risk of early menopause in women exposed to chemotherapy, even when regular menstrual cycles and ovulation have resumed post-treatment. It shows that the initial recurrence of regular cycles and ability to conceive can give false reassurance to patients and their oncologists. Long term follow-ups show that young women still experience ovarian side-effects and a

narrowed window of opportunity to conceive as well as an increased risk of early menopause (Rose & Davis, 1997).

1.6.5 Age and chemotherapy effects

As mentioned earlier, women who regain spontaneous menstrual cycles following chemotherapy have usually suffered some degree of reduction in ovarian reserve and are therefore at risk of premature menopause (Bath *et al.*, 2003; Larsen *et al.*, 2003b; Partridge *et al.*, 2010b). The effect is age dependent. Meirow et al. 2010 highlighted that ovarian damage happens to women of all ages exposed to cytotoxic drugs but is manifested sooner in older women as they already have reduced ovarian reserve at the time of commencing the treatment (Meirow *et al.*, 2010).

In a retrospective survey of 1041 American women who were diagnosed with cancer at an age of 18-40 years, 3-10% of them reported symptoms of acute ovarian failure (defined as amenorrhea for 12 months which started at/post treatment). The percentage of cancer survivors experiencing acute ovarian failure increased with age and, for example, was found to be 6% at age 20 years and 30% at age 35 years at diagnosis in patients with Hodgkin lymphoma ($p< 0.001$). For breast cancer patients, the incidence was 32% at 35 years and 55% at the age of 40 at diagnosis ($p< 0.001$). When infertility (defined as inability to conceive for 12 months or more) was studied, in the Hodgkin disease group, 57% of patients at age 35 had difficulty conceiving even if their menses returned

within 12 months of cancer treatment. Similarly, within the breast cancer group the incidence of subfertility was 32% at age 35 and 80% at age 40. Interestingly, within the group of women whose menses returned within 12 months from the time of chemotherapy, younger women had a higher probability of early menopause than older women closer to menopause (Letourneau *et al.*, 2012). Overall, those results were consistent with some previous reports and raised the issue of a narrowed window of opportunity to conceive even in very young women undergoing chemotherapy.

Table 2 Estimates of the chemotherapy induced risk of early menopause (from Meirow *et al.,* 2010).

Breast cancer	**Treatment**	**Age**	**Ovarian failure**
(Lower *et al.*, 1999)	Various regimens	Pre-menopause	45%
		<35y	28%
(Bines *et al.*, 1996)	Various regimens	Pre-menopause	68%
(Goodwin *et al.*, 1999)	CMF	43.7y	65%
(Meirow, 1999)		<44y	50%
(Burstein & Winer, 2000)	CMF	30y	19%
		30-39y	30-40%
	CAF	<30y	0%
		30-39y	10-25%
	AC	<30y	0%
		30-39y	13%
(Petrek *et al.*, 2006)	Various regimens	<35	15%
		35-39	39-55%
		>39	>55%
Hodgkin disease	**Treatment**	**Age**	**Ovarian failure**
(Howell & Shalet, 1998)	Aggressive treatment		38-57%
(Meirow, 1999)	Relapse post 1st treatment		32%
(Bokemeyer *et al.*, 1994)	Infradiaphragmatic radiotherapy		50%
(Brusamolino *et al.*, 2000)	'ovarian sparing protocol.'	<25y	0%
		<45y	30%
(Behringer *et al.*, 2005)	Dose escalated BEACOPP	30y	51%
		>30y	95%

AC –cyclophosphamide, CAF- cyclophosphamide doxorubicin and fluorouracil, CMF- cyclophosphamide, methotrexate and fluorouracil.

1.6.6 Radiotherapy and obstetric outcomes in cancer patients

It has been shown that obstetric complications are more common in cancer survivors. This depends on the type and dosage of treatment, but overall chances of miscarriage, preterm delivery, and neonatal low birth weight are higher in cancer survivors (Meirow *et al.*, 2010).

Chemotherapy does not seem to have a significant, long-lasting effect upon uterine function, however when accompanied by total body radiation or radiotherapy to the pelvis, impact on both ovarian and uterine tissue is evident (Meirow *et al.*, 2010).

The occurrence of premature ovarian failure following total body irradiation and abdominal/pelvis radiation were 90% and 97% respectively in long-term follow-up studies. Similarly to chemotherapy, the degree of damage depended on the patients' age and the cumulative dose of radiotherapy (Meirow *et al.*, 2010). Ideally, the gonads should be shielded from the radiation field, but this is not always possible. Based on mathematical models it has been estimated that the pool of non-growing follicles is halved by a radiation dose of 2Gy (Wallace *et al.*, 2003).

The damaging effect of radiotherapy on uterine function results in a lower implantation rate, higher number of premature deliveries and miscarriages, (which is assumed to be a result of abnormal vascularisation,) decrease in

uterine size and thinning of the endometrium following high dose ionizing radiation (Meirow *et al.*, 2010). In patients who received direct pelvic radiotherapy, increased risks of preeclampsia, foetal malposition and post-partum haemorrhage have been observed (Demeestere *et al.*, 2012). The same has been observed in women with history of childhood cancer exposed to abdominal radiotherapy, where association with a higher rate of preterm deliveries and low birth rates in offspring was found (Reulen *et al.*, 2009). The same was not found in in girls and young omen exposed to chemotherapy only. (Reulen *et al.*, 2009)

Interestingly, only a very few papers report on the impact of radio/chemotherapy on the hypothalamus and pituitary gland in relation to fertility. One of the cohort retrospective studies examined fertility outcomes in 3,619 female Childhood Cancer Survivors and compared them with participants' siblings. Based on a multivariable regression model including race, smoking status, marital status, education, and age at diagnosis; hypothalamic/pituitary exposure to doses of radiotherapy >22 Gy was found to be associated with reduced fertility in the cancer group (Green *et al.*, 2011).

1.6.7 Teratogenic impact of chemotherapy on future pregnancy

There is no evidence of increased chromosomal abnormalities in children born from mothers previously exposed to chemo- and radiotherapy (Winther *et al.*, 2004). However, many authors stressed that the time between the end of

chemotherapy and the start of pregnancy is crucial in preventing potential teratogenic effects. It is estimated that a break of at least 6 months after the last dose of chemotherapy should be recommended to avoid the potentially dangerous exposure of maturing oocytes to chemotherapy (Meirow *et al.*, 2010). Understandably, a longer period of 1 to 5 years is recommended in order to avoid the risk of recurrence of the malignant condition during pregnancy.

Animal studies suggest that exposure to chemotherapy just before conception or during early pregnancy increases fetal malformations and miscarriages. Consequently, it is considered important to avoid cryopreservation of oocytes immediately after a first course of chemotherapy, even if it was not offered earlier, because the impact on future offspring could be significant (Meirow *et al.*, 2010).

1.6.8 Long term clinical impacts of chemotherapy-induced loss of ovarian function

Apart from infertility, there are a number of other clinical consequences of premature menopause (before age 40) or early menopause (age 40-45) (Nelson, 2009). The terms premature ovarian failure, primary gonadal dysfunction or primary ovarian insufficiency (POI) are often use to describe secondary amenorrhoea as an effect of a significant reduction in the primordial follicle pool in young women aged < 40. Most commonly primary ovarian insufficiency is used to describe amenorrhea for more than 4 months and two

readings of FSH at menopausal levels in women below 40. Nevertheless, intermittent ovarian function can still occur in half of women diagnosed with POI (Goswami & Conway, 2005) and about 5-10% of women still conceive after a diagnosis of primary ovarian insufficiency (Anderson & Cameron, 2011; Nelson, 2009). POI differs from menopause which is considered more definitive. In patients with cancer, the situation is more complex, and assessment is difficult. Secondary amenorrhoea can be permanent and may start immediately at the time of gonadotoxic treatment in patients with already diminished ovarian reserve. In younger patients, usually having a larger stock of follicles, menses may stop as an effect of chemotherapy-induced loss of growing follicles but then resume as some of the surviving primordial follicles start to grow and mature. Chemotherapy-related amenorrhea (CRA), describes acuter and sometimes temporary changes which take place within the first few months after starting chemotherapy rather than the long term consequences, however it has also been considered as a marker of ovarian function (Anderson & Cameron, 2011; Petrek *et al.*, 2006). The understanding of those terms and their definitions seems to vary among infertility experts and oncologists (Anderson & Cameron, 2011).

1.6.9 Consequences of induced premature menopause

The consequences of premature menopause relate to the loss of circulating follicle-derived hormones, and include low bone density, earlier onset of osteoporosis and bone fractures, increased risk of cardiovascular morbidity and mortality, higher rates of depression and anxiety and psychosexual dysfunction (Daan et al., 2015). Other common complaints include vasomotor symptoms, vaginal dryness and dyspareunia (Rocca *et al.*, 2009; Shuster, 2010).

One of the commonly used methods of assessing the association between early menopause and health problems is to compare women who underwent oophorectomy for benign causes with the general population of women who had a natural menopause. In one such population based cohort studies, an increased mortality was observed in women who underwent total abdominal hysterectomy and bilateral oophorectomy for benign causes before the age of 45 and who had not received estrogen replacement therapy as compared with the general population (Rocca *et al.*, 2006).

Another study looked into the potential effects of estrogen decline on cognitive function in women who had ovaries removed for reasons other than cancer. It reported impaired performance and a lower score in Mini Mental State examination and Wechsler Memory Scale in women post-

oophorectomy as compared to age- and education-matched controls. Post-surgery results in women with estrogen levels dropping more than 50% at 3 and 6 months follow-ups, the changes in visual and logical memory loss were more pronounced (Farrag *et al.*, 2002).

In conclusion, the effects of chemotherapy on future fertility are age-, agent- and dose-dependent. Different combinations of agents used, and repeated treatments make the classification of the degree of gonadotoxicity difficult.

The mechanisms of acute injury to ovaries and the long term impact on ovarian function are complex and may vary significantly among different agents. There is a lack of clear guidance for counselling women having gonadotoxic treatment. It is the time that well-designed longitudinal prospective studies with end points that include pregnancy and premature menopause should be undertaken.

1.7 Fertility preservation options

There are a number of choices for fertility preservation in young women with cancer. Some oncologists decide not to refer for a preservation discussion with a fertility specialist and are guided by their own experience and beliefs (Quinn *et al.*, 2009). Not all oncologists may have up to date knowledge on the timing, funding and treatment options and their side effects, as there has been significant progress in this field recently (Gilbert *et al.*, 2011).

Patients' expectations, however, may be different. In a large web-based survey conducted in the United States amongst young female survivors of early stage breast cancer, over 50% of respondents stated that their 'fertility concerns'; were not addressed properly at the time of cancer diagnosis and treatment. More than 70% of them expressed concerns about future fertility (Partridge *et al.*, 2004). In the UK, a study conducted by the University of Sheffield at a Teenage Cancer Trust meeting showed that only 40% of females diagnosed with cancer as a teenager were happy with the fertility information and fertility preservation options that they had received (Yeomanson *et al.*, 2013). In the UK, the most established method of fertility preservation for women is embryo freezing while a newer option is vitrification of oocytes. Cryopreservation of ovarian tissue is still performed only in research settings in selected centres, due to regulations and a lack of funding, therefore not allowing easy accessible across the UK (Ajala *et al.*, 2010; NICE, 2013a). The decision regarding an appropriate fertility

preservation method is ideally guided by the type of cancer, timing of chemotherapy/radiotherapy, age of the patient, relationship status and the patient's wishes (Donnez & Dolmans, 2011) .

Embryo cryopreservation is a widely used technique in IVF centres and it is the most commonly offered fertility preservation option in the UK. This method requires a male partner willing to consent to embryo storage and its use in the future. However, the psychological and emotional impact of the life-threatening illness on a relationship at the time of diagnosis, and a few years delay until embryos are actually used with a view to establishing pregnancy may cause difficulty in obtaining or maintaining the partner's consent. There have been high profile cases where the male partner had withdrawn consent when the couple separated, not allowing their ex-partner to use the cryopreserved embryos, which had been her last chance of pregnancy with her own biological offspring (English, 2004). Cryopreserving mature oocytes could solve this problem. However, the quoted pregnancy rates vary greatly in centres worldwide.

Since delay with commencing cancer treatment needs to be avoided, many centres offer a short stimulation protocol for oocyte collection for fertility preservation. In this situation, stimulation uses a GnRH antagonist as opposed to so-called long protocols requiring time consuming down-regulation with GnRH agonist. If the patient is referred immediately after diagnosis and

happens to be in the early follicular stage of the menstrual cycle, short protocol could result in oocyte collection within 10-14 days.

In cases of estrogen receptor positive breast cancer, stimulation with high doses of FSH and LH, leading estrogen levels to increase, may cause concern amongst oncologists. There is no evidence of a negative effect of transiently high estrogen levels, just before chemotherapy is commenced, in terms of long-term mortality and the response to chemotherapy in estrogen receptor positive breast cancer patients (Anderson & Wallace, 2011). However, there is also a lack of trials confirming its safety. Some research groups have therefore used modified ovarian stimulation protocols in such cases, adding Letrozole or Tamoxifen alongside FSH for the duration of the stimulation cycle, although this has raised issues of safety and impact on the development of embryos (Oktay *et al.*, 2005). Health professionals may find it difficult to quote success rates and safety of such modified protocols during their discussions with patients (Kim *et al.*, 2011). National statistics on pregnancy rates, specific for women with cancers who underwent fertility preservation, are not available. Some authors question its efficacy. Additionally, some women who generate frozen embryos may never wish to use them due to natural conception or ongoing illness, and some may die from their cancer (Anderson & Wallace, 2011; Kim *et al.*, 2011).

Cryopreservation of oocytes could avoid the creation of unused embryos, which may raise ethical concerns. The success of oocyte cryopreservation for young

women is becoming closer to that with fresh oocytes as stated in the American Society for Reproductive Medicine Guidelines for Practice, 'Mature oocyte cryopreservation' (ASRM, 2013). Using vitrification instead of slow freezing has improved success rates. However, the method is technically challenging and requires a skilled embryologist. It is estimated that over 900 babies have been born worldwide from frozen oocytes (mainly using slow-freezing) and there has been no increase in chromosomal abnormalities or birth defects reported as compared with natural conception (Noyes *et al.*, 2009). Oocyte cryopreservation still requires ovarian stimulation and some research groups have proposed that cryopreservation of immature oocytes at the germinal vesicle (GV) stage may be an appropriate alternative for women with estrogen receptor positive breast cancer or where any delay of cancer therapy is not recommended. It may also be considered in women at a high risk of ovarian hyperstimulation syndrome (Kim *et al.*, 2011). However, this approach is still experimental because it requires maturation of oocytes in vitro, which is associated with lower success rates than the use of mature oocytes (Donnez & Dolmans, 2011).

A further option for fertility preservation that helps to avoid delays in cancer treatment is cryopreservation of ovarian tissue. This approach is still considered to be experimental in the UK and requires registration with the Human Tissue Authority. In Denmark and Belgium, the method is more commonly used, and satellite centres transport laparoscopically retrieved samples of ovarian cortex for cryopreservation in a central certified laboratory. Subsequent re-implantation

of cortical ovarian tissues into the pelvic cavity (orthotopic site), once patients were recovered from their cancer, has been reported to result in 15 live births (Donnez & Dolmans, 2011).

In the Donnez research group, ovarian function was restored within 3-6 months after surgery in all cases of orthotopic re-implantation of the cryopreserved ovarian cortex. This therapy could therefore potentially reverse the symptoms of premature menopause, however, such grafts usually last for only short periods of time (2 years maximum). Re-implanting additional slices of ovarian cortex requires repeated surgery. So far, the method was found to be successful only in women below the age of 30, in whom a substantial ovarian reserve would be expected (Donnez & Dolmans, 2011). Interestingly, in Denmark, in reported series of 18 patients undergoing ovarian cortex orthotransplant, all of the recruited women resumed production of sex hormones. Authors declare that on average it took 20-25 weeks to develop first pre-ovulatory follicle (Andersen *et al.*, 2012).

An important disadvantage of this option is that re-implantation of ovarian tissue may reintroduce malignant cells. It has been reported that malignant cells are present in ovaries of patients with leukaemia but seems less likely in cases of lymphoma and breast cancer (Donnez & Dolmans, 2011). A review on the risk of re-implantation of malignant cells suggested that performing the procedure in patients in complete remission could minimise the risk. Based on current

literature, guidance on the risk of ovarian metastasis in all common cancers classified Hodgkin lymphoma and stage I-III ductal breast cancer as low risk, while leukaemia, Burkitt lymphoma and neuroblastoma were categorised as high risk (Dolmans *et al.*, 2013). For this reason, alternative methods are being developed such as the re-implantation of isolated follicles within an artificial ovary or scaffold (Amorim *et al.*, 2009) or maturation of oocytes from primordial follicles from cryopreserved ovarian tissue (Hovatta, 2004).

A systematic literature review of studies examining the presence of malignant cells in cryopreserve human ovarian tissue was conducted in 2013. Based on 42 included papers, ovarian tissue from overall 422 women have been examined using different methods: histology, immunochemistry, PCR or transplantation of tissue to animals. In around 7% of cases, some evidence or suspicion of the presence of malignant cells has been reported. The impractical observation made by authors was that transplantation of a representative tissue to a suitable host animal for a period of 5 months is the most reliable method of testing for presence of malignant cells (Rosendahl *et al.*, 2013). Other methods were not as reliable as the one using host animals.

Another option of fertility preservation comprises the use of GnRH agonists alongside chemotherapy, to suppress ovarian follicular activity. The suppressing effect of GnRH -agonists on the hypothalamic-pituitary-gonadal axis, slowing rate of folliculogenesis with potential protective effect on ovarian

tissue was initially suggested based on observational studies in women receiving chemotherapy for breast cancer (Blumenfeld *et al.*, 2008; Recchia F, 2006). Although, the exact mechanism of the potential protective action of GnRH agonists in humans remains uncertain. Imai et al., (2007) reported positive results while using GnRH - agonist against doxorubicin in in-vitro studies on granulosa cells obtained from patients undergoing egg collection for IVF (Imai *et al.*, 2007). The effect was restricted to cells with GnRH receptors only. Others have suggested that GnRH agonists may reduce the blood flow to ovarian tissue and, therefore, minimise exposure to gonadotoxic drugs (Morgan *et al.*, 2012).

A meta-analysis of 11 prospective studies on co-treatment with GnRHa showed some improved ovarian function after treatment, but subanalysis of 3 randomised trials failed to show any statistical difference (Kim *et al.*, 2010). Additionally, two recently published, multicentre randomised trials have given opposing results, and it still remains uncertain if GnRH agonist treatment has any protective effect. The ZORO study did not provide evidence for any protective action of goserelin in hormone-insensitive breast cancer patients below the age of 46 receiving adjuvant chemotherapy (Gerber *et al.*, 2011). The end point of the study was a recurrence of menses 6 months after the end of chemotherapy. However, this has been considered too short a follow-up.

In contrast, PROMIS-GIM6 showed a reduced incidence of early menopause by 17% in premenopausal women with breast cancer taking triptorelin during adjuvant or neoadjuvant chemotherapy (Del Mastro *et al.*, 2011). The outcome was measured as the resumption of menses by 12-months follow-up and FSH levels. One could argue against the validity of such an assessment since a true measure of ovarian reserve would be long-term follow-up including pregnancy rates and age at menopause in all participants, and objective tests of ovarian reserve by AFC and AMH.

The last published Cochrane review suggests considering GnRH agonist use in form of intramuscular or subcutaneous injections in reproductive age women undergoing chemotherapy despite a lack of evidence for improvement in pregnancy rates (Chen *et al.*, 2011). Overall, GnRH agonist is currently an option for women who have declined or are unsuitable for other forms of fertility preservation and wish to use GnRHa (Kim *et al.*, 2012)

In summary, there are options for fertility preservation available for women prior to gonadotoxic treatment. The success rates and safety for cancer patients have been widely investigated, and provide hope for the future. None of the options is perfect, but women should be allowed to make an informed decision based on an individualised assessment, using ovarian reserve tests, and discussion with a fertility specialist.

1.8 Background to the research: Which is the most appropriate ovarian reserve test for cancer patients and selection of participants?

Following a literature review of the available ovarian reserve tests, I selected serum AMH measurements as the most suited for my project. AMH is a useful test of 'reproductive capacity' in women pre- and post-chemotherapy (Anderson & Cameron, 2011). Recent studies have shown that amongst several ovarian reserve markers currently available, serum AMH measurement is of high clinical value (La Marca *et al.*, 2009). The AMH concentration is relatively independent of the menstrual cycle and more accurate amongst women with irregular menstrual cycles in comparison to other markers such as FSH, E2, inhibin B (Cook *et al.*, 2000b). In all the published comparisons, the performance of other endocrine ovarian reserve tests was never better than AMH (Broekmans *et al.*, 2006).

A non-endocrine ovarian reserve test considered equally reliable is transvaginal ultrasound assessment of AFC. However, for a multicentre study, ultrasound assessment was found to be less suitable in view of inter-observer variation. If AFC were selected as the ovarian reserve test for this study, the same ultrasonographers using the same USS equipment to specific standards should ideally have performed it. This would, therefore, involve, for some patients,

travelling to a distant clinic. Additionally, there is a lack of international standards as to which size range of follicles should be classed as antral follicles for AFC. Transvaginal ultrasound offered no advantage, as compared to serum AMH measurements, in research setting where only the ovarian reserve was to be tested.

In some young women newly diagnosed with cancer, a requirement for transvaginal ultrasound could also cause additional stress and would require an additional appointment in this difficult time when they try to come to terms with their diagnosis. Additionally due to religious beliefs or previous traumatic experiences, some women may not be willing to have a transvaginal ultrasound for research purposes. Therefore, although a comparison of both AFC and AMH would have been interesting and valuable in my selected patient group, I decided to use only AMH, a test that can be done on a blood sample taken at any time. In the future, it could readily be included as part of the pre-chemotherapy work-up and post-chemotherapy follow-up blood tests, carried out in all cancer patients. In contrast, a transvaginal ultrasound is not a routine procedure for this patient group and could present logistical and compliance challenges.

Based on incidence rates from World Cancer report 2008, young women most commonly would be diagnosed with breast cancer, melanoma, cervical cancer, Hodgkin and non-Hodgkin lymphoma and colorectal cancer (Boyle & Levin, 2008).

In patients with early stages of melanoma, treatment would be localized without any systemic therapy. Treatment of cervical and colorectal cancer may include extensive pelvic/abdominal surgery and radiotherapy which would make it difficult to distinguish the impact of chemotherapy from the rest of treatment. Therefore, in this study, I have concentrated on two main groups: breast cancer and lymphoma. As study design is observational and I am using a new assay in previously not validated group of patients, following discussion with my Supervisors, I have decided to allow participation of women with other haematological condition (malignancy) or other cancer (e.g. colorectal) if patients self-referred. Due to small numbers, they are not included in the main statistical analysis and may be described only as case reports which could potentially provide interesting initial observations for other researchers working in the same field.

1.9 Research questions and objectives

In the next chapter, I present a literature review of ovarian reserve tests (including AMH) in female cancer patients, in order to identify currently available evidence and optimal practice for the assessment of ovarian function in women with severe illness undergoing gonadotoxic treatment.

As an initial step of my experiment, I conducted a small pilot study to assess how different blood sample handling and storage may impact the AMH results. It was an important step in setting up multicentre study which required transport of samples from various hospitals.

The objectives of my prospective study were, firstly, to compare baseline ovarian reserve using AMH in volunteer women and female cancer patients in their reproductive years. This part of the study aimed to determine whether there was evidence of an impact of severe illness on ovarian reserve, prior to chemotherapy. In this study, I aimed to ensure that AMH levels were analysed in full knowledge of women's menstrual cycle stages, contraception, BMI, smoking habits, parity and past medical history.

For the main part of the study, I assessed the effects of different types, dosages and durations of chemotherapy regimens on the ovarian reserve using AMH measurements as a marker, in young women receiving treatment for breast cancer or lymphoma. The effects of chemotherapy on the ovarian reserve in

cancer patients and healthy volunteers of similar ages were compared over a one year period. At the same time, I analysed young cancer patients' views of pregnancy at the time of cancer diagnosis, at 6, 9 and 12 months using a medical questionnaire. Additional information was obtained directly from PIs and research nurses about each patient's referral to Reproductive Medicine Consultant for discussion about fertility preservation options. It showed how patient's wishes for pregnancy changes (if) from the time of diagnosis till 12 month follow-up and how many female adult patients are currently referred to specialist for discussion about fertility preservation in the UK. In a volunteer group, I aimed to analyse AMH levels in relation to women's menstrual cycles, contraception used, BMI, smoking habits and past medical history. I tested the reliability of second generation assay using a new methodology including a pre-dilution step.

The longitudinal design of this study, which will include a 5-year follow-up, has allowed me to assess the accuracy of a pre-chemotherapy serum AMH measurement in predicting future reproductive capacity (end points: pregnancy, live birth, early menopause) in cancer patients. Such a study has not previously been reported. If such a link is established, serum AMH, as a reliable test of ovarian function, could provide important information for the work-up and counselling of young women before chemotherapy. Due to the time frame of a research degree, the 5-year follow-up was not included in this dissertation.

FLOW CHART OF STUDY KEY ACTIVITIES

Registration for Research Degree at University of Warwick

Writing literature review and attendance on the following courses

Understanding research and critical appraisal

Epidemiology and Statistics module – Warwick Medical School

SPSS (PASW) Statistics 17- University of Warwick - IT Services

Subfertility and Reproductive Endocrinology course RCOG

How to write a literature review – research skills module for PhD student

Chief investigator course-Warwick Medical School Clinical Trial Units

Protocol writing, collaboration with PIs (13 sites) and training of research nurses, opinion from patient group and experts: Professors Adam Balen, Richard Anderson and Nigel Stallard

Ethical approval, support from Breast Cancer Research Study Group and inclusion into NHS portfolio

Laboratory training in sample handling and AMH immunoassay. Pilot study on stability of AMH assay

R&D approvals on all sites, confirmation of local protocol and forms, establishing local procedures, building team contacts and setting up oncology clinics for this research.

Recruitment of patients and volunteers across all sites and coordination of timely arrival of samples, distribution of results letters to healthy volunteers.

Processing samples and AMH assay, analysis of medical questionnaires, data entry and matching control group, ensuring recruitment and sample collection remained on track at each setting, assisting as required by patients or PIs. Follow up of patient details and treatments. Arranging regular delivery of forms, packages to each clinic. Assuring properly timed continuation of blood sampling, questionnaires and patient information, as subjects reach 12 months from recruitment. Continually monitoring that recruitment is on track with appropriate size sub-groups (breast cancer and lymphoma patients), and age-matched control samples.

Attendance at course Theses and Dissertations- Microsoft Word, Statistical analysis and presentation of results at BFS, ASRM, ESHRE meetings

Thesis submission, commencement of final stage of study (5 year follow-up) prior to publication of study results.

CHAPTER 2

2 Literature review on ovarian reserve tests (ORT) in reproductive age women with cancer

2.1 Introduction

The increasing chances of survival of young women with cancer emphasise questions about their quality of life following successful treatment. Fertility, or prospects of it, are considered to be a very important aspect of quality of life amongst young cancer survivors (Ganz, 2000).

Assessment of remaining ovarian function after chemotherapy is not straightforward. Chemotherapy-induced ovarian follicle loss depends on several factors including the type of chemotherapy, its dosage, duration, and the age of the patients, i.e. how large their ovarian reserve is before treatment begins (Lower *et al.*, 1999; Meirow & Nugent, 2001).

A number of studies have used the recurrence of menses at 6 or 12 month follow-ups after chemotherapy as an end point for re-initiation of fertility (Lower *et al.*, 1999, Behringer *et al.*, 2005; Berliere *et al.*, 2008). However, it is known today that this may not be the best measure of ovarian function because the

time for recovery from gonadotoxic therapy may be longer than 6-12 months and women who regain menses still may be at risk of premature menopause (Reh *et al.*, 2008).

Ovarian reserve tests could provide more tailored and detailed information for the individual patient. However, a number of questions remain outstanding. Which test, or a combination of tests, is suitable for measuring changes in ovarian reserve, in reproductive age women before and after chemotherapy? Do those tests correlate with clinical outcomes: live birth, infertility, early menopause? Could ORT be used as a guide as to whether or not patients offer fertility preservation treatment?

This systematic review of literature of ovarian reserve testing in young women undergoing chemotherapy seeks to answer those questions.

2.2 Objectives

My aim was to provide a review of all studies measuring ovarian reserve by methods that included AMH, in women exposed to gonadotoxic agents and to assess the validity of the findings. I sought to identify a single ovarian reserve test or a combination of tests which may be used reliably before, during and after chemotherapy, to identify women at high risk of early menopause and chemotherapy induced infertility.

2.3 Methodology

2.3.1 Protocol

I used a prospective protocol following the widely recommended approach to a systematic review (PRISMA-http://www.prisma-statement.org). No pre-existing systematic literature review on ORT in the young female population affected by cancer was identified at the time of my initial analysis. The current guidelines and other reviews have been searched to assess their currency and to identify new research questions. I cross-checked systematic reviews on ovarian reserve tests in general and subfertile populations to identify if any data analysis on subgroups of cancer patients had been performed, which helped to identify one literature review. No existing review protocol on ovarian reserve tests in reproductive age women with malignancy was found.

2.3.2 Eligibility criteria

Given the rarity of the condition, databases were searched using PICOS questions (http://www.prisma-statement.org) without any additional restrictions (e.g. language), and with any length of follow-up. The search included all published studies until January 2010 without any language restriction. Subsequently, a further literature search has been performed prior to submission of this thesis, which included studies published between January 2010 and January 2014.

The following PICOS question set was used:

P (population/patient) - reproductive age women with malignancy

I (intervention) - cancer therapy: chemotherapy

C (comparison) - healthy volunteers

O (outcome) - change in ovarian reserve prior and past chemotherapy, subfertility, early menopause

S (study design) - prospective study design, cohort study or case control

2.3.3 Information sources and study selection

The online search included studies of commonly used ovarian reserve tests (FSH, LH, estradiol, inhibin B, AMH, AFC, ovarian volume) in young female cancer patients treated with chemotherapy. Following advice from the Academic Support Librarian, Samantha Johnson, the databases were searched using relevant text words, Medical Subject Headings (MeSH) including all subheadings and their word variants. Terms representing the concept (ovarian reserve tests OR ovarian function OR ovarian reserve markers OR 'specific ovarian reserve marker') and (AMH OR Anti-Mullerian hormone) AND (cancer OR neoplasm OR malignancy) AND (chemotherapy OR drug therapy) AND (female) and humans were used to search Medline (2000 to 2014), Embase (2000 to 2014) and the Cochrane Library Database (2000 to 2014). Studies evaluating ovarian reserve tests in childhood cancer survivors were not included.

2.3.4 Data collection process, data items

Information was extracted from each selected study on the population and test characteristics including the time was taken to perform the tests. Methodological quality and accuracy of the results were assessed by two independent reviewers, myself and Dr Honest Honest (Consultant Obstetrician and Gynaecologist at Good Hope Hospital). We defined quality as the confidence that the study design, conduct and analysis minimized bias in the estimation of

test accuracy. We considered a study to be of good quality if it reported a prospective design, consecutive patient enrolment, an adequate test description and information on *a priori* estimation of the sample size. All variables for which papers were examined are included in a proforma 'Data extraction' form **(Appendix 1)**.

2.3.5 Risk of bias

Understandably, most studies assessing the impact of chemotherapy upon fertility have an observational character such as case control, cohort or case series. With that in mind, the following criteria for assessment of the risk of bias in individual studies have been selected:

selection bias – assessing whether the same selection criteria have been used for all participants (patients and control group)

the loss to follow-up –assessing whether the loss to follow-up differs between the exposed and the unexposed the group

information bias – assessing whether the same quality and extent of information is available from the exposed and the unexposed groups

2.3.6 Statistical analysis

Due to the large heterogeneity of studies measuring ovarian reserve in young women with cancer, I was unable to perform quantitative synthesis of the results. Instead, I have provided a descriptive summary of the selected studies.

2.4 Results

2.4.1 Study selection

I screened 42 papers, 35 full text articles were assessed for eligibility, and finally 15 studies were included in qualitative synthesis **(Figure 5).**

Excluded studies were excluded for the following reasons: not-systematic review (Letourneau *et al.*, 2013), studies including a predominantly peri-menopausal age group were fertility potential could not be readily assessed (Meng *et al.*, 2013), or co-treatment with a GnRH agonist (Franco *et al.*, 2012; Giuseppe *et al.*, 2007; Park *et al.*, 2010; Su *et al.*, 2013), one review article on fertility conservation (Lawrenz *et al.*, 2011), studies describing childhood cancer (Bath *et al.*, 2003; Larsen *et al.*, 2003b; Beek *et al.*, 2007, Lie Fong *et al.*, 2009; Brougham *et al.*, 2012; Gracia *et al.*, 2012; El-Shalakany *et al.*, 2013; Nielsen *et al.*, 2013), gynaecological cancers (Iwase *et al., 2012*), a small study (N=6) which included patients on hormonal therapy (Dieudonne *et al.*, 2011), and

studies assessing only baseline AMH prior to any gonadotoxic therapy (Lawrenz *et al.*, 2012; Su *et al.*, 2013)

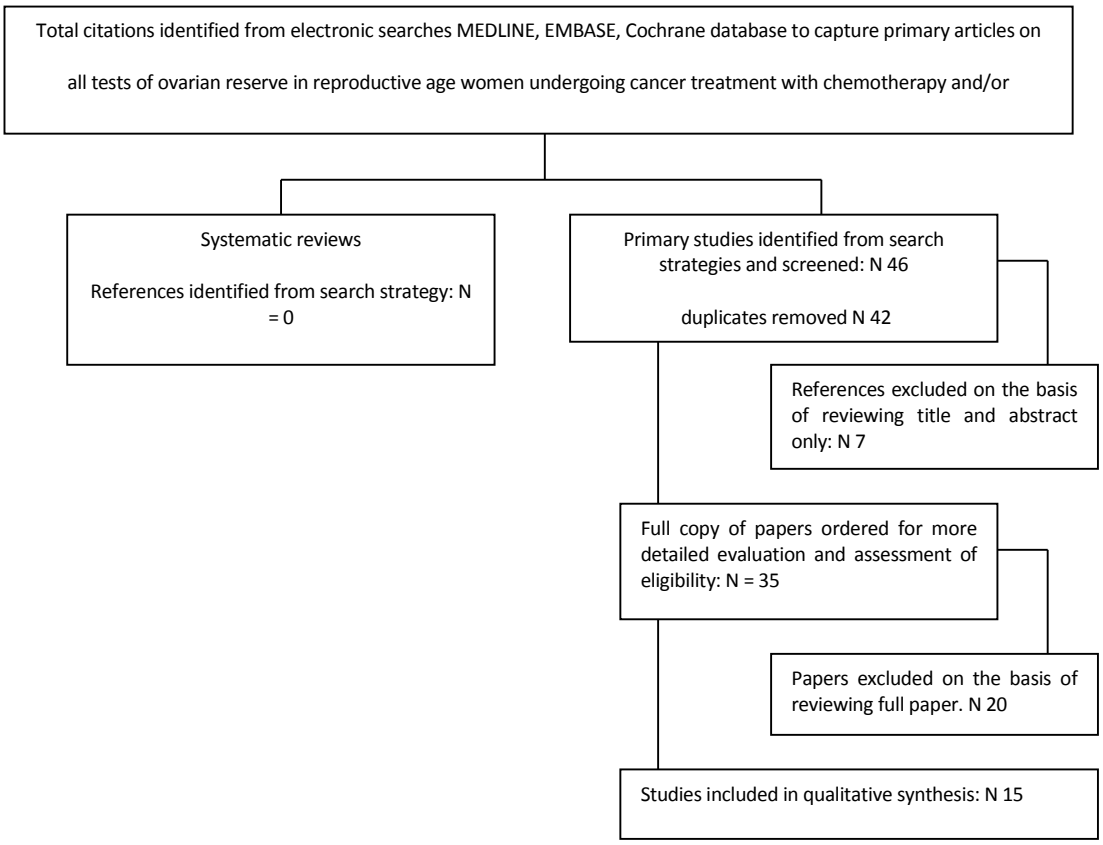


Figure 5 Identification of literature for systematic review of ORTs in reproductive age women with malignancy treated with chemotherapy.

Table 3 Study characteristics: number of cancer patients, mean age, cancer type and inclusion/exclusion criteria are presented.

References	N	Mean age	Cancer type	Inclusion	Exclusion
Anders et al. 2008	44	40 years	Breast Ca	>2 menses in 180days, Operable cancer	Pregnancy, Contraception, Ovarian tumour
Anderson et al. 2006	42 +14	41 years	Breast Ca	Regular menses or normal FSH, Operable cancer	Metastases
Anderson et al. 2011	42	42 years	Breast Ca	Regular menses or normal FSH, Operable cancer	Hormonal contraception
Anderson et al. 2013	59	42 years	Breast Ca	Regular menses or normal FSH, Operable cancer	Previous chemotherapy, oophorectomy
Behringer et al. 2012	562	32 years	Hodgkin lymphoma	Age less than 40, remission >1 year	Other trial medications
Decanter et al. 2010	30	24	Hodgkin, non-Hodgkin lymphoma	Not specified	Removal of ovary for cryopreservation
Dhillon et al.	46	26	Any type of cancer	11-35 year old with presence of uterus	Pregnancy/post partum, lactation, chemotherapy
Di Paola et al. 2013	63	31	Haematological malignancy	Remission> 1 year	Oophorectomy/ hysterectomy, amenorrhea
Henry et al. 2014	27 (26)	41	Breast Ca	Menstruating women with breast Ca	Oophorectomy/ hysterectomy, treatment with radiotherapy/ GnRHa
Lie Fong et al. 2008	25	29 years	Haematological	Described for volunteers only	Described for volunteers only
Lutchman-Singh et al. 2007	22	Range 22-42	Breast Ca	Pre-menopausal women with breast Ca	Tamoxifen or GnRH agonist use
Partridge et al. 2010	20	36	Breast Ca	Menstruating women post-chemotherapy	Recurrence of Ca Use of tamoxifen
Rosendahl et al.2009	17	30	Any type of cancer	Age 18-35 prior to chemotherapy	Not specified
Su et al. 2010	127	45.3 years	Breast Ca	Premenopausal women with breast Ca	Hysterectomy/ oophorectomy
Yu et al. 2010	26	Range 27-40	Breast Ca	Premenopausal Women with breast Ca	Not specified

Table 4 Type of ovarian reserve test used in the study and time of follow-ups.

Study	Type of ORT	ORT before treatment	ORT after treatment	Time of ORT since cancer therapy
Anders et al. 2008	FSH, inhibin B, E2, AMH	Yes	Yes	3-7 weeks, 6 months and 12months
Anderson et al. 2006	FSH, LH, E2, inh B, AMH AFC	Yes	Yes	3, 6, 9 and 12 months
Anderson et al. 2011	FSH, LH, E2, inh B, AMH AFC	Yes	Yes	At 2, 3, 4, 5 years
Anderson et al. 2013	FSH, inhibin B, AMH	Yes	Yes	At 1 and 2 years from start of chemotherapy
Behringer et al. 2012	FSH, LH, inhibin B, AMH	No	Yes	12- 46 months
Decanter et al. 2010	AMH	Yes	Yes	3, 6, 9, 12 months form start of chemotherapy
Dhillon et al.	FSH, LH, E2, inh B, AMH AFC	Yes	Yes	Up to 12 months from chemotherapy
Di Paola et al. 2013	FSH, AMH AFC	No	Yes	1 to >11 years form chemotherapy
Henry et al. 2014	FSH, E2, inh B, AMH,	Yes	Yes	Up to 15 months post completion of chemotherapy
Lie Fong et al. 2008	FSH, E2, inh B, AMH, AFC	Yes	Yes	Between 1 and 11 years
Lutchman-Singh et al. 2007	FSH, E2, inh B, AFC, AMH, dynamic tests of OR	Yes	Yes	Immediately after completion of chemotherapy
Partridge et al. 2010	FSH, E2, inhB AMH, AFC	No	Yes	>1 year post-chemotherapy
Rosendahl et al.2009	FSH, E2, inhB AMH, AFC	Yes	Yes	Up to 5-12 moths from treatment dose
Su et al. 2010	FSH, E2, inhibin B, AMH	No	Yes	Median 2.1 years from chemotherapy
Yu et al. 2010	FSH, E2, AMH	Yes	Yes	Up to 52 weeks

Table 5 Study design, control group, description of chemotherapy and outcome measures.

References	Study design	Control group	Chemotherapy regimen/dose provided	Outcomes measure
Anders et al. 2008	Prospective observational	No	Yes/no	Changes in levels of ORTs
Anderson et al. 2006	Prospective observational	No	Yes/Yes	Changes in levels of ORTs
Anderson et al. 2011	Prospective longitudinal	Yes, women with breast Ca, no chemotherapy	Yes/Yes	Changes in levels of ORTs Amenorrhea
Anderson et al. 2013	Prospective longitudinal	No	Yes/Yes	Changes in levels of ORTs Amenorrhea
Behringer et al. 2012	Cross sectional	No	Yes/Yes	Differences in ORTs between 3 chemotherapy groups
Decanter et al. 2010	Prospective longitudinal	No	Yes/Yes	Differences in ORTs between 2 chemotherapy groups
Dhillon et al.	Prospective longitudinal	No	Yes/No	Changes in levels of ORTs
Di Paola et al. 2013	Cross sectional	Yes	Yes/yes	Comparison of ORT with controls
Henry et al. 2014	Prospective observational	No	No/no	Changes in levels of ORTs
Lie Fong et al. 2008	Retrospective (cohort)	42 healthy volunteers with proven fertility	No details	Changes in levels of ORTs, Amenorrhea
Lutchman-Singh et al 2007	Longitudinal, n=8, Cross-sectional n=14	Healthy volunteers with proven fertility	Yes/Yes	Changes in levels comparison with healthy volunteers
Partridge et al. 2010	Cross sectional	Yes	Yes/	Comparison of ORTs with healthy volunteers
Rosendahl et al.2009	Prospective observational	No	Yes/Yes	Changes in levels of ORTs,
Su et al. 2010	Case control study	Yes	Yes/Yes	Menses/no menses Comparison of AMH, in 2 groups
Yu et al. 2010	Prospective cohort	Yes, subfertilite (male factor)	No/no	Changes in levels of ORTs,

2.4.2 Overview of the selected studies

In the study by Lie Fong et al. (2008), historical serum samples obtained prior to and post-chemotherapy from young women diagnosed with any haematological malignancy were thawed to analyse FSH, LH, E2, inhibin and AMH levels. The patient group consists of ten patients who received treatment with alkylating agents, three who had different chemotherapy regimens and 12 who received total body irradiation. The control and cancer patient groups had AMH levels tested using two different assays. The methods of handling serum samples were not described although this is a currently known factor affecting AMH measurements. The timing of follow-up samples was between 1 and 11 years. The research group concluded that all patients who underwent total body irradiation had undetectable AMH and developed premature ovarian failure. In the remaining group of 13 patients treated with chemotherapy alone, AMH levels as well as AFC were lower compared to a group of healthy volunteers with regular menstrual cycles and proven fertility. Considering the small sample size, and use of a 'correction factor' to compare AMH levels in both groups, the heterogeneity of haematological conditions, and the wide window within which follow-up samples were measured, then the precision of the results and their reproducibility could be questioned (Lie Fong *et al.*, 2008).

Gonadal function sub-analysis within the German Hodgkin Study Group HD13 and HD15 Trial is one of the biggest studies describing differences in hormone

levels between Hodgkin lymphoma survivors receiving different chemotherapy regimens. Although a significant proportion of women was on GnRH analogues during chemotherapy and also, hormone levels were tested at any time between 12 and 46 months follow-up, the results are still worth taking into consideration. In the enrolled group of Hodgkin lymphoma survivors, 562 were female. The study results showed differences in AMH levels between women who received only ABVD and women on the BEACOPP protocol. In the group of women who received BEACOPP, AMH levels were undetectable regardless of age. In contrast, women on ABVD and aged less than 30 years had higher AMH levels (>2 µg/L) as compared with women above 30 years old. The data lacks a longitudinal analysis and comparison with healthy volunteers and/or with hormone levels prior to treatment. The large number of patients studied gave definitive confirmation of the reduced gonadotoxicity of the ABVD regimen as compared to other regimens (Behringer *et al.*, 2013a).

Similarly, Decanter et al. (2010) measured AMH levels in a very young group of women (mean age 24), treated with ABVD and non-ABVD regimens for lymphoma. Serum AMH levels were measured prior to and up to 1 year after treatment. In the ABVD group n=17, AMH was found to be significantly higher than in the non-ABVD group n= 13 at 3, 6, 9 and 12 months of follow-up. Although the study was conducted on a small group of patients without specified inclusion criteria, it highlights the value of AMH measurements and once more confirms the lower gonadotoxicity of ABVD regimen (Decanter *et al.*, 2010).

Di Paola et al. (2013) conducted a study of ovarian reserve tests amongst haematological cancer survivors compared with healthy volunteers. The study included 63 patients with various haematological conditions at remission between 1 and >11 years. It showed that ORTs results (FSH, AMH, AFC) were significantly different in the cancer group and healthy controls. Although the sample size is much higher than most of the studies presented earlier, concerns may be raised regarding the wide range of times when AMH was tested in relation to chemotherapy. Moreover some patients underwent total body irradiation which may have affected the AMH results (Di Paola *et al.*, 2013) .

Dillon et al. (2013) evaluated ovarian reserve markers including FSH, LH, E2 inhibin B and AMH in a heterogeneous population comprising 46 women with different types of cancers including breast cancer, brain tumour, sarcoma and haematological malignancies. Patients receiving different types of chemotherapy were followed up every 3 months. The rate of post-chemotherapy recovery of ovarian function was based on the estimated monthly recovery of AMH in two groups of patients with pre-chemotherapy AMH of ≤2 ng/ml and ≥ 2 ng/ml. The research group concluded that the rate of recovery of AMH post-chemotherapy was associated with pre-chemotherapy levels of AMH. Those findings, if proven in a large well designed study, could provide a basis for AMH measurements during consultations about fertility preservation, although the heterogeneity of the population makes clinical application of results of this particular study difficult (Dillon *et al.*, 2013).

Rosendahl et al. (2010) provided interesting data, in line with the above findings. The authors investigated 17 women with breast Ca or haematological malignancy before, during and up to 1 year after chemotherapy. It was shown that higher pre-treatment serum concentrations of AMH were predictive of higher post-treatment levels. It was also one of the first studies providing a description of acute hormonal changes during chemotherapy. The study provided interesting theories on the impact of chemotherapeutic agents on granulosa cells in the first week post chemotherapy, however, longitudinal data analysis of this study may be affected by the fact that most of the patients underwent unilateral oophorectomy prior to or after chemotherapy which itself lowered the ovarian reserve (Rosendahl *et al.*, 2009).

In patients with breast cancer, there is more substantial evidence for the usefulness of ovarian reserve tests, especially AMH. In his pioneer study, Anderson et al. (2006) included women with operable breast cancer aged 28-52. The ORTs were performed before and 3, 6, 9 and 12 months after chemotherapy. Amongst the recruited women, 42 received one of the four chemotherapy regimens (the remaining 14 had GnRH and/or Tamoxifen only). In the chemotherapy group, AMH fell rapidly during chemotherapy and remained low while inhibin B levels dropped only by around 50%. E2 levels were relatively well maintained while gonadotropins were significantly raised. AFC fell, but the changes remained modest. The authors concluded that their data confirmed the value of AMH as a marker of ovarian aging induced by

chemotherapy and its usefulness in assessing the degree of gonadotoxicity caused by different chemotherapeutic agents (Anderson *et al.*, 2006a).

In the study by Anders et al. (2008), a similar group of patients was recruited. Forty four menstruating women with operable breast Ca were examined prior commencement of chemotherapy; 32 of them had post-chemotherapy levels checked, with only 21 at final 12 month follow-up. The limitations of this study included the small samples size, large loss at follow-ups and heterogeneity of chemotherapy regimens. In summary, the study illustrated that pre-chemotherapy concentrations of AMH and inhibin B are lower in women who subsequently develop chemotherapy-induced amenorrhea. The authors suggested that AMH could be used as a marker to assess individual risks of amenorrhea for patients undergoing chemotherapy (Anders *et al.*, 2008).

Yu et al. (2010) evaluated ovarian reserve markers in 26 women aged <40 with breast Ca prior to chemotherapy and follow-up for up to 52 weeks post chemotherapy. In contrast to the results provided by Anders et al. 2008 and Anderson et al. 2006, this research group did not find a correlation between pre-chemotherapy AMH levels and the risk of chemotherapy-related amenorrhea (CRA) at the final 52 weeks follow-up. Both groups, women with CRA and menstruating women, had similar AMH levels prior to chemotherapy. Levels of E2 and FSH had plateaued at final follow-up while AMH levels remained lower as compared to pre-chemotherapy levels (Yu *et al.*, 2010).

Su et al. (2010) enrolled women ~2 years post chemotherapy for breast Ca and measured AMH, FSH and inhibin B. The results were compared with an age-matched control group. Overall, the treatment group was found to have lower AMH (p=00.4) and inhibin B (p<0.01) than healthy volunteers. Women who were still menstruating following chemotherapy were found to have higher AMH (p=0.03) than those who developed CRA. The researcher group postulated the use of AMH and inhibin B to predict post-chemotherapy ovarian function (Su *et al.*, 2010).

In a small study by Lutchman Singh et al. (2007), 8 patients (age 22-24) with breast Ca were recruited prior to chemotherapy and had FSH, E2, inhibin B, AFC/ovarian volume and AMH tested at baseline. Immediately following completion of chemotherapy, the ORTs were repeated. Although, AMH was found to be lower post-chemotherapy, the results did not reach statistical significance. Similarly, the differences in levels of the remaining biochemical markers did not reach statistical significance. The second cross-sectional arm of the study included healthy controls. FSH, AMH and inhibin B were found to be significantly different between women post-chemotherapy and the volunteer group. Additionally, the research group postulated use of dynamic ovarian reserve testing because baseline markers were found to be significantly different between post-chemotherapy patients and healthy controls (p<0.05) (Lutchman Singh *et al.*, 2007).

In a study by Partridge et al. (2010), ovarian reserve markers including FSH, E2, inhibin B, AMH, AFC were evaluated in 20 breast cancer survivors and compared with 20 healthy volunteers. The inclusion criterion in the breast cancer group were that women were still menstruating post-chemotherapy (at least one menstrual bleeding since chemotherapy). The results confirmed a statistically significant difference in levels of biochemical markers of ovarian reserve and AFC between the two groups. It confirms that women who regain menstrual cycles post-chemotherapy may still have a diminished ovarian reserve. In this study, AMH and AFC were found to be the most sensitive markers of reduced ovarian reserve in the cancer population ($p<0.0004$) (Partridge *et al.*, 2010a).

Henry et al. (2014) examined ORTs in 27 women aged 25-50 with newly diagnosed breast cancer. Prior to chemotherapy, 19 out of 26 patients had detectable AMH. Additionally, 1 patient underwent bilateral oophorectomy and was excluded from follow-ups. Only 13% of patients had detectable AMH post-chemotherapy. The authors concluded that older age and undetectable pre-treatment levels of AMH are predictive of premature ovarian failure at 18 months of follow-up (Henry *et al.*, 2014).

In a prospective longitudinal study, Anderson and Cameron (2011) prospectively assessed ovarian function in women with breast cancer before, and 2, 3 and 5 years after chemotherapy. Pre-chemotherapy concentrations of

AMH were higher in women still menstruating at 5 year follow-up as compared to women reporting amenorrhea (2.5 ± 0.4 vs 0.7 ± 0.1 ng/ml, $p < 0.0001$). All participants with AMH less than 1.9 ng/ml prior to chemotherapy reported amenorrhea at final follow-up. A multivariate logistic regression model assessing the value of age and other ovarian reserve markers in the prediction of ongoing menses post-chemotherapy showed that only AMH was a statistically significant predictor (Anderson & Cameron, 2011).

In his next study Anderson et al. (2013) prospectively examined AMH in women with breast cancer and followed them up for 2 years. The authors compared mean AMH in women with the return of menses (9 patients) versus women with no menses (30 patients) at their 2 year follow-up. Women with menses at follow-up had statistically higher mean AMH pre-chemotherapy as compared to women with no menses. It is worth noting that all women had undetectable AMH at 1 year of follow-up (Anderson *et al.*, 2013).

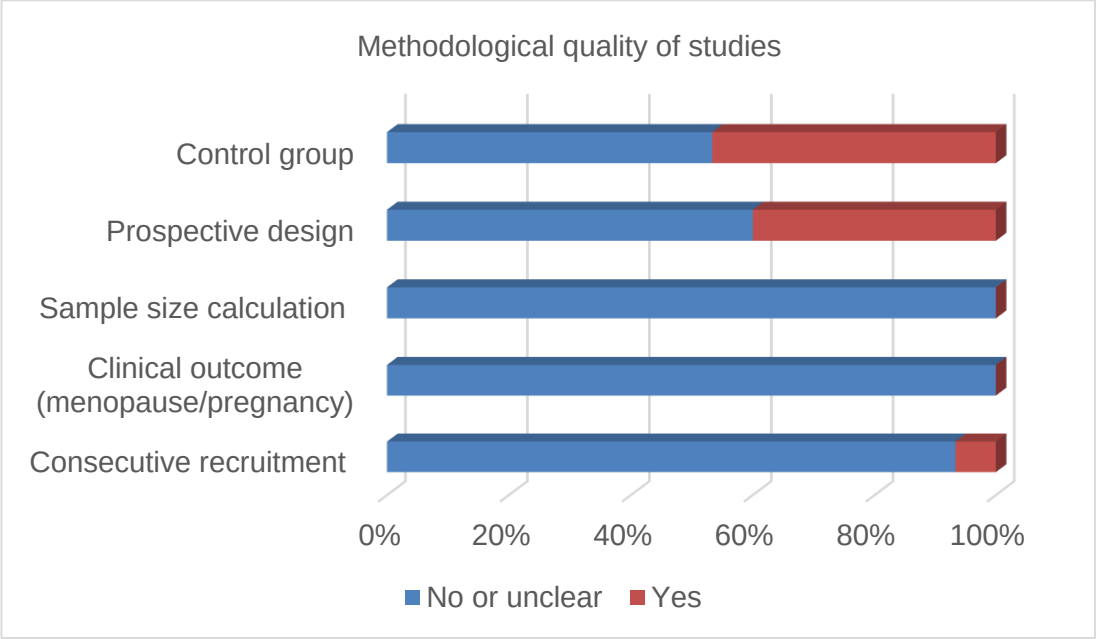


Figure 6 Methodological quality of studies included in the systematic review of ORTs in women treated with chemo/radiotherapy.-

2.5 Discussion

2.5.1 Summary of main findings

There were only a limited number of studies, mainly small cross sectional and observational studies, assessing ovarian function in pre-menopausal women with malignancy undergoing gonadotoxic treatment. In all studies, ethical aspects and the rarity of the condition/exposure restricted the design and data collection. Overall, the selection criteria for the cancer populations were wide and not clearly specified in most of the studies. Chemotherapy regimens were

poorly described in some of the studies (e.g. no information about the cumulative dose of chemotherapy used or its duration, repeated courses of chemotherapy). The quality of the various studies was also affected by poor reporting in the respective methods' sections. I found no agreed test(s) of ovarian reserve that could be used as a gold standard in the cancer population. Although AMH is the most widely recommended test, the timing of testing in relation to chemotherapy varied amongst the selected studies.

None of the manuscripts correlated their ORT results prospectively with tangible outcomes such as premature menopause, pregnancy or live birth rates. The control groups varied in the studies and included healthy volunteers with proven fertility, cancer patients not receiving chemotherapy, or patients seeking fertility treatment for male factor infertility. Additionally there was no agreed time interval for when to assess or test the ovarian reserve in the context of cancer therapy.

In summary, the results showed that there is no single widely agreed test of ovarian reserve or combination of tests in reproductive age women undergoing chemotherapy/radiotherapy, which might be used as a gold standard and producing results that would be reliable in predicting early menopause and chances of pregnancy. In this review, an accurate comparison of ovarian reserve tests and assessment of their accuracy for pregnancy or live birth prediction in cancer patients was not possible due to lack of data. Moreover, the

lack of consecutive recruitment and wide inclusion criteria make clinical interpretation and application of such results difficult.

2.5.2 Limitations

The clinical applications of such tests have been rare, and most of the published studies were pioneering. Published studies vary significantly in design and population selection which makes a synthesis of the results difficult. The ORT were measured within a wide window of follow-up periods (from weeks to 11 years post-chemotherapy).

2.5.3 Conclusion

To date, the clinical application of ORTs has been rare. However, young women with cancer should be individually counselled about their treatment's gonadotoxicity, the risk of early menopause and reduced ovarian reserve. Given the disparate evaluation of ORT in young women with cancers, it is timely that a rigorous prospective longitudinal study evaluating the different tests be carried out so that an accurate test or a combination of tests could be used in clinical practice.

CHAPTER 3

3 Methodology

3.1 Introduction

In this chapter, I provide details of protocol development, followed by approvals and participants' recruitment procedures. Next, the methodology used for assay of AMH in serum is described in detail together with the techniques used to assure quality in handling blood samples. Finally, the methods of data and record management are presented, and the statistical methods that were applied following expert advice from professional statisticians, Professor Nigel Stallard and Dr Nick Parsons, of Warwick Medical School.

3.2 Protocol development

The protocol was developed following a review of the literature and having solicited expert opinion from a range of stakeholders, including patients.

Stakeholders included Haematology and Oncology Consultants, especially Dr Fiona Clark based at University Hospitals Birmingham NHS Foundation Trust and Professor Chris Poole based at University Hospitals Coventry and Warwickshire NHS Trust (UHCW). We discussed the impact of the study on patients' care, especially in the context of minimizing any form of intervention at the difficult time of cancer diagnosis. We planned follow-up blood samples according to the 'routine' follow-ups for cancer patients and decided that it would be best to avoid patients being asked to travel for additional tests (e.g. transvaginal ultrasound).

As the study was funded by a private IVF clinic, we had to make sure that our project was not seen as an advertisement for fertility preservation services. Additionally, the study design was observational, and we aimed to assess current practice in the UK without increasing any fertility preservation interventions through participation in our study.

I discussed with my Supervisors if I should be directly approaching and recruiting patients into the study. Due to the relative rarity of malignant conditions in young women, we agreed that it would be more practical and

would allow more patients to be recruited if I had support from research nurses at multiple sites. Therefore, I asked for support from the UK Clinical Research Network (UKCRN). In order to include my study into the UKCRN portfolio, I needed support from the Breast Cancer Studies Group first. I was invited to attend their meeting in London **(meeting protocol CSG/BC 10/41)** where the following particular issues were discussed.

- The need to limit the number of different chemotherapy regimens, where possible, to allow subgroup analysis of the data.
- The control group should consist of healthy volunteers, as distinct from patients with fertility problems.
- Restricting the study to mainly breast cancer and lymphoma would improve statistical analysis and reduce heterogeneity. In the final version protocol, I included mainly breast cancer and lymphoma patients, however the participation of patients with other malignancies (e.g. other haematological conditions) was allowed due to the pioneering character of the study in the UK. Rare cases of other cancers and the impact on fertility of their treatment could be reported as case studies/case series which could provide initial information for future research in this field.

I conducted multiple presentations about the research project for oncology nurses. The nurses raised some additional points (e.g. not repeating the same question about number of pregnancies with each medical questionnaire at 6, 9

and 12 month follow-ups because patients undergoing chemotherapy may find it inappropriate to be asked sensitive questions repeatedly). Therefore, I requested an amendment so that the question about pregnancy was asked at the time of recruitment and at 12 month follow-up only.

Further changes to the study design were a result of discussions at my upgrading meeting (as per University regulations, the initial registration of research degree students is for a masters degree). Professor Janet Dunn, who was the expert present at my upgrade, suggested that for the longitudinal observational design of the study, the most appropriate ratio of patients to controls would be 1:2. Therefore, I contacted the R&D Department and the research nurses at UHCW, requesting additional support with recruitment of volunteers, to increase the numbers and improve age matching between cases and controls.

The stakeholders agreed that there was a need for more studies in the field of oncofertility in order to improve information counselling prior to chemotherapy and to assess the risk of infertility and early menopause post chemotherapy. Most of the clinicians (Principal Investigators) thought that women generally wished for more accurate and detailed information tailored for themselves as individuals prior to chemotherapy.

The majority of the cancer patients who were consulted prior to the study recruitment stage said that having an additional blood sample taken before and

after chemotherapy to check hormone levels would not be too invasive or interruptive at this difficult and busy time of cancer diagnosis. However, some of them felt that travelling to an IVF unit for transvaginal ultrasound scanning of the ovaries for research purposes only, would not be appropriate.

An independent peer review was obtained from Professor Richard Anderson, University of Edinburgh, an expert in the field of oncofertility. One of the main points he discussed was supporting the decision not to exclude women with endocrine pathology (I was planning to exclude women with e.g. thyroid problems), but instead obtaining information on any medical conditions via questionnaire.

All of the above was discussed during meetings with my clinical and academic supervisors. The implemented changes were fed back to the oncology and haematology teams. The acceptance of the study protocol and the involvement of a number of regional oncology and haematology teams was widely discussed over a 10 month period **Appendix 2 (study protocol version 5).**

3.3 NHS Research Ethics Committee approval

At the time of my application for ethical approval, the Integrated Research Application System on line (IRAS) had recently been introduced (IRAS project ID 12828). The on-line application form completed by myself included questions on the research and its design, ethical issues, research procedures, risks and benefits, details of recruitment, management and informed consent.

The project was evaluated by Warwickshire Research Ethics Committee, and its view was that cancer patients should not be given the results of their AMH tests because the assay was not validated for this particular group of patients. Additionally, an abnormal result following chemotherapy could cause stress in cancer patients. Additionally, a drop in AMH levels during or immediately after chemotherapy may be transient and could recover with time.

Pending minor changes in the Oncology Team Form and consent form, conditional approval was granted by the Ethics Committee. Final confirmation was received on 01/12/2009.

3.4 R&D approval and UKCRN Portfolio approval

Once ethical approval had been granted, R&D approval was sought for each research site. This process proved to be time consuming because of recent centralisation of R&D approvals. As a result, many research nurses and/or Principal Investigators (PIs) were not familiar with IRAS site-specific information (SSI) forms. In order to expedite the R&D approval process, I completed draft SSI forms for each R&D site.

I approached and met the PIs on each of 13 sites and discussed details of recruitment as well as the protocol. The sites and PIs were as follows:

- Dr Fiona Clarke, Dr Andrea Stevens (Good Hope Hospital, The Heart of England NHS Foundation Trust and Queen Elizabeth Hospital, University Hospitals Birmingham NHS Foundation Trust),
- Dr Guy Pratt (Heartlands Hospital, The Heart of England NHS Foundation Trust),
- Professor Christopher Poole (University Hospital, University Hospitals Coventry and Warwickshire NHS Trust),
- Dr Rozenn Allerton (New Cross Hospital, The Royal Wolverhampton NHS Trust and Russells Hall Hospital, The Dudley Group NHS Foundation Trust),

- Miss Raghavan Vidya (Stafford Hospital, Mid Staffordshire Foundation Trust),
- Dr Jane Worlding (George Elliot Hospital NHS Trust),
- Dr Shazza Rehman (Airedale General Hospital, Airedale NHS Foundation Trust),
- Mrs Lynda Wagstaff (Walsall Manor Hospital, Walsall Hospitals NHS Trust),
- Mrs Jan Dodge (The Great Western Hospital, The Great Western Hospitals NHS Foundation Trust),
- Mrs Rebecca Foster (Pinderfields Hospital, Mid Yorkshire Hospitals NHS Trust),
- Dr Fiona Clark (Worcestershire Royal Hospital, Worcestershire Acute Hospitals NHS Trust),
- Dr Gillian Lockwood (Midland Fertility, Tamworth).

At the same time, I started the process of including my study into the UK Clinical Research Network Study Portfolio (UKCRN). As mentioned before, malignant conditions are relatively rare in young women. Therefore, it was important to recruit across several centres. NHS Portfolio approval was essential to obtain support from research nurses and help with recruitment. The NCRI Breast Cancer Studies Group supported the project and subsequently my study was included in the UK Clinical Research Network (UKCRN) Portfolio. An NIHR Clinical Research Network Portfolio account was set up for details to be viewed

by the public or other research teams under number UKCRN: 8445. The study trial registration number is: International Standard Randomised Controlled Trial Number Register: ISRCTN28988709

I attended a course on Good Clinical Practice for Chief Investigators (CI) at the University of Warwick. Since all students are registered as Masters Students for their first year, I could not become a CI (University of Warwick regulations). My supervisor, Professor Geraldine Hartshorne assumed the role of CI, allowing me to be relatively independent in coordination and administration of the project.

3.5 Training of trial centre staff

When all approvals were in place, I conducted training sessions for all the research nurses on AMH and ovarian reserve. I also discussed the details of recruitment. The main points included, for example, log books, transport of samples in a timely manner and the need for completion of the medical questionnaire at the time of the blood test. I provided folders including flow charts, recruitment charts, the study protocol and general information on AMH. The research teams on each site were provided with research packs which included an instruction letter, medical questionnaire, MFS blood analysis request form, blood tube, plastic bag (DGP PATHOSEAL), boxes for safe transport of biological substances (biological substance category B, UN3373), pre-paid envelopes and oncology team forms.

I discussed the need for timely follow-ups with each research nurse and PI. It was agreed that breast cancer patients would have routine follow-ups at 6, 9 and 12 months in most centres, which was why these intervals were selected for my protocol. In general, haematology teams tended to see patients more frequently for their follow-ups and the exact dates are decided on an individual basis. The haematology teams agreed to follow the same protocol for follow-ups as the breast cancer teams. I encouraged each team to have a system in place to remind them about follow-ups.

To minimize any burden of additional appointments for participants, 6 weeks leeway for appointments was accepted.

3.6 Study design

A prospective cohort study design was used to measure the effect of chemotherapy on ovarian function over 5 years. However, for this thesis, the assays and analysis of AMH have been performed over a shorter period (1 year), in keeping with an MD time scale. I plan to follow up the research participants for at least 5 years, to assess their post-chemotherapy fertility, using pregnancy, live birth and early menopause as end points for the whole project.

Serum AMH was measured in patients before starting the treatment with potentially gonadotoxic chemotherapy and at follow-up intervals of 6, 9 months and 1 year. The results were compared with an age-matched group of healthy control women without known fertility problems.

The following eligibility criteria were applied:

- Patients aged 18-43 with newly diagnosed cancer, prior to any chemotherapy/radiotherapy
- For the control group, volunteer women of the same ages were recruited, without known significant medical problems or previous exposure to chemotherapy/radiotherapy

The following exclusion criteria were applied:

- Previous chemotherapy/radiotherapy
- Age below 18
- Age above 43
- End stage cancer patients with very poor prognosis
- Known significant ovarian pathology

Patients over the age of 43 were excluded because they were reaching the end of their reproductive life and had reduced prospects of pregnancy (less than 5%). Patients with a poor prognosis (less than 10% chance of 1 year survival) might find it inappropriate to be asked to take part in a project which assesses their potential future fertility. The oncology teams had flexibility (based on their experience and clinical judgement) to decide when to approach patients. Women with known significant ovarian pathology were excluded because it might prove difficult to distinguish the effects of chemotherapy from the natural progression of ovarian pathology. Women with known PCOS (or PCOS diagnosed as a result of participation in our study) or oophorectomy were not included in the analysis. Information on any additional medical conditions was collected via medical questionnaire and the oncology team form. The latter was completed by PIs or research nurses directly from the patients' medical records.

3.7 Recruitment

3.7.1 Multicentre recruitment of cancer patients

Female patients meeting the eligibility criteria, who were about to proceed with chemotherapy, (mainly with breast Ca and lymphoma) were identified by participating oncology and haematology research teams within the West Midlands and Yorkshire. An information leaflet describing the study and its objectives was circulated to all prospective patients by oncology teams **Appendix 3 (Patient/volunteer information leaflet and consent form).** A consent form and a medical questionnaire including obstetric and gynaecological history, were completed in the oncology clinic together with a research nurse. Alternatively, patients were requested to complete the forms at home and return them using pre-paid envelopes.

Data collected from the medical questionnaire included: age, regularity of menstrual cycles, parity, history of gynaecological or fertility problems, family history of early menopause, body mass index (BMI), smoking habits, hormonal contraception and medications **Appendix 4 (Medical questionnaire/Follow-up medical questionnaire)**. An additional question was asked about patients' wishes for pregnancy. Patients described their desire for pregnancy on a scale

from 1-4 (1–no, 2-neutral, 3-maybe, 4-strong). Patients were given the option to write comments.

All site PIs and research nurses who were direct patients' clinicians, provided written information on the number of patients who were referred to a specialist for discussion about fertility preservation options. Additional information on the type and stage of cancer, dose, duration, and the number of courses of chemotherapy and radiotherapy was provided by the oncology teams from the medical records. All participants had a blood sample taken together with a detailed questionnaire before starting any chemotherapy.

Recruitment of the first patients began in UHCW and QE in the last week of May 2010, while the R&D approval process continued in other centres. The last R&D approval was obtained on 20th August 2011 for Pinderfields Hospital (Mid Yorkshire).

3.7.2 Recruitment of volunteers

An age-matched control group was recruited into the study in order to compare the changes in AMH levels between patients undergoing chemotherapy and those receiving no gonadotoxic treatment over the same period.

The control group consisted of healthy, reproductive age women, aged 18-43 years, who responded to an advertisement at UHCW. The advertisement was

conducted via intranet following approval from Trust R&D and Medical Director. More detailed information about the study was given verbally by research nurses at UHCW and by providing a written information leaflet. Volunteers wishing to proceed were asked to complete a consent form.

3.7.3 Provision of results to volunteers

Following the initial submission to NHS REC, a new, second generation AMH assay was for the first time registered for diagnostic use. The previous, first generation assay had been registered for research use only. Following discussion with my supervisors, I submitted an amendment, approved on 05/03/2011, allowing the AMH results to be disclosed to healthy volunteers only. As agreed earlier at the ethics committee meeting, AMH results were not provided to cancer patients because the assay was not validated for that particular group.

The results letter for volunteers was approved by the UHCW R&D department, following discussion with the local research nurses, specialists in Reproductive Medicine, and Consultant Obstetrician and Gynaecologist, Mr Tarek Ghobara. In order to support the volunteers in dealing with abnormal AMH results, the following protocol was agreed with UHCW R&D:

- Volunteers wishing to discuss their results contacted the research nurse, who passed their details to the CI, Professor Hartshorne.
- Professor Hartshorne contacted those in receipt of an abnormal result to discuss their concerns and to offer them the opportunity to be referred to an Infertility Specialist, for further investigations.

- If required, an appointment with Mr Tarek Ghobara, Consultant Obstetrician and Gynaecologist, was arranged at the Centre for Reproductive Medicine at UHCW, after prior agreement by the volunteer's GP. During that consultation, the woman had the opportunity to discuss the implications of the abnormal AMH results and if necessary, additional blood tests and a transvaginal ultrasound scan were offered, to further investigate the ovarian reserve. Reassurance was provided, and a course of action was discussed and documented. Where appropriate, the GP was provided with guidance on further management.

3.8 Data and record management

Upon arrival at the MF Laboratory, identifiable data about the participants (name, date of birth, address, and contact telephone number) were recorded on a separate (paper based) database and a unique research reference number was applied to each blood sample. Identifiable data were stored in the MF medical records area and were accessible only by responsible persons in connection with the research. Anonymised clinical data were analysed and stored on a University of Warwick database. A log book at each research site was completed by the PI and dedicated research nurse.

3.9 Blood sample handling and assay methodology

All participants underwent venepuncture at an unspecified time of the menstrual cycle. Cancer patients had their samples obtained before the first dose of chemotherapy. Whole blood was collected in serum tubes and sent to MFS by first class post. Samples that arrived more than 48 hours after collection were not used, and venepuncture was repeated if possible. Samples were processed on the day of receipt, rendered acellular by centrifugation and then the serum was stored in 3-4 aliquots at -20°C. The cellular fraction was discarded. Serum aliquots were thawed on the day of analysis. AMH was measured according to MFS protocol LO77 using the AMH Gen II assay. MF is an approved laboratory for AMH assay, and regular quality checks were performed. Each assay procedure included both AMH Gen II ELISA (enzyme-linked immunosorbent assay) controls and independent external controls provided by the Assay and Control Company Ltd. Controls were used across the plate to assure quality for all samples measured with each kit. Researchers and laboratory personnel were blinded to either patient clinical progress or AMH concentration. The results were not used to manage the patients.

Blood samples (biological substance, category B) sent by first class post from any of the 13 centres, were normally received at MFS within 24 hours. In rare cases of delay (more than 48 hours), the research nurse was contacted to request a repeat sample.

During the initial phase of recruitment, I was actively involved in handling the samples and performing AMH assay at the MF laboratory. In the later stages of my study, I concentrated on coordinating sample arrival, administrative tasks related to blinding, filing of patients' consent forms, medical questionnaires and oncology team forms and transferring data to electronic database. During this period, assays were performed by Jo Milner and Lynne Harrison (Biomedical Scientists at MF Laboratory). For each assay, a full calibration curve, high and low level controls were documented, and a summary results form completed.

3.10 Changes in AMH assay methodology

For the purposes of this study, all participants' samples were analysed using Beckman Coulter Active AMH Gen II ELISA assay. At the initiation of the study, this assay had replaced the discontinued DSL Active AMH ELISA assay (first generation). The second generation assay used the same antibodies but was CE marked and intended for in vitro diagnostic use. Second generation assay values were 40% higher than the DSL assay as confirmed in a multicentre study (Wallace *et al.*, 2011).

Sometime later, a field safety notice, FSN 17971, was issued by Beckman Coulter (November 2011), recommending a change from linear to cubic regression curve fit protocol for reporting AMH values. In response, 74 study samples were re-analysed using both regression plots. The results for cubic

regression were found to be 12% lower than linear regression. Therefore, all ranges were recalculated **Appendix 5 (Linear versus cubic regression models)**.

A second safety field notice was issued in June 2013 when all the study samples were already analysed, and statistical analysis was being undertaken. The report suggested that results using the second generation assay may be lower than expected due to complement interference. The company provided instructions for a modification of the protocol including an initial 'dilution' step.

Following this safety notice, I conducted a pilot study assessing the correlation between the new 'diluted protocol' (adding 300 µl of assay buffer prior to sample analysis) and old method using selected study serum samples (ensuring that the samples of serum analysed had not previously been thawed). It showed that the new method of analysis gave consistently higher AMH results than the previous method but fixed corrective additive adjustment would be sufficient **Appendix 6 (Comparison of two methods of AMH assay**). Eventually, following discussion with a representative of Beckman Coulter, my supervisors and a statistician, all the study samples were re-analysed in duplicate using the new diluted protocol to assure reliability of the study results.

3.11 Assay methodology

Below, I have described the final assay methodology used in this dissertation. The AMH assay was a sandwich enzyme linked immunoassay. The microtitration wells were coated with anti AMH antibody which bound the AMH. A second biotin labelled antibody bound to the AMH at a different site. Streptavidin enzyme conjugate attached to the biotin which acted as a catalyst when the tetramethylbenzidine (TMB) substrate was added. The addition of stopping solution terminated the reaction due to acidification. Dual wavelength absorbance measured at 450 nm and 600-630 nm was proportional to the AMH concentration. The calibration curve of absorbance was plotted against AMH concentrations.

The first step was to allow the contents of the reagent kit, calibrators, controls and samples to reach room temperature. Following preparation of the worksheet recording batch numbers and conducting the necessary checks, dilution of 60 µl of calibrators, controls and samples with 300 µl of assay buffer into labelled centrifuge tubes took place (pre-dilution was the new step added following safety notice REF 79765). In the next step 120 µl of diluted standards, controls and samples were added and incubated on the plate shaker at 600rpm at room temperature for 1 hour. The procedure was repeated a total of 5 times.

Subsequently, 100 µl of the antibody-biotin conjugate was added to each well and incubated on the shaker for 1 hour, as before. After that, 100 µl streptavidin-enzyme conjugate was used, following incubation on the shaker for 30 min before the next wash. Final steps included adding 100 µl of TMB chromogen solution and incubating as before for no longer than 7 min and using forward pipetting to improve mixing of 100 µl of stopping solution. After stopping the reaction with sulphuric acid, the degree of enzymatic turnover of the substrate was evaluated using a spectrophotometer. All steps and timings were recorded.

3.12 Assay Characteristics

The characteristics outlined below are based on work by Kumar et al., 2010 on the development of the second generation AMH assay.

The limit of detection, described as the lowest amount of AMH in a serum sample, which could be detected with a 95% probability, was 0.08 ng/mL (0.57 pmol/L). The limit of quantification (also called the functional sensitivity) i.e. the lowest dose that could be measured quantitatively, was 0.16 ng/mL (1.14 pmol/L) (Kumar *et al.*, 2010).

There was no evidence of cross reactivity between AMH and FSH, LH, inhibin A, activin A found in an experimental pilot study (Kumar *et al.*, 2010). Interestingly, human antispecies antibodies have been reported to interfere with first generation immunoassays, by generating false positive results. This could

be particularly important in patients with breast cancer taking Herceptin (monoclonal antibodies) and could generate unusually high results in this group of patients. To avoid the problem, in the second generation assay heterophilic blockers were used (Kumar *et al.*, 2010).

When I was setting up the study, there was little information published on the optimal handling of samples to ensure the stability of AMH measurements. Therefore, I conducted a pilot study on sample handling which is presented in Chapter 4, Results.

3.13 Statistical methods

Initial statistical support for the study design and power calculation was provided by Prof Nigel Stallard, Professor of Statistics, Warwick Medical School.

3.13.1 Power calculation

The power calculation was performed based on the sample size and previously reported mean AMH levels (±SD). The calculation was performed using the online power calculator http://www.quantitativeskills.com recommended by the University of Warwick. The calculation was based on the estimated study sample (50 cancer patients, 100 volunteers). The sample mean was derived from published papers (Anderson *et al.*, 2006b; Steiner *et al.*, 2011). Based on the study sample size, with 80% power and alpha error of 0.05, the study was powered to detect a difference in AMH levels of 0.14ng/ml and to detect a difference of 0.16ng/ml with 90% power.

3.13.2 Statistical Analysis

Final analysis of the AMH assay results was performed with assistance from Dr Nick Parsons from the Statistics and Epidemiology Group at Warwick Medical School. Statistical analysis was conducted using R freeware by Dr Parsons. I have performed the more basic statistical analysis using SPSS.

The mean ages of the control and cancer groups were calculated. As some cases did not have exactly age-matched controls, following discussion with Dr Parsons, the decision was made to analyse data as an unmatched case control study and adjust for age imbalance during the statistical analysis.

The AMH levels were log transformed to normalise a right skewed distribution. Continuous variables were compared using the Tukey multiple comparisons of means while categorical variables were presented as a proportion and compared using Pearson's Chi-squared test.

A logistic regression model was built where the outcome (dependant variable) was used as an indicator for the groups: cases and controls. The univariate analysis allowed the selection of statistically significant variables ($p<0.05$) and construction of multiple logistic regression models adjusting for any confounding factors. The decline in AMH concentration with age in three subgroups: volunteers, breast cancer and lymphoma patients, was presented using fitted regression models. Boxplots were used to present log transformed AMH concentrations in relation to the stage of breast cancer.

A prediction model was built to estimate differences in serum AMH concentrations between the healthy volunteer group and the cancer patients according to their age at the point of recruitment.

For the longitudinal part of the study, a paired t-test was used to compare values between baseline AMH concentrations and follow-ups in both groups. The recovery of ovarian function post chemotherapy was presented as a percentage of detectable serum AMH in each group. A Pearson correlation coefficient was used to examine if pre-chemotherapy levels of AMH were associated with levels post-chemotherapy.

Assessment of cancer patients' desire at different stages of treatment to have children was analysed descriptively and presented as percentages.

McNemar test was used to test whether the level of desire to have children had changed during the follow-up period amongst cancer patients.

For the final part of the analysis, serum AMH concentrations in the healthy volunteer group were examined in the context of stages of the menstrual cycle, contraception use, BMI and smoking status by using linear regression model. Reliability of AMH assay was tested by using the coefficient of variation.

CHAPTER 4

4 Results: assay validation and sample handling

4.1 Introduction

Sample handling and storage for AMH were not described in detail in the assay manufacturer's instructions. Setting up a multicentre study, which required transportation of samples between sites, raised the question of reliability of results if blood samples were not processed immediately. Therefore, the first step of my study was to conduct a pilot study for assay validation and sample handling. Further, I provide details of patient and volunteer demographics including past gynaecological history, menstrual cycles, contraception, smoking status, BMI and family history of early menopause. The first part of my data analysis was focused on serum AMH concentration in the healthy population and those with a diagnosis of cancer at point 0. Next, longitudinal data on AMH was examined to assess its potential value as an ovarian reserve marker in young female cancer patients. Finally, I analysed changes in AMH levels in association with menstrual cycles, parity, hormonal contraception, BMI and smoking status.

4.2 Assay validation

I personally performed a pilot study in order to optimise and validate the planned procedures for serum assay after collection of blood samples at each site. This validation was performed using the DSL assay which was the only AMH assay available at the time. The ultrasensitive AMH/MIS ELISA was performed as per manufacturer instructions (Active MIS AMH ELISA, PCL-10-14400A-Beckam Coulter, Inc performing a DSL 10-14400 AMH assay). The lowest detectable level of MIS/AMH distinguishable from zero with 95% confidence was 0.06 ng/ml as determined by the manufacturer.

The intra-assay precision of active AMH/MIS ELISA has been calculated from two different AMH concentrations by assaying two serum samples 8 times (duplicate 4 times) within the same assay.

Table 6 The intra-assay precision of active AMH/MIS ELISA in MF Laboratory

Serum sample (low and high in house controls)	N=times tested	Mean (ng/ml)	Standard deviation (ng/ml)	Coefficient of variation (%)
1	8	0.5	0.01	2
2	8	7.2	0.07	0.97

The inter-assay coefficient of variation was also determined for two different AMH concentrations by assaying two serum samples in duplicate in 15 independent assays

Table 7 The intra-assay precision of active AMH/MIS Elisa in MF Laboratory

Serum sample	N=times tested	Mean(ng/ml)	Standard deviation(ng/ml)	Coefficient of variation (%)
1	15	1.27	0.12	9.4
2	15	4.89	0.45	9.2

Following the introduction of the new assay the intra and inter-assay precision was determined for AMH second generation Elisa with a detection level of >1.2 pmol/L.

The intra-assay precision of active AMH second generation Elisa has been calculated from two different AMH concentrations (low and high) by assaying two serum samples 8 times (duplicate four times) within the same assay. The inter-assay coefficient of variation was also determined for two different AMH concentrations by assaying two serum samples in duplicate in 11 independent assays run on different days.

Table 8 The intra-assay precision of AMH second generation ELISA in MF Laboratory.

Serum sample (low and high in house controls)	Number of times tested	Mean (ng/ml)	Standard deviation (ng/ml)	Coefficient of Variation (%)
1 (low)	8	2.635	0.05	2
2 (high)	8	6.689	0.228	3.4

Table 9 The inter-assay coefficient of variation of AMH second generation ELISA in MF Laboratory.

Serum sample	Number of times tested (in duplicates)	Mean (ng/ml)	Standard deviation (ng/ml)	Coefficient of Variation (%)
1	11	2.673	0.15	5.6
2	11	7.068	0.408	5.7

4.3 Conclusion

The intra and inter-assay precision was found to be within recommended values. The second generation assay had higher intra and inter-assay precision as compared with DSL assay. Ongoing monitoring of inter-person, intra assay and inter-assay precision have been conducted during the duration of the study. The external quality assurance scheme is performed by UK NEQAS.

4.4 Pilot study on sample handling

4.4.1 Introduction

AMH is a known marker for ovarian reserve, frequently assayed as part of sub-fertility investigation and treatment using commercial assay with a standardised assay technique. It is not clear how the initial sample handling, storage temperature, delay in processing can affect final results. There is no agreed standard methodology for AMH sample storage, and there are no guidelines which hinders the comparison of results between different clinical and research laboratories. The assay manufacturer recommends storage of samples in a tightly stoppered container for no longer than 24 hours. In some situations, this may not be practical e.g. when there is not a batch of samples be analysed or if transferred to a laboratory some distance away from the clinics where samples were obtained. If equivalent results were observed, e.g. after thawing freshly frozen serum samples, this shortcoming could be overcome. To address those

uncertainties, I performed a pilot study comparing serum AMH measurements in different storage condition of blood samples obtained from 15 volunteers.

4.4.2 Methods

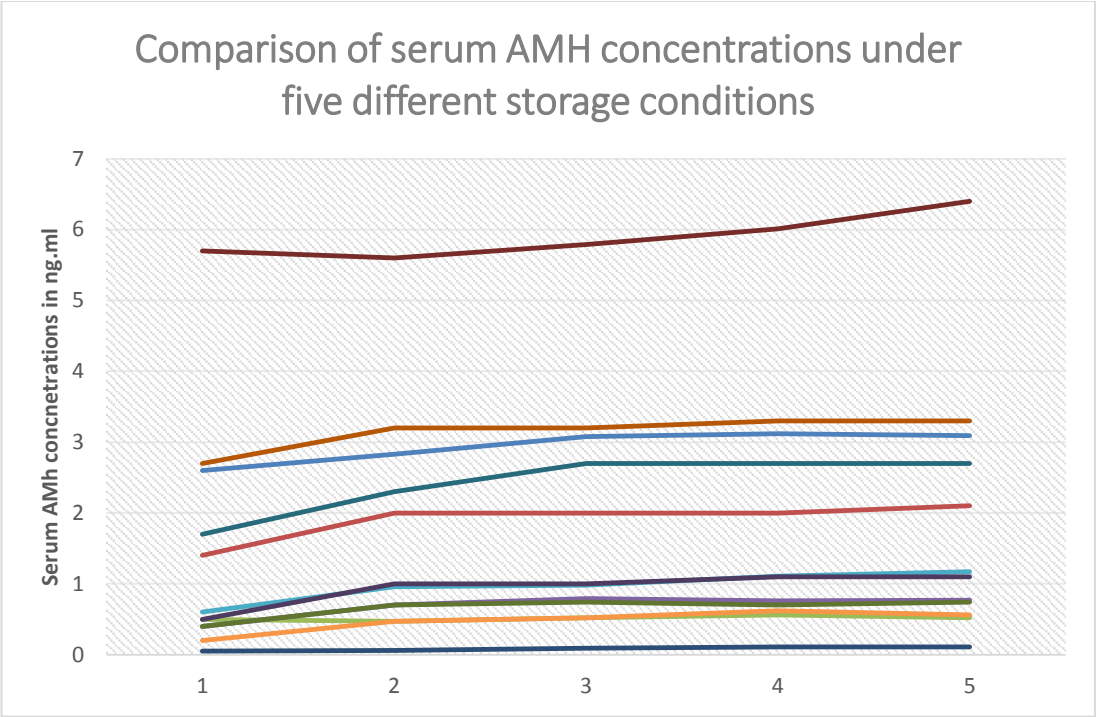
Institutional review board agreement was obtained for this pilot study. Fifteen consecutive healthy volunteers provided blood specimens via venepuncture for AMH assay. Two identical blood samples were obtained from each volunteer on one occasion, without consideration of the individual's menstrual cycle. One fraction of serum was stored at room temperature (20°C) only and the second was frozen at-20°C in three different time frames: immediately after venepuncture, 24 hours after venepuncture and 48 hours after venepuncture. The frozen specimen was thawed gradually at room temperature, and AMH assay was performed when the specimen had completely defrosted and been thoroughly mixed as per manufacturer instructions. In summary, the samples were analysed under 5 different storage conditions for each participant: within 2 hours from venepuncture as per manufacturer instructions (storage at room temperature only); after 24 hours (storage at room temperature only); following immediate freezing after venepuncture; following freezing after 24 hour storage at room temperature; following freezing after 48 hour storage at room temperature.

The mean serum concentrations of AMH in blood samples processed immediately and after 24 hours storage at room temperature were compared using paired t-test (STATA 8.2).

4.4.3 Results

The mean age of women who provided the samples was 37.8 (range 20.6 - 45.2), and their mean parity was 1.2 (range 0 - 3). All of them were Caucasian, Mean BMI was 24.6 (range 19 – 32). 33% (5/15) of them were smokers, and 60% (9/15) of them use combined hormonal contraception.

The changes in AMH serum concentration in 5 different storage conditions for each participant were plotted on the graph below (Figure 8). The mean concentration of AMH in freshly frozen serum was 1.48 ng/ml (immediately after venepuncture) and at 24 hours of whole blood storage AMH was 1.719 ng/ml. Paired t-test did not show a statistically significant difference (p=0.82) between the concentrations of AMH for samples stored using either method (Figure 9).



Sample storage conditions:

1. within 2 hours from venepuncture as per manufacturer instructions

2. after 24 hours storage in room temperature only

3. following immediate freezing (after venepuncture)

4. following freezing after 24 hour storage in room temperature

5. following freezing after 48 hour storage in room temperature

Figure 8 Comparison of serum AMH concentrations under five different storage conditions.

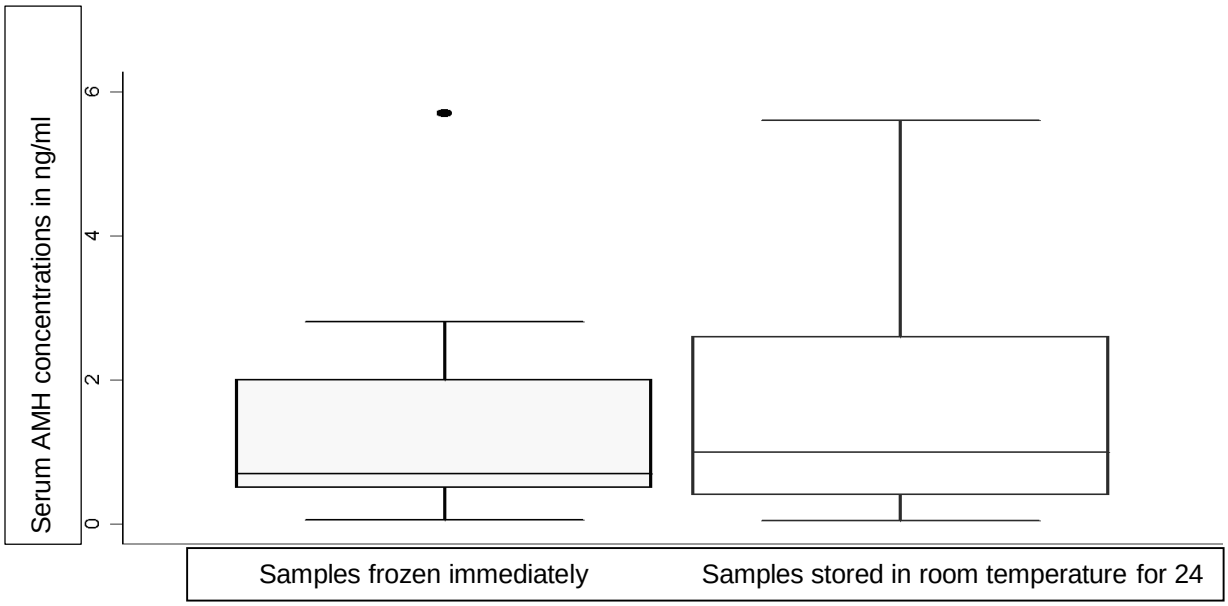


Figure 9 Comparison of AMH concentrations (ng/ml) measured in 2 different storage conditions: serum frozen immediately after venepuncture (sample frozen immediately) and whole blood stored for 24 hours at room temperature before processing (storage at room temperature for 24 hours). Paired t-test did not show a statistically significant difference (p=0.82) between the mean concentrations of AMH for samples stored using either method. Boxes represent the interquartile ranges (IQR), horizontal bars within the boxes represent the medians, and whiskers represent the lowest / highest data points within 1.5 IQR of the lower/upper quartiles.

4.4.4 Conclusion

The two analysed methods of storage including freezing the serum samples immediately after venepuncture and after 24 hour storage of full blood at room temperature were broadly comparable. Small differences in actual results would not affect AMH-guided clinical decisions in the assisted conception setting and would indicate that transfer of samples from distant Laboratory for AMH testing should be an acceptable method in my research setting. Additional measurement of samples frozen after 48 hours from venepuncture, have been higher but did not reach statistical significance. Further validation studies using a larger population at a variety of temperatures would be valuable unless an agreed storage procedure for samples transferred from distant Laboratories or IVF units is established by a relevant national or international body.

CHAPTER 5

5 Results: Demographics in cancer patients and healthy volunteers

5.1 Introduction

A total of 68 patients between the ages of 18 and 43, recently diagnosed with malignancy, were recruited into the study. The majority of participants, 54/68 (79.4%) were diagnosed with breast cancer. The second large group, 12/68 (17.6%) were women with haematological conditions, namely Hodgkin Lymphoma (HL) and non- Hodgkin Lymphoma (NHL). Two patients: one with adenocarcinoma of the caecum and one with Ig kappa myeloma were not included in the main analysis 2/68 (2.9%).

The volunteer group comprised 130 healthy women aged between 18 and 43 years, with no previous history of exposure to chemotherapy. Women with a confirmed history of polycystic ovarian syndrome (PCOS) or diagnosed with PCOS as a result of this study were not included in the analysis. Thus, 6/130 (4.6%) volunteers were excluded.

Overview of participants' demographics and past medical history is presented below. Overall, a group of women diagnosed with cancer had higher parity as

compared with controls (**Table 10**). A similar percentage of women in both groups reported a family history of early menopause ('first line relative'). In the cancer group, only 4.4% of women were using combined oral contraceptive pill while in the control group nearly 18% (**Table 11**). No data were available on the duration of contraception. In the group of women recently diagnosed with cancer the preferred form of contraception was Mirena coil. While combined oral contraceptive pill was the most commonly used form of hormonal contraception in healthy volunteers. Obesity (BMI >30) was noted in 13.4% of cancer patients and 14.4% of control group respectively. A higher percentage of active smokers was noticed in the cancer group (**Table 12**).

5.2 Overview of participants' demographics

Table 10 Past obstetric and gynaecological history in volunteers and cancer patients including age at menarche, family history of early menopause, parity.

Age of menarche	**Case n (%)**	**Control n (%)**
Below age of 9	0/68	0/130
Below 11	2/68 (2.9%)	6/130 (4.6%)
11-15	62/68 (91.1%)	118/130 (90.7%)
16 and above	2/68 (2.9%)	5/130 (3.8 %)
Not stated	2/68 (2.9%)	1/130 (0.7 %)
FH of early menopause (first line relative)	**Case n (%)**	**Control n (%)**
Yes	5/68 (7.3%)	7/130 (5.3 %)
No	61/68 (89.7%)	119/130 (91.5 %)
Not stated	2/68 (2.9 %)	4/130 (3 %)
Parity	**Case n (%)**	**Control (%)**
Nulliparous	21/68 (30.8%)	71/130 (54.6%)
Para 1	14/68 (20.5%)	22/130 (16.9%)
Para 2	20/68 (29.4%)	30/130 (23%)
Para ≥3	13/68 (19%)	7/130 (5.3%)

Table 11 Type of contraception used and timing of the natural menstrual cycle at the time of the blood test in non-users in both volunteers and cancer patients.

Type of contraception	**Case n (%)**	**Control n (%)**
Mini-pill	3/68 (4.4%)	3/130 (2.3%)
Combined oral contraceptive pill	**3/68 (4.4%)**	**23/130 (17.6%)**
Mirena coil	8/68 (11.7%)	14/130 (10.7%)
Implanon	2/68 (2.9%)	10/130 (7.7%)
Depo-Provera injections	2/68 (2.9%)	2/130 (1.5%)
Unknown	1/68 (1.5%)	6/130 (4.6%)
No hormonal contraception	**49/68 (72%)**	**72/130 (55.3%)**
Time of natural menstrual cycle		
Follicular phase	19/68 (27.9%)	32/130 (24.6%)
Mid-menstrual cycle	11/68 (26.2%)	6/130 (4.6%)
Luteal phase	18/68 (26.2%)	30/130 (23%)
Not known	1/68 (1.5%)	4/130 (3.0%)

Table 12 Body mass index and smoking status at time 0 in volunteers and cancer patients.

Body mass index	**Case n (%)**	**Control n (%)**
Below 18.5	3/68 (4.4%)	3/130 (2.3%)
18.5-24.9	34/68 (50%)	61/130 (46.9%)
25-29.9	18/68 (26.4%)	46/130 (35.3%)
30-34.9	3/68 (4.4%)	10/130 (7.6%)
35-39.9	5/68 (7.3%)	8/130 (6.1%)
Above 40	1/58 (1.7%)	1/130 (0.7%)
Not stated	4/68 (5.8%)	1/130 (0.7%)
Exposure to nicotine	**Case n (%)**	**Control n (%)**
Smoker	8/68 (11.7%)	8/130 (6.1%)
Non smoker	48/68 (70.5%)	102/130 (78.4%)
Ex-smoker	8/68 (11.7%)	20/130 (15.3%)
Passive smoker	4/68 (5.8%)	0/130
Not stated	0/68	0/130

5.3 Type of malignancy and cancer therapy

Invasive ductal carcinoma accounted for 70.5% of all breast cancer cases (n=48), ductal carcinoma in situ for 1.4%. Only one patient was diagnosed with invasive lobular carcinoma. Rare types of cancer were represented by inflammatory breast cancer (n=3) and medullary breast cancer (n=1).

In the group of patients with haematological malignancies, there were nine women diagnosed with Hodgkin lymphoma, 9/68 (13.2%) and three with non-Hodgkin lymphoma, 3/68 (4.4%): namely mediastinal large B-cell lymphoma (n=2) and MALT Lymphoma also called extranodal marginal zone B-cell lymphoma (n=1). Additionally, there was one participant suffering from IgG kappa myeloma. One patient was recruited with adenocarcinoma of the caecum, as she expressed great interest in taking part in the study. The latter two participants were not included in the analysis.

Detailed information on the type of cancer, staging, and treatment are presented below (**Table 13-16**). In breast cancer group, only one patient was known to have BRCA mutation. Nearly 60% of patients had oestrogen positive breast cancer. In breast cancer group, more than 50% of patients had treatment with FEC and Docetaxel. In lymphoma group, the most commonly used regimen was ABVD only (58.3%).

Table 13 Type of diagnosed malignancy in patients.

Type of cancer	Number of cases	Percentage (%)
Invasive ductal carcinoma	48/68	70.5%
Ductal carcinoma in situ	1/68	1.4%
Invasive lobular carcinoma	1/68	1.4%
Inflammatory breast cancer	3/68	4.4%
Medullary carcinoma of breast	1/68	1.4%
Hodgkin lymphoma	9/68	13.2%
Non-Hogdkin lymphoma NHL	3/68	4.4%
• MALT lymphoma (NHL)	1/68	1.4%
• Mediastinal large B cell lymphoma (NHL)	2/68	2.9%
IgG kappa myeloma	1/68	1.4%
Carcinoma of caecum	1/68	1.4%

Table 14 Staging and other characteristics in breast cancer patients.

Stage of breast cancer (n= 54)	**Number of cases n (%)**
In-situ	1/54 (1.8%)
I	14/54 (25.9%)
II	32/54 (59.2%)
III	6/54 (11.1%)
IV	1/54 (1.8%)
Estrogen receptor positive	31/54 (57.4%)
Known BRCA mutation	1/54 (1.8%)

Table 15 Chemotherapy regimen and hormonal therapy used in breast cancer patients.

Chemotherapy regimen	**N (%)**
*FEC	9/54 (16.6%)
FEC+Docetaxel	29/54 (53.7%)
**EC	2/54 (3.7%)
EC +Docetaxel	9/54 (16.6%)
CMF***+Epirubicin	3/54 (5.5%)
CMF, Epirubicin, Docetaxel	2/54 (3.7%)
Additional Paclitaxel or Carboplatin	8/54 (14.8%)
Use of hormonal therapy Tamoxifen GnRH agonist	 30/54 (55.5%) 2/54 (3.7%)

**FEC (Fluorouracil(5FU), Epirubicin, Cyclophoshamide)*

***EC (Epirubicin, Cyclophoshamide)*

****CMF (Cyclophosphamide, Methotrexate, Fluorouracil)*

Table 16 Staging and chemotherapy regimen in haematology group.

Haematological malignancies (n=13)	**Stage (I-IV)**	**Cases (n=)**
Hodgkin lymphoma	I	0
Hodgkin lymphoma	II	5/12 (41.6%)
Hodgkin lymphoma	III	1/12 (8.3%)
Hodgkin lymphoma	IV	3/12 (25%)
Mediastinal large B-cell lymphoma	I	1/12 (8.3%)
Mediastinal large B-cell lymphoma	IV	1/12 (8.3%)
MALT lymphoma	IV	1/12 (7.6%)
Type of lymphoma	**Treatment**	**N (%)**
Hodgkin lymphoma (HL)	*ABVD/**AVD	7/12 (58.3%)
Hodgkin lymphoma (HL)	ABVD+***BEACOPP	2/12 (16.6%)
MALT lymphoma (NHL)	Chlorambucil	1/12 (8.3%)
Mediastinal large B cell lymphoma (NHL)	****R-CHOP	2/12 (16.6%)

***ABVD** *(Adriamycin, Bleomycin, Vinblastine, Dacarbazine)* ***AVD** *(Adriamycin, Vinblastine, Dacarbazine)*

****BEACOPP** *(Bleomycin, Etoposide, Adriamycin, Cyclophosphamide, Vincristine, Procarbazine, Prednisolone)*

*****RCHOP** *(Rituximab, Cyclophosphamide, Doxorubicin, Vincristine, Prednisolone)*

Table 17 Patients with other malignancies and chemotherapy regimen.

Type of cancer	Chemotherapy regimen	Percentage of patients
Ig kappa myeloma	*CTD+Mephalan	1/68 (1.4%)
Adenocarcinoma of caecum	Oxaliplatin Capecitabine	1/68 (1.4%)

**CTD (Cyclophosphomide, Thalidomide, Dexamethazone)*

In the haematology group, none of the patients received total body radiotherapy during the 12 month period of follow-up. Some of the patients had localised radiotherapy, but none had pelvic radiation. Therefore, information about radiotherapy was not included in the analysis. The patient with IgG kappa myeloma had Mephalan based autograft, followed by a maintenance dose of Lenalidomide (immunomodulator), but she was not included in the main analysis.

CHAPTER 6

6 Results: Analysis of pre-treatment serum AMH concentrations in women with malignancy compared with healthy controls

6.1 Introduction

It is debatable whether women who develop a malignancy have the same ovarian reserve as a general population before they begin cancer treatment. It is not clear if malignancy itself or systematic illness has a significant impact on results of ovarian reserve tests. In previous studies, 38 lymphoma patients had significantly lower serum AMH concentrations prior to chemotherapy compared to a healthy population (Lawrenz *et al.*, 2012) while the same was not confirmed in young (aged <37) women with breast cancer (Su *et al.*, 2013).

Knowing pre-chemotherapy ovarian reserve would be particularly useful for counselling patients regarding: their individual risk of subfertility; fertility preservation options and doses of stimulation drugs needed in ovarian stimulation cycles. At present, only limited data are available to guide clinicians

about individuals' likelihood of early menopause before starting cancer therapy (Kim *et al.*, 2012).

Additionally, it is important to assess patients' views and expectations about future fertility prospectively, at the time of cancer diagnosis and after treatment. Previous reports from the USA have shown dissatisfaction with fertility counselling and significant concerns for future fertility among young female cancer survivors, reported retrospectively (Partridge *et al.*, 2004). Therefore, I assessed the desire for pregnancy *at the time* of cancer diagnosis as well as the number of referrals to fertility specialists in young cancer patients in this multicentre UK based study.

6.2 Statistical analysis

The analysis of serum AMH concentration at time of recruitment is presented below. Serum concentrations of AMH were compared between the cancer population (breast cancer and lymphoma) and healthy volunteers before any gonadotoxic treatment was commenced. Measured results for AMH were log transformed to normalize the right skewed distribution. A linear regression model was built with log transformed values of AMH concentrations as the response variable and age as the explanatory variable. A logistic regression model was used to assess if the outcome (log transformed AMH value) is an indicator for cases or controls. Univariate analysis was used to indicate if factors such as smoking status, previous live births, type of contraception used, time of

the menstrual cycle (in non-users) and BMI are predictive of group membership (cases versus controls). The multiple logistic regression model was built by Dr Nick Parsons to confirm whether AMH was significantly different in cancer patients and volunteers after adjusting for all of the confounding factors. Assessment of the patient's wishes for pregnancy at the time of cancer diagnosis was analysed descriptively and presented as percentage (scale 1-4). The percentage of patients being referred to a fertility specialist for consultation regarding fertility preservation was calculated.

6.3 Results

Overall, a total of 66 patients and 124 volunteers were included in this analysis with a mean age of 35.9 (SD±5.9) and 33.8 (SD±6.4) years respectively. Overall mean serum AMH concentration of patients in the cancer group was 13.6 (SEM±2.1) pmol/L. The mean AMH of the breast cancer subgroup (n=54) was 12.8 pmol/L while haematology subgroup (n=12), 16.8 pmol/L (SEM±2.1). The control group consisted of 124 healthy volunteers with a mean AMH of 20.6 pmol/L (SEM±1.6).

Table 18 Demographic factors, obstetric and gynaecological history in volunteers and cancer patients (p value calculated using Tukey comparison of means/Pearson's Chi-squared test.

Parameter	Breast cancer group (n=54)	Lymphoma group(n=12)	Volunteer group (n=124)	(p value)
Mean age	37.8 (SD±4.2)	27.6 (SD±5.5)	33.8 (SD±6.4)	p<0.001
Age at menarche	12.7 (SD±1.6)	13.1 (SD±1.6)	12.7 (SD±1.5)	p=0.71
Family hx of menopause<45	5/52 (9.6%)	0/12	7/119 (5.8%)	p=0.32
Parity				p<0.001
0	12/54 (22.2%)	8/12 (66.6%)	67/124 (54%)	
1	12/54 (22.2%)	2/12 (16.6%)	21/124 (16.9%)	
2	17/54 (31.4%)	1/12 (8.3%)	29/124 (23.2%)	
≥3	13/53 (24.1%)	1/12 (8.3%)	7/124 (5.6%)	
Time of cycle				p=0.2
Follicular	16/40 (40%)	3/9 (33.3%)	30/64 (46.8%)	
Mid-cycle	8/40 (20%)	3/9 (33.3%)	5/64 (7.8%)	
Luteal	16/40 (40%)	3/9 (33.3%)	29/64 (45.3%)	
Type of hormonal contraception				p=0.01
Combined oral	0/54	2/12 (16.6%)	23/124 (18.5%)	
Progestin based	14/54 (25.9%)	1/12 (8.3%)	29/124 (23.2%)	
No contraception	40/54 (74%)	9/12 (75%)	72/124 (58%)	
Smoking				p=0.15
Active smokers	8/54 (14.8%)	0	7/124 (5.6%)	
Ex-smokers	5/54 (9.2%)	1/12 (8.3%)	19/124 (15.3%)	
No smokers	41/54 (75.9%)	11/12 (91.6%)	98/124 (79%)	
BMI	26.2 (SD±5.6)	23.2 (SD±5.9)	25.7 (SD±4.8)	p=0.83

AMH data was extremely right –skewed. Distributional properties of AMH were improved significantly by log transformation.

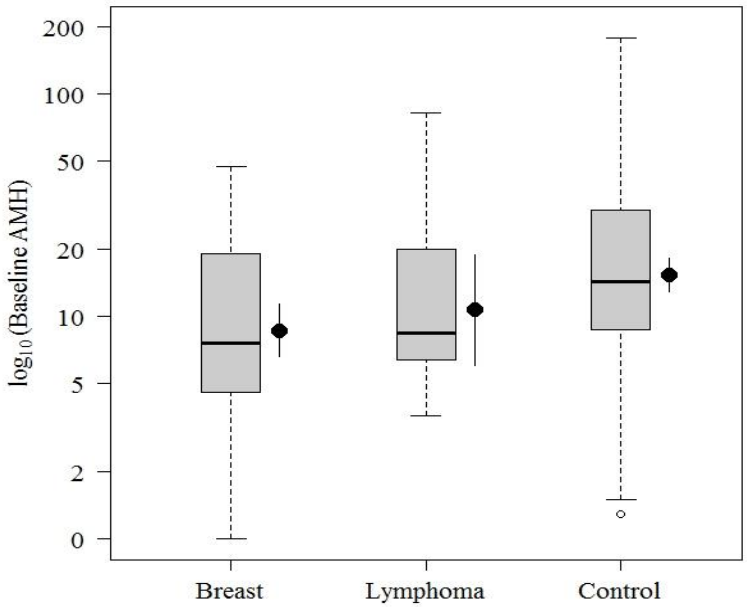


Figure 10 Log transformed AMH values in 3 groups: breast cancer, lymphoma and control. Boxplot: black dots represent means, boxes represent the interquartile ranges (IQR), horizontal bars within the boxes represent the medians, and whiskers represent the lowest / highest data points within 1.5 IQR of the lower/upper quartiles.

Tukey multiple comparisons of means in 3 groups, shown that patients with breast cancer had significantly lower serum AMH concentrations than the

control group (95% CI -0.41. -0.01, p<0.006). Lymphoma group had serum AMH concentrations that appeared lower but were not found to be significantly different to the volunteer group (95% CI -0.23, 0.47, p=0.57).

Based on the linear regression model, after adjusting for age, cancer patients as a whole had significantly lower serum AMH concentrations as compared to same age volunteers ($F_{2,175}$=21.3, p=0.01). AMH declined with age in both cancer and volunteer groups.

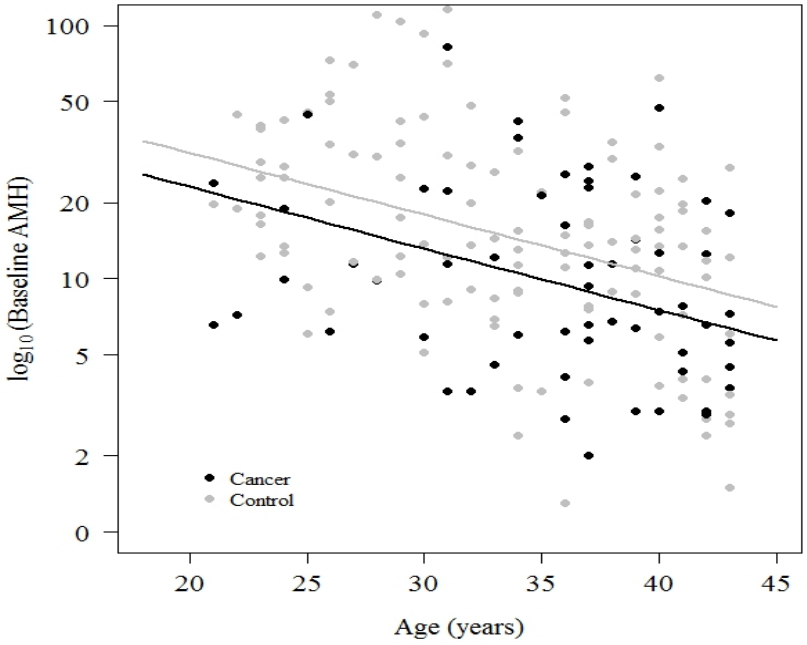


Figure 11 Correlation between log10 transformed serum AMH concentrations (y-axis) and age (x-axis) in the control group and cancer patients. A linear correlation was found to be the line of best fit. There was no evidence that the slope of the line was different between the two groups (p=0.135).

After adding cancer type to the regression model and adjusting for age, AMH was found to be statistically different in all three subgroups: breast cancer, lymphoma and volunteers ($F_{5,174}$=14.5, p=0.01).

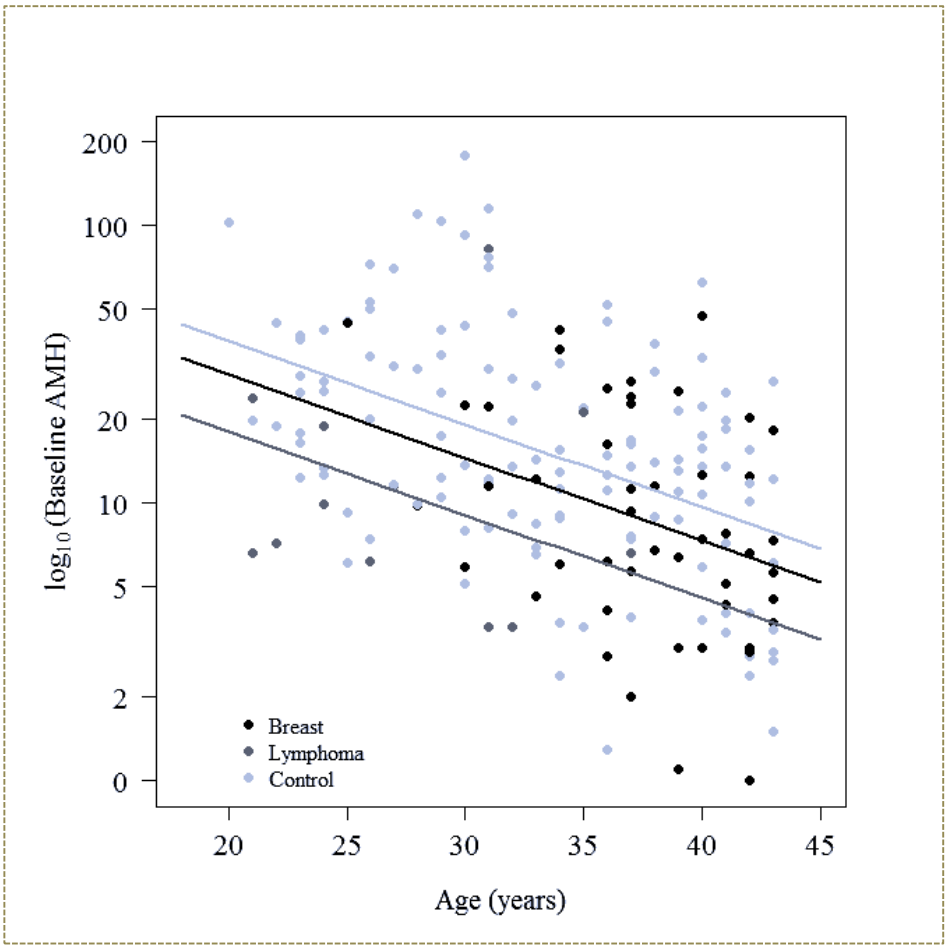


Figure 12 Linear correlation between log10 transformed serum AMH concentrations (y-axis) and age (x-axis) in breast cancer, lymphoma and control groups.

After adjusting for age, serum AMH concentrations differed significantly between 3 groups: breast cancer, lymphoma (Hodgkin and non-Hodgkin) and

healthy volunteers. The prediction model built by Dr Nick Parsons which was based on AMH values in age-matched volunteers and cancer patients, estimated that a cancer patient had AMH level equivalent to a 6 years older volunteer, prior to chemotherapy. There was no evidence that the extent of the difference changed with age (interaction term was not significant).

Univariate analysis was used to indicate if factors such as smoking, previous live births, number of pregnancies, type of contraception used, time of the menstrual cycle, BMI, etc. were predictive of group membership (cases versus controls).

Table 19 Univariate analysis indicates whether the dependent variable is an indicator for volunteers and cancer. Variables: log transformed AMH, age, age at menarche, length of menstrual cycle, number of pregnancies, number of life births, body mass index (BMI), time of menstrual cycle in non-users of hormonal contraception, regularity of menstrual cycles (1-regular 2-no regular), gynae problems* (gynaecological history: ovarian cystectomy or endometriosis), hormonal therapy other than hormonal contraception, desire for children, smoking status. Df* degrees of freedom.

Dependent Variable	Df*	Deviance	P value	Rank
Age	1	5.166	0.023	5
log10(AMH) at baseline	1	9.256	0.002	3
Cycle length	1	0.454	0.501	11
Age menarche	1	0.087	0.768	14
No pregnancies	1	13.966	<0.001	2
LB at baseline	1	15.096	<0.001	1
BMI	1	0.041	0.839	15
Time of cycle	1	9.793	0.002	4
Regularity of cycles	2	0.671	0.7155	12
Gynae problems	1	0.014	0.905	16
Other medical problems	1	1.68	0.195	7
Family early menopause	1	0.567	0.452	10
Contraception type	5	11.959	0.021	6
Hormonal Therapy	1	0.180	0.671	13
Desire for children	3	4.506	0.212	8
Smoking	1	0.946	0.331	9

Based on multiple logistic regression models (built by Dr Nick Parsons), AMH was significantly different between cancer patients and volunteers after adjusting for the variables ranked above (p=0.01).

Baseline serum AMH concentration was not found to be significantly different between groups with negative and positive estrogen receptor breast cancer (p=0.61). The trend suggestive of lower serum AMH concentrations in more advanced stages of breast cancer was observed, but no significant difference was found in between patients with breast cancer stage I, II and III (p=0.57).

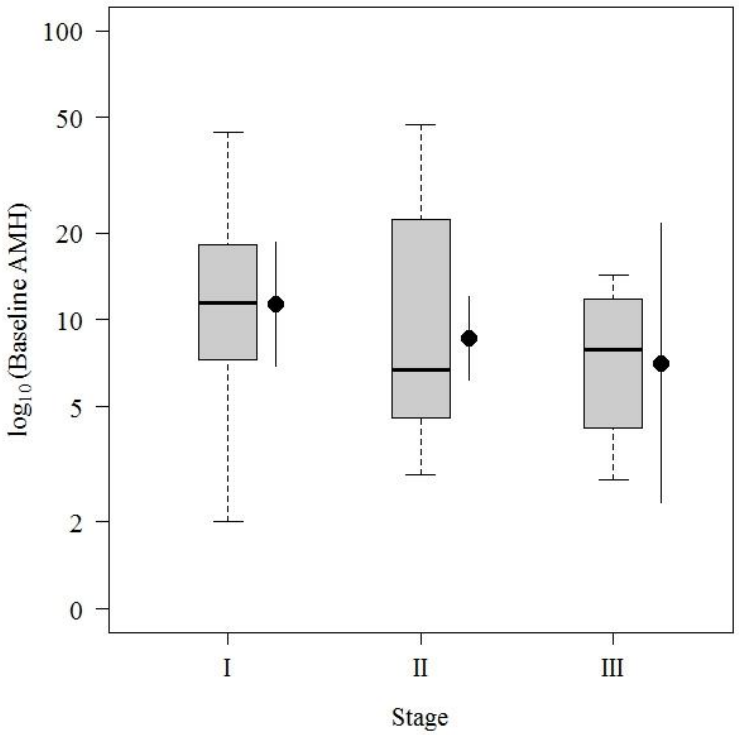


Figure 13 Differences in log transformed AMH levels (y-axis) by stage of breast cancer (x-axis). Boxplot: black dots represent means, boxes represent the interquartile ranges (IQR), horizontal bars within the boxes represent the medians, and whiskers represent the lowest / highest data points within 1.5 IQR of the lower/upper quartiles. No statistically significant difference was found in log transformed AMH levels between patients with breast cancer stage I, II and III (p=0.57).

Of the total group of women newly diagnosed with cancer, 35% still expressed some desire to have children. When considering a younger subgroup of women (those aged ≤39), 48.8% (21/43) desired children in future. In total, 9% of women had been seen by a fertility specialist for discussion about fertility preservation options.

Table 20 Assessment of women's desire to have children at the time of cancer diagnosis before commencing chemotherapy in the age group 18-43.

Desire to have children (marked as strong or maybe on medical questionnaire at the time of cancer diagnosis)	**All patients** N (%)	**Breast cancer group** N (%)	**Lymphoma group** N (%)
Age range 18-43	24/68 (35.3%)	14/54 (25.9%)	10/12 (83.3%)
Age ≤39	21/43 (48.8%)	11/31 (35.5%)	10/12 (83.3%)

6.4 Summary of results

- Serum AMH concentrations decrease with female age.
- AMH levels were found to be lower in the cancer group as a whole, prior to any gonadotoxic treatment, compared to healthy volunteers. Based upon a multiple logistic regression model, after adjusting for confounders: i.e. age, smoking status, BMI, parity, use of contraception, time of the cycle; AMH levels were still found to be significantly lower in the cancer group.

- A prediction model estimated a 6 year difference in ‘fertility age’ between groups. In other words, on average a 25 year old cancer patient was found to have a serum AMH concentration equivalent to that of a 31 year old healthy volunteer.
- There was no significant difference in baseline AMH concentrations, according to the stage of breast cancer. The trend was suggestive of lower AMH in patients with more advanced stages of breast cancer, but a larger study would be needed to provide conclusive results.
- Nearly 50% of women, newly diagnosed with cancer at an age ≤ 39 years, expressed some desire (either ‘strong’ or ‘maybe’) to have children.
- Less than 10% of women recruited into the study had had a consultation with a fertility specialist.

CHAPTER 7

7 Results: Longitudinal data analysis

7.1 Introduction

An accurate assessment of remaining ovarian function and fertility potential after chemotherapy is not straightforward. Women who regain menses may appear fertile but are still at risk of premature menopause and have reduced window of potential fertility (Bath *et al.*, 2003; Larsen *et al.*, 2003a; Reh *et al.*, 2008).

The exact long-term effects of the different types of chemotherapeutic agents and regimens upon ovarian reserve in a woman of reproductive age are still difficult to determine on an individual basis. The degree of chemotherapy-induced ovarian follicle loss depends on the type of chemotherapy, its dosage, duration and the age of the patients i.e. how large their ovarian reserve is before treatment begins (Lower *et al.*, 1999; Meirow & Nugent, 2001).

Additionally, information on the menopausal status of women with breast cancer is very valuable for oncologists. Post chemotherapy, it helps to determine what type and duration of adjuvant hormonal therapy would be most suited for the group of young women with breast cancer (Henry *et al.*, 2014). At present, an accurate determination of menopausal status in patients around the age of 40

who have completed chemotherapy is not straightforward. A number of studies have used as an indicator, the recurrence of menses at 6 or 12 months follow-ups post chemotherapy, but amenorrhea was not found to be a reliable indicator of menopause (Smith *et al.*, 2006). Biochemical markers, such as FSH and estradiol, were not found to be reliable markers of ovarian reserve in patients post chemotherapy, especially in breast cancer patients taking hormonal therapy e.g. Tamoxifen (Hadji *et al.*, 2012; Lum *et al.*, 1997).

Currently, measurement of serum AMH is considered the best biomarker of ovarian reserve (Broekmans *et al.*, 2006). AMH was previously found to be an accurate indicator of ovarian function even amongst women without regular menstrual cycles and was found not to be strictly dependent of the menstrual cycle (La Marca *et al.*, 2006a). Interestingly, in a small pilot study, in patients with breast cancer using Tamoxifen, no significant impact of Tamoxifen on AMH levels was observed during 8 months of follow-up (Oktay *et al.*, 2013).

These characteristics make an AMH a good candidate for ovarian reserve assessment and measurements of the degree of changes in ovarian reserve in childbearing age women with cancer. Those women who may not have regular menstrual cycles following chemotherapy for months. Additionally, in premenopausal women with estrogen positive breast cancer information about definitive menopausal status following chemotherapy, is crucial for planning any hormonal therapy.

7.2 Statistical analysis

In the initial analysis, serum AMH concentrations were measured at the time of recruitment (Time 0). For the longitudinal analysis, additional samples were obtained at 6, 9 and 12 months later in both groups: cancer patients (who had been undergoing treatment for their condition) and healthy volunteers over the same period. These samples were analysed simultaneously using the same assay protocols as the Time 0 samples.

Statistical analysis was performed using log transformed AMH values in volunteer and cancer groups using 4 repeated values. Paired t-test was used to compare values between baseline AMH and follow-ups in both groups. A Pearson correlation coefficient was used to asses if pre-chemotherapy levels of AMH were correlated with levels post-chemotherapy. Logistic regression analysis was used to check if serum concentrations of AMH at the time of cancer diagnosis (explanatory variable) were predictive of return of menstrual cycle (binary variable) at 12 month follow-up.

Mc Nemar test was used to test whether the desire to have children before and 12 months after completing chemotherapy had changed significantly in cancer patients.

7.3 Results

AMH concentrations reduced with the onset of chemotherapy, as was expected since growing follicles in the ovary would become atretic. However, after 9 or 12 months, some recovery was expected unless the patient was still undergoing chemotherapy at this time. Considering the age group 18-43 years of recruited patients, the degree of recovery of AMH concentrations with time was lower than anticipated.

Overall, in the cancer group, at 6 months follow-up, significant fall in serum AMH was observed. Only 7.4% of women had detectable serum AMH concentrations at that point while at 9 months this proportion increased to 18%. Recovery of ovarian function increased slightly at the time of final follow-up when 21.8% (12/55) of women had detectable AMH concentrations in serum samples. Five of these were in the Hodgkin lymphoma group, in which 62.5% (5/8) of patients who completed 12 month follow-ups had a noticeable recovery of ovarian function. It was observed that all of them (5/8) had received ABVD regimen only and were aged ≤32. Additionally one patient with same characteristics had detectable AMH at the 9 month follow-up but did not complete the final 12 month follow-up. Interestingly, some recovery of ovarian function was observed even in cases in which AMH at point 0 was as low as 3.6 pmol/L if standard ABVD regimen was used. In all patients (5/8) with detectable AMH concentrations at the time of 12 month follow-up, the concentration was similar to or higher than

that measured at time 0. Two patients in Hodgkin lymphoma group (2/8) were given Cyclophosphamide (non ABVD regimen), and both of them had undetectable AMH in the follow-up period. Overall, in the haematology group, the return of menses, either regular or irregular, was reported in 88.8% of patients at the time of final follow-up appointment. At the time of final follow-up, only 16.6% (2/12) of patients were on combined oral contraceptive pill. Interestingly, two young patients diagnosed with Hodgkin lymphoma, aged 21 and 31, conceived within three months from their 12 month follow-up. The final serum AMH measurement was low (3.6 pmol/L) in one patient and undetectable in the other.

Among breast cancer patients, 12 months from starting chemotherapy, in 15.5% (7/45) of them, some degree of recovery of ovarian function was noted. All patients with measurable AMH concentrations at 12 months were aged ≤40 with a baseline serum AMH concentration ≥18 pmol/L and had received regimen FEC/EC with or without Docetaxel. Overall, in the breast cancer group, 58.1% (25/43) of patients were amenorrheic at 12 months from commencing chemotherapy. A further 23.2% (10/43) had irregular bleeding, and 18.6% (8/43) reported regular menstrual cycles. Women who were taking hormonal contraception or were known to have a non-hormonal coil have been excluded from this final sub-analysis.

Overall, based on logistic regression analysis, after adjusting for age, pre-chemotherapy serum concentrations of AMH in reproductive age women with

malignancy were not found to be associated with presence or absence of menstrual bleeding at the 12 month follow-up (p=0.219). Same was confirmed for a subgroup of patients with breast cancer (p=0.108).

A younger age of the patient was confirmed to be statistically significant in predicting return of menses at 12 months after initiation of chemotherapy (*EXP (B)* =0.86*, 95% CI* 0.76, 0.98*, p*=0.033).

Based on formula (build by Dr Richard Crossman) for any age and pre-chemotherapy AMH for a patient, one can calculate the probability of return of menses at 12-month follow-up:

p = (exp (4.407 -0.144A+1.020L))/(1+exp(4.407 -0.144A+1.1020L))

where p is the probability of menses returning at 12 months from first dose of chemotherapy, A is equal to the age of a patient, L is equal to the log-transformed pre-treatment AMH concentration:

e.g. patient aged 30 with pre-chemotherapy AMH of 9 pmol/L would have 74% chance of menstruation by 12 months.

Exp (4.407 – 0.144*30 + 1.020*LOG(9)) =74.3%

Using a Pearson correlation coefficient, no association between pre-treatment and 12-month follow-up serum concentrations of AMH in cancer patients as a

whole group was found (p=0.18). Same was confirmed in the group of women with breast cancer (p=0.408). In Hodgkin lymphoma, higher pre-treatment levels were correlated with better recovery of ovarian function (p=0.02). However, the group size is small, and those results need to be interpreted with caution.

In the volunteer group, serum concentrations of AMH at point 0 were not found to be significantly different from AMH at 6, 9, and 12-month follow–ups. This finding was in contrast to the cancer group where pre-chemotherapy AMH concentrations were significantly higher than at 9-month and 12-month follow-ups (p=0.01, p=0.014 respectively).

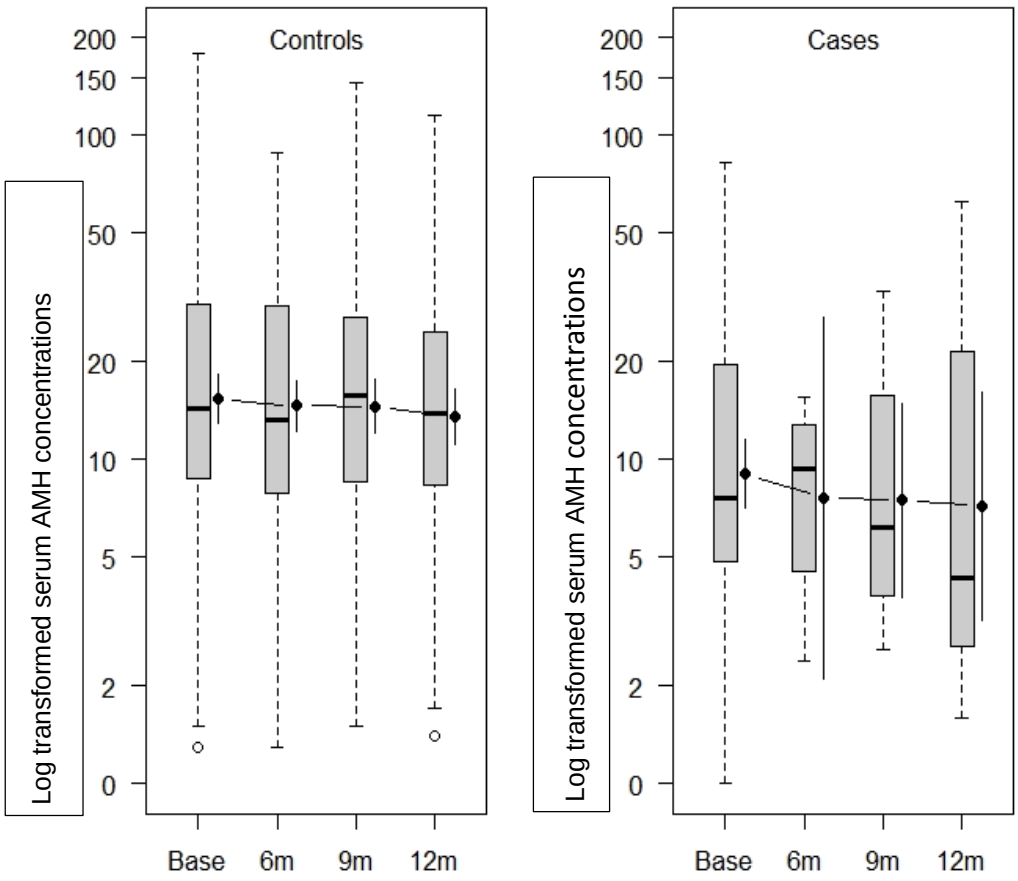


Figure 14 Boxplot for temporal tends in serum AMH concentrations (y-axis) in control group and cancer patients (cases) at different time points (x-axis): point 0 (base), 6 months (6m), 9 months (9m) and 12 months (12m). Black dots next to the boxes represent means, boxes represent the interquartile ranges (IQR), horizontal bars within the boxes represent the medians, whiskers represent the lowest/ highest data points within 1.5 IQR of the lower/upper quartiles and dots represent outside the boxes represent outliers.

Table 21 Changes in serum AMH concentrations (on log transformed scale) over a 12 month period using paired t-test in the control group. AMH concentrations at time 0 (baseline) compared with measurements at 6, 9 and 12 month follow-up. No evidence for significant temporal changes in serum AMH concentrations in the control group. Df*- degrees of freedom. U.CI** upper limit of the confidence interval, L.CI*** lower limit of the confidence interval.

Log transformed AMH at time 0 and 6, 9 and 12 month follow-up in volunteers						
Time of the samples	Mean Difference	U.CI95%**	L.CI95%***	t.statistic	Df*	p value
Baseline versus 6 month	0.023	-0.014	0.061	1.23	102	0.221
Baseline versus 9 month	0.045	-0.002	0.092	1.88	98	0.063
Baseline versus 12 month	0.046	-0.001	0.094	1.95	98	0.054
6 month versus 9 months	0.011	-0.026	0.048	0.6	93	0.551
6 month versus 12 month	0.02	-0.024	0.063	0.9	89	0.371
9 month versus 12 month	0.016	-0.029	0.061	0.71	90	0.479

Table 22 Changes in serum AMH concentrations (on log transformed scale) over a 12 month period using paired t-test in cancer patients. AMH concentrations at time 0 (baseline) compared with measurements post-chemotherapy at 6, 9 and 12 month follow-up. Evidence of statistically significant differences between pre and post-chemotherapy AMH was observed. P value of 0.014 and 0.001 is considered statistically significant, Df*-degrees of freedom. U.CI** upper limit of the confidence interval, L.CI*** lower limit of the confidence interval.

Log transformed AMH at time 0 and post-chemotherapy in cancer patients						
Time of the samples	Mean Difference	U.CI95%**	L.CI95%***	t-statistic	Df**	p value
Baseline versus 6 month	0.433	-0.326	1.191	1.81	3	0.167
Baseline versus 9 month	0.525	0.288	0.762	5.11	8	**0.001**
Baseline versus 12 month	0.447	0.108	0.786	2.9	11	**0.014**
6 month versus 9 month	-0.435	-1.274	0.403	-6.6	1	0.096
6 month versus 12 month	-0.286	-0.942	0.369	-1.39	3	0.258
9 month versus 12 month	0.059	-0.214	0.333	0.56	5	0.602

After adjusting for age, no significant difference was found between mean AMH concentrations in breast cancer patients using Tamoxifen versus non-users of Tamoxifen (p=0.053) at 12 month follow-up.

The comparison of patients' wishes before and after chemotherapy regarding future pregnancy is presented. In the haematology group, all but 2 women provided the same answers to the question about wishes for future pregnancy (scale 1-4) both before commencing chemotherapy and at the 12-month follow-ups.

In the breast cancer group, 20% of patients marked a higher score (1-4 scale) in comparison to their pre-treatment answers, suggesting a trend of increasing desire to have children after completion of chemotherapy. However, this result was not statistically significant (p=0.291). Those views were not analysed in the context of the progression of the disease and need for repeated doses of chemotherapy.

7.4 Summary of results

- Twelve months after starting chemotherapy nearly 80% of women still had undetectable AMH levels. Therefore, measurement of serum AMH at 12 month follow-up could not be reliably used to assess the degree of damage to ovarian function caused by specific chemotherapy regimen and dosage.
- In comparison, in the same age group of healthy volunteers, AMH concentrations were found to be stable over 12 months.
- In a young group of patients aged ≤ 32, with Hodgkin lymphoma on ABVD protocol, over 60% had a noticeable degree of recovery of ovarian

reserve at 12 months. Nearly 90% of those women reported the return of menstrual bleedings.

- In breast cancer group, only a small number of women had detectable AMH at 12 month follow-up – those women were overall younger (age ≤40) and had ‘good ovarian reserve’ before chemotherapy.
- During the follow-up period, in the haematology group, desire to have children remained as high as prior to chemotherapy. In the breast cancer group, 20% of women reported a greater wish for future pregnancy at 12 month follow-up than at the time of cancer diagnosis.
- Pre-chemotherapy concentrations of AMH were not found to be correlated with the recurrence of menses at 12 month follow-up. However, a prediction model was built, using age and pre-chemotherapy AMH which allowed the chances of return of menstrual periods at 12 months from starting chemotherapy to be estimated.

CHAPTER 8

8 Results: AMH as a marker of ovarian reserve in a healthy population

8.1 Introduction

Anti-Müllerian hormone (AMH) testing has been widely investigated for its use in a range of fertility applications. However, it remains controversial due to issues with the reliability of the assay, including changes in methodology, different thresholds, interference caused by complement in serum samples causing artificially low AMH measurements. These problems are compounded by the prevalence of small published studies, mainly addressing the subfertile population. All of these issues have resulted in contradictory evidence regarding the clinical uses of serum AMH assays (Dewailly *et al.*, 2014).

Among the controversial aspects are, for example, impact of the use of hormonal contraception, smoking and BMI on serum concentrations of AMH. Some research groups have reported a negative relationship between high body mass index and AMH in women age 35-47 (Freeman *et al.*, 2007), but this finding was not confirmed by others (Halawaty *et al.*, 2010). Similarly, the AMH concentration was found to be lower in active smokers (Plante *et al.*, 2010) but a different study showed that smoking status had no impact (Dafopoulos *et al.*,

2010). As described in my introduction chapter, serum concentrations of AMH are generally considered to be relatively independent of the menstrual cycle (Hehenkamp *et al.*, 2006; La Marca *et al.*, 2006b; Tsepelidis *et al.*, 2007), however, some studies have reported statistically significant inter- and intra-cycle fluctuations in AMH concentrations (Cook *et al.*, 2000b; Streuli *et al.*, 2009). Additionally, the reproducibility of AMH measurements using the second generation assay has been questioned (Rustamov *et al.*, 2012). Consequently, a new method of testing including pre-dilution step was recommended to improve stability of the assay (described in methodology chapter)

In order to address these problems, I have analysed in detail the results of AMH assays in the group of healthy controls. I have conducted an analysis of the stability of AMH concentrations measured at four specified points in time, including an assessment of cycle dependence, the impact of hormonal contraception, smoking and BMI on the measured AMH concentrations. The most recently recommended 'pre-dilution' method for the assay was employed.

8.2 Statistical analysis

For this part of the analysis, previously recruited 124 volunteers, without any significant medical illness, were selected

Four repeated AMH measurements were used to calculate within-subject variability using the coefficient of variation. Correlation between AMH changes

during stages of the menstrual cycle, contraception use, BMI and smoking status were assessed using a linear regression model with AMH as the response variable.

8.3 Results

Overall 124 volunteers with a median age of 34 were included in this analysis. Mean AMH for the group was 20.6 pmol/L (SD±18.9) at time 0, 20.5 pmol/L (SD±18.7) at 6 months follow-up, 19.1 (SD±16.4) at 9 months and 20.7 (SD±20.7) at final blood test. Amongst healthy volunteers, 18.5% (23/124) have been on combined oral contraceptive pill while 23.3% (29/124) were using progestin based contraceptives. Assessment of past obstetric history showed that 45% (57/124) of women were para≥1. Less than 5% of patients reported a family history of early menopause at age <45 in a first line relative. Mean BMI was 25.7 (SD±4.8). In the volunteer group, 5.6% (7/124) were active smokers.

Mean CV (coefficient of variation) in the group of volunteers on 4 repeated measurements of AMH at time 0, 6, 9 and 12 months was 15% which confirms acceptable within-subject (sample to sample) variability of second generation assay using new method of testing (including pre-dilution step).

Univariate analysis of relationship between the time of natural menstrual cycle and serum measures of AMH showed no statistically significant correlation at the time of recruitment (p=0.868) and at repeated analysis at time of 6 month

follow-up (p=0.638), 9 moth-follow-up (p=0.531) and at final follow-up (p=0.960). After adjusting for other confounding factors: time of the menstrual cycle, type of contraception used, parity, smoking status, and BMI were not found to be significantly correlated with serum AMH concentrations in healthy volunteers.

Table 23 Linear regression model: the relationship between log transformed AMH values (dependent variable) and explanatory variables listed below. R representing correlation coefficient, R square showing the amount of variation in AMH value that can be explained by age.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	0.470	0.221	0.184	0.33725

ANOVA

Model		Sum of Squares	Degrees of freedom	Mean Square	Sig.
1	Regression	3.422	5	0.684	p=0.001
	Residual	12.056	106	0.114	
	Total	15.478	111		

Table 24 Linear regression model- the relationship between AMH and explanatory variables. AMH log transformed values which is dependent variable and explanatory variables: age at the time of recruitment, time of the menstrual cycle (1-follicular, 2-mid-cycle, 3-luteal), type of contraception used (1-none hormonal, 2-combined oral contraceptive pill, 3-progestin based contraceptives), BMI (calculated as body mass divided by the square of height), smoking status (1-smoker, 2-non-smoker, 3-ex-smoker) and number of life births >24 weeks (parity). After adjusting for other confounding factors, only age was found to be correlated with serum AMH concentrations (p=0.001). AMH concentrations decrease with age (β=-0.453, 95% CI -0.38, -0.18, p=0.001).

Model	**Unstandardized Coefficients**		**Standardized Coefficients**	**Tscore**	**P value**
	B	Standard error	Beta		
Constant	2.341	0.308		7.596	p=0.001
Age	-0.027	0.006	-0.0460	-4.148	p=0.001
Day of natural cycle	-0.006	0.036	-0.21	-0.172	p=0.864
Type of contraception	-0.012	0.031	-0.048	-0.397	p=0.692
Parity	0.011	0.040	0.029	0.268	p=0.789
BMI	-0.008	0.007	-0.098	-1.111	p=0.269
Smoking status	-0.029	0.075	-0.033	-0.382	p=0.703

After adjusting for age, serum AMH concentrations were not found to be significantly correlated with the type of hormonal contraception used by healthy volunteers. Additional analysis on two subgroups, users of combined oral contraception versus non users found no correlation between AMH and use of combined contraceptives (p=0.545).

Table 25 Multivariate model-correlation between different types of hormonal contraception and log transformed values of serum AMH concentrations.

Model	Estimate (log odds ratio)	Standard error	T value	P value
Intercept	0.770554	0.348293		0.0296
Age	-0.026576	0.005413	-4.910	4.35e-06
Combined oral contraception	-0.130497	0.082616	1.400	0.1179
Mini pill	0.447458	0.319669	-1.580	0.1652
Mirena coil	-0.426522	0.172015	-2.480	0.151
Implanon	0.026186	0.324170	0.081	0.9358
Depo-provera injection	-0.244766	0.321456	-0.761	0.4485

Residual standard error: 0.3171 on 85 degrees of freedom, multiple R-squared: 0.4049, adjusted R-squared: 0.3558 , F-statistic: 8.26 on 7 and 85 DF, p-value: 1.174e-07

8.4 Summary of results

- The study confirmed acceptable, within-subject (sample to sample) variability of the second generation assay using a new method of testing (including pre-dilution step).

- Smoking status, BMI, timing of menstrual cycle and type of contraception used were not found to be correlated with serum AMH concentrations in the healthy population.

CHAPTER 9

9 Conclusions and Discussion

There are now, considerable data in support of ovarian reserve testing using AMH in a cancer population (Anders *et al.*, 2008; Lie Fong *et al.*, 2009; Decanter *et al.*, 2010, Partridge *et al.*, 2010b; Anderson & Cameron, 2011; Dillon *et al.*, 2013, Su *et al.*, 2013, Su *et al.*, 2014, Hamy *et al.,* 2014, Peigne *et al.,* 2014, Bozza *et al.,* 2014). By contrast, there are fewer studies that have data on AMH in reproductive age women at the time just before they initiate cancer therapy (Lawrenz *et al.*, 2012; Su *et al.*, 2013). This dissertation provides good evidence of diminished ovarian reserve in women with malignancy even before any gonadotoxic treatment is commenced. AMH levels were lower in the cancer group as a whole, prior to any gonadotoxic treatment being commenced, as compared with the same age group of healthy volunteers. After adjusting for other confounders: smoking status; BMI; parity; use of contraception; time of the cycle; AMH was still found to be significantly lower in the cancer group and differed between breast cancer, lymphoma and control groups.

This finding is similar to that of the FertiPROTEKT Network report (Lawrenz *et al.*, 2012) who studied ovarian reserve in 38 female patients with Lymphoma prior to cancer treatment and compared it with age-matched healthy controls. In combined Hodgkin and non-Hodgkin lymphoma groups, the mean AMH was

2.06 ng/mL while a significantly higher mean concentration of 3.20 ng/mL was reported in volunteers. The study population of 38 patients is considered to be relatively small, and the authors do not mention if they adjusted their analysis for any confounding factors. Strict selection criteria for the control group have been described, but it was not stated whether the same was applied to the cancer group. Nearly 60% of controls were on hormonal contraception while the same information was not reported for the cancer population. This is one of the first studies presenting results in the context of age-matched healthy volunteers as opposed to previous reports including subfertile patients as controls (Yu *et al.*, 2010).

The only other study presenting results of pre-chemotherapy ovarian reserve in reproductive age women diagnosed with cancer, provided opposing results (Su *et al.*, 2013). A total of 108 patients with a breast cancer and a median age of 40.2 years were found to have similar serum concentrations of AMH in comparison with healthy controls with a median age of 33.9 years. In contrast to this dissertation, the research group did not exclude women with PCOS who are known to have significantly higher serum AMH concentrations. Additionally, in their analysis, they have not adjusted for confounding factors including usage of hormonal contraception, time of the menstrual cycle and previous history of infertility. The multiple logistic regression models built for this dissertation population has included all of the above. Su et al. (2013) also provided only a limited description of how the blood samples were handled moreover, it is not

clear whether a pre-mixing step for assaying the serum using the second generation assay was included, as currently recommended by manufacturer. If the old methodology was used, this might have affected their AMH results.

In summary, this dissertation is the first one to confirm lowered ovarian reserve in women with malignancy prior to any gonadotoxic treatment using a new, more reliable AMH assay method and adjusting for any confounding factors in statistical analysis.

A biological explanation of diminished ovarian reserve in cancer sufferers, even before gonadotoxic therapy, is difficult. At present, it is unclear whether there is a cause and effect relationship, for example, does the lower ovarian reserve in some way predispose to breast cancer initiation, or does it arise as a response to the malignant disease? Titus et *al.*, reported that women with BRCA1 mutation may have lower levels of serum AMH as a result of impaired DNA repair mechanisms in oocytes. This idea may imply that loss of more oocytes might be expected due to a deficit of DNA repair. Expression of BRCA1 gene in oocytes was found to be important in the process of ovarian aging related to DNA double–strand break repair (Titus *et al.*, 2013). Others have reported that BRCA mutation may be associated with a lower age at natural menopause that would support the finding of reduced ovarian reserve in this group (Finch *et al.*, 2013; Lin *et al.*, 2013). By contrast, others have recently found similar serum AMH concentrations in healthy BRCA1/2 mutation carriers age 26-40, as compared with the general population (Michaelson-Cohen *et al.*, 2014).

Apart from widespread metastatic disease, the mechanisms that may be responsible for decreased fertility potential in women with other malignancy are uncertain. The consumptive character of systemic illness, increased catabolism, and elevated levels of stress hormones causing an increase in prolactin levels, which may impact on ovulation have all been proposed as possible pathways affecting ovarian reserve (Agarwal & Said, 2004). Additionally, poor health at the time of cancer diagnosis measured using serum markers e.g. increased C-Reactive Protein (CRP), high body temperature and low haemoglobin have been shown to correlate with low serum AMH concentration in girls age 0-18 years newly diagnosed with cancer (van Dorp *et al.*, 2014). The nature of this relationship is unclear, but the author has hypothesised that factors other than the size of the pool of follicles in the ovary might be contributing to low serum AMH in cancer patients.

In basic science research, it has been suggested that AMH is one of the mediators of growth regulatory signals in breast tissue (Hoshiya *et al.*, 2003). Exposure of breast cancer cells to AMH was found to inhibit growth and induce apoptosis. These authors suggested that lowered circulating AMH concentrations may be one of the predisposing factors for developing breast cancer (Hoshiya *et al.*, 2003). To explore this hypothesis further, the author has tested to find if there is a significant difference in pre-treatment serum AMH concentrations according to the stage of breast cancer at the time of diagnosis. The slight trend was suggestive of lower AMH in patients with more advanced

stages of breast cancer, but a larger study would be needed to provide conclusive results.

The data in this dissertation confirmed previous reports that AMH concentrations in serum decrease with female age (Wallace & Kelsey, 2010) in both healthy volunteers and the cancer group. Interestingly, a prediction model estimated a six year difference in ‘fertility age’ between groups. In other words, on average, a 25 year old cancer patient was found to have a serum AMH concentration equivalent to that of a 31 year old healthy volunteer. There was no evidence that the extent of the AMH difference between groups changed with age. At present, this is the first dissertation to report such a prediction model that can be used in clinical practice.

The longitudinal results of this dissertation show that, within 12 months from the first dose of chemotherapy, in nearly 80% of patients, serum AMH concentrations remained undetectable.

Measurements of AMH, 12 months from first dose of chemotherapy, may not provide a valuable clinical indication of the risk of early menopause. Undetectable AMH at this point cannot confirm the diagnosis of early menopause unless proved by long term studies with end points including pregnancy and permanent amenorrhea. Until today there has been insufficient data to define clearly the time needed for recovery of ovarian function post-chemotherapy and the best time for AMH measurements to be undertaken (Lie Fong *et al.*, 2008, Decanter *et al.*, 2010; Dillon *et al.*, 2013; Anderson *et al.*, 2013; Behringer *et al.*, 2013a).

The few studies that describe the recovery of ovarian function, based on pre- and post-chemotherapy AMH levels have some limitations. Dillon et *al.*, (2013) included a heterogeneous patient population comprising 46 women with many different types of cancers including breast cancer, brain tumour, sarcoma, leukaemia etc., requiring different treatment types and dosages and followed them up every three months. The rate of post-chemotherapy recovery of ovarian function was based on the estimated monthly recovery of AMH in two adjusted group of patients with pre-chemotherapy AMH of ≤2 ng/ml and ≥ 2 ng/ml. This statistical model is based on assumptions and, due to the heterogeneity of the population, might not be relevant for a given subgroup of patients. In opposition,

Anderson *et al.,* (2013), recruited only women with breast cancer and followed them up to two years. The authors compared mean AMH in women with the return of menses (nine patients) versus women with no menses (30 patients) at two year follow-up. Women with the presence of menses at follow-up had statistically higher mean AMH pre-chemotherapy versus women with no menses. It is worth noting that the mean age of the participants was 42.6 years, and all cancer patients had undetectable AMH at one year of follow-up.

In this dissertation, in the breast cancer group, only a small number of women had detectable AMH at 12 months of follow-up. Overall, those women were younger (age ≤40) and had higher average AMH concentrations before chemotherapy. The results show that in the group of patients with breast cancer, pre-treatment levels of AMH were not predictive of post-treatment levels of AMH, however, such a correlation was found in the small sub-group of patients with Hodgkin lymphoma. Additionally, pre-chemotherapy serum concentrations of AMH were not predictive of return of menses at 12 month follow-up. Interestingly, the age of patients at the time of first dose of chemotherapy was associated with their risk of amenorrhea within 12 months and was found to be a better indicator than AMH alone.

In the Hodgkin lymphoma group, better recovery of ovarian function was observed in patients who received the ABVD regimen only and were aged ≤32. Although, some recovery of ovarian function was observed even in cases where

the first AMH measurements were as low as 3.6 pmol/L if ABVD regimen only was used.

The same was not found in patients using the non-ABVD regimens including alkylating agents. Analysis of the results in for this dissertation was performed on a small subgroup of haematology patients but is consistent with results from a large cross sectional study on Hodgkin lymphoma survivors (Behringer *et al.*, 2013b). The authors observed statistically higher mean AMH concentrations in women who were younger (30 year old) and who received the ABVD regimen. Use of a cyclophosphamide-based protocol resulted in undetectable serum AMH concentrations. In the same study, 90% of participants experienced a recurrence of menses which is high and similar to the results in this dissertation (88%). As confirmation, another prospective study on 30 patients diagnosed with lymphoma reported good recovery of ovarian function in the group of patients on the ABVD regimen, which was observed to be significantly better than in patients on the non-ABVD protocol (Decanter *et al.*, 2010)

Interestingly, in this dissertation, two cancer patients with very low or undetectable levels of AMH conceived with three months of the final blood test. It shows that, despite an undetectable or low serum AMH concentration at 12 months, conception can still take place. The occurrence of pregnancy despite undetectable serum AMH levels has been previously presented in a case report (Fraisse *et al.*, 2008). Similar findings were reported from a large cross sectional study where some cancer survivors with undetectable AMH concentrations in

serum reported pregnancy (Behringer *et al.*, 2013a), although the authors provided no clinical details nor time between pregnancy occurrence and testing for AMH.

All the results presented above raise questions about the time needed for recovery of ovarian function post-chemotherapy and the findings also challenge the sensitivity of the currently available second generation AMH assay. A new ultrasensitive AMH assay (Ansh) with a lower limit of detection of 0.2 pmol/L has been developed. Some pilot studies have provided evidence supporting its use in certain circumstances, e.g. reduced ovarian reserve post-chemotherapy (Fleming *et al.*, 2013b). It is unclear how many non-growing follicles are still remaining in the ovary when serum AMH concentrations become undetectable (using detection limits of the currently available assay) post-chemotherapy because AMH is produced mainly by small, growing follicles. However, this population is believed to be lost first when chemotherapy is applied. It is estimated that the postmenopausal ovary would, on average, contain fewer than 1000 non-growing follicles that are considered too few to initiate recruitment and growth that could lead to pregnancy (Kelsey *et al.*, 2012). One research group estimated that serum AMH concentrations below 0.39 ng/mL (2.7 pmol/L) are predictive of menopause in the next six years following serum measurements (Tehrani *et al.*, 2013).

This dissertation shows that, in breast cancer patients taking Tamoxifen as compared to non-users of Tamoxifen, after adjusting for age, no difference was

found in serum AMH concentrations at 12 months follow-up. In contrast to a report from a small study of 20 patients, which suggested lowered levels of AMH in Tamoxifen users (Partridge *et al.*, 2010a).The sample size of Partridge's study was small, and the authors did not adjust their results according to the age of the patients. However, the results of this dissertation are consistent with a report from Oktay *et al.,* (Oktay *et al.*, 2013). Their study recruited 210 menstruating women and measured AMH prior to the use of Tamoxifen at 4 and eight months follow-up that showed unchanged levels during the follow-up period.

This dissertation has provided important data on patients' wishes to become a biological mother assessed at the time of cancer diagnosis and 12 months later after completion of cancer therapy. In women aged up to 43 years, facing a cancer diagnosis, overall 35% still expressed desire (either 'strong' or 'possibly/maybe') to have children, while this was true for nearly 50% in the group aged ≤ 39 years. In the group of patients with lymphoma, more than 80% of them expressed wishes for pregnancy. As reported by the oncology group recruiting into this study, less than 10% of participants had had a consultation with a fertility specialist. This finding is reported prospectively for the first time in this multicentre, UK based study. The results indicate that the often forgotten aspect of fertility is important for cancer patients. An effort should be made to build protocols that would help to refer young women of reproductive age for fertility counselling before commencing chemotherapy.

In this study, during the follow-up period, the desire to have children remained the same in the haematology patients while in the breast cancer group 20% of women reported a more favourable view on future motherhood at 12 month follow-up as compared with initial assessment prior to chemotherapy at the time of diagnosis. Overall, in the whole group of cancer patients, wishes for future pregnancy had not changed as a result of treatment burden.

Serum AMH concentration was undetectable at 12 months of follow-up in the majority of patients. Consequently, assessment of the impact on fertility in subgroups of patients receiving different types and dosages of chemotherapy was suboptimal. The time frame of this MD study was too short to correlate changes in ovarian reserve with clinical outcomes such as pregnancy rate and early or premature menopause. It was, therefore, aimed to complete a 5 year follow-up (as per protocol) to describe the longer term impact of chemotherapy and the value of AMH measurements in this group of women.

There is ongoing support for using AMH as a marker of ovarian reserve (Fleming *et al.,* 2015, Iliodromiti *et al.,* 2015), however most of publications present data on AMH results obtained from subfertile population (La Marca *et al., 2007,* Broer *et al.*, 2009; Nelson *et al.*, 2009 Anderson *et al.*, 2012). Among the published studies, there is conflicting evidence on the degree of cycle dependence of serum AMH concentrations and also disagreement regarding the effects of other factors such as hormonal contraception, BMI or smoking status on its levels (Dewailly *et al.*, 2014).

In this study, a sub-analysis of serum AMH measurements was conducted in a population of healthy women. It was found that, in healthy volunteers aged 18-43 without hormonal contraception, AMH was not correlated to the stage of the menstrual cycle (categorised as follicular, mid-cycle and luteal phase). Blood samples for this study were obtained at unspecified times of the cycle and AMH results were analysed according to the self-reported time of the cycle at venepuncture. To assess the intra-cycle variability of AMH, a different design, with repeated measures of blood samples within the same menstrual cycle would be more appropriate. Some researchers have found fluctuations in the mean AMH increasing up to 20% in the late follicular phase and decreasing 20% after ovulation and in early luteal phase (Cook *et al.*, 2000a; Wunder *et al.*, 2008a, Sowers *et al.*, 2010a;) in contrast to others (La Marca *et al.*, 2006b; Tsepelidis *et al.*, 2007). Some authors have suggested that changes in AMH concentrations were more prominent in younger women, while only minimal changes were observed in the older population, which the authors explained in terms of changes related to ovarian aging (Sowers *et al.*, 2010b; Wunder *et al.*, 2008a).

The first two well-designed, prospective studies assessing intra-individual variation of AMH were conducted in the subfertile population (Fanchin *et al.*, 2005; van Disseldorp *et al.*, 2010). The author has assessed intra-subject fluctuation of AMH on 4 repeated measures and found that the mean coefficient of variation for the groups was 15% which is consistent with a previous report

from Van Disseldorp *et al.,* (2010). Overall, as previously recommended (La Marca *et al.*, 2009, Dewailly *et al.*, 2014;) by a group of experts, AMH measurements at unspecified times of the menstrual cycle are acceptable. This dissertation confirmed those findings in a group of healthy volunteers using the most up to date and accurate method of assaying AMH, which included a pre-dilution step. It is a very important finding for clinicians working in the field of Reproductive Medicine, who may have decided to stop using AMH in their clinical practice as a result of recent questions about reproducibility and reliability of AMH results.

The impact of the use of hormonal contraception on AMH levels was debated (Somunkiran *et al.*, 2007; Streuli *et al.*, 2008; van den Berg *et al.*, 2010, Li *et al.*, 2011) until a large, prospective study on nearly 900 participants reported significantly lowered AMH concentrations (by 29%) in combined contraception users (Bentzen *et al.*, 2012) in comparison with non-users. In contrast, this study, after adjusting for other confounding factors, found no significant correlation between the use of combined contraception and AMH concentrations, although, only a small number of participants (18%) was using combined contraception which might have contributed to lack of statistical power to detect significant differences. The theory behind decreasing levels of AMH during the use of hormonal contraception is that the number of small antral follicles responsive to FSH, which are known to have a relatively large mass of granulosa cells responsible for AMH production, is decreased. Additionally

estradiol may play a role in AMH production which may be altered by use of combined hormonal contraception (Kallio *et al.*, 2013). Therefore, the duration and type of hormonal contraception and the timing of the blood tests, potentially, have had an impact on the degree of changes in AMH serum concentrations. This study design did not allow to look into all those aspects.

After adjusting for age, in the group of healthy childbearing age women, this dissertation found no association between BMI and serum AMH measurements. By contrast, one study (Freeman *et al.*, 2007), reported lowered AMH levels in obese women in late reproductive age. This study's group had a wider age range which could provide different results because AMH declines throughout the reproductive life span and some AMH changes provoked by lifestyle factors could be more prominent in late reproductive age. In agreement with this dissertation's results, another research group found no correlation between AMH and obesity, however, the mean age in their examined population was 46 (Halawaty *et al.*, 2010). Also, a large study on 461 healthy women, after adjusting for age found no correlation between serum AMH concentrations and BMI (La Marca *et al.*, 2012). Others found negative correlation between AMH and high BMI in group of Caucasian women only (Moy *et al.,* 2015) but not in Asian and African-American.

In a cross sectional study on 284 women, active smoking was found to be correlated with lowered serum AMH concentrations in late reproductive age women, aged 38-42 (Plante *et al.*, 2010). This is consistent with studies

suggesting an impact of smoking on the age at natural menopause (Sun *et al.*, 2012), although the authors did not observe the same correlation in women who were ex-smokers. In this dissertation, which included a younger population of patients with median age of 34, no relationship between smoking status and AMH concentrations was found. The findings are similar, however, to a large study on a healthy population with a similar age range (La Marca *et al.*, 2012). As mentioned earlier, selection of an older age group for analysis could have an impact on AMH results in the light of lifestyle factors.

Overall, measuring serum AMH concentrations at unspecified times of the menstrual cycle was found to be reliable. This dissertation adds conclusive results to improve upon a large number of small studies with contradictory results regarding the relationship of serum AMH concentrations with different lifestyle factors.

10 Recommendations for clinical practice and limitations of the study

10.1 Recommendations for clinical practice

The AMH assay has historically had a number of concerns. However, the results of this dissertation are among the first to utilise the manufacturer's most recent instructions of applying a pre-mix step and, thus, are more reliable than most of the previous studies. These results have verified the adequate intra-subject (sample to sample) reproducibility of the second generation AMH assay. It has confirmed that serum AMH measurements can be reliably measured at unspecified times of the menstrual cycle, which is in concordance with current recommendations (La Marca *et al.*, 2009, Dewailly *et al.*, 2014). It gives a very useful information for clinicians who may have questioned the practical use of AMH test following recently published data (Rustamov *et al.*, 2012).

Measurement of AMH concentrations at 12 months from starting chemotherapy in pre-menopausal patients with breast cancer, as a means of determining the patient's future reproductive function, cannot be supported at present as there is not enough evidence that undetectable levels of AMH would confirm menopause. Pre-treatment AMH measurements in this group were not predictive of post-treatment AMH or amenorrhea at the 12 month follow-up. A longer period of follow-up may be needed to find such a correlation.

This study has resulted in a predictive model where, based on pre-treatment AMH and age of the patient, the chance of return of menses can be calculated. Using this model, one can calculate, for example, an average 30 year old with pre-treatment AMH of 9 pmol/L would have a 74% probability of return of menses within the 12 months after the first dose of chemotherapy. It can be used as a tool in counselling young, reproductive age women with cancer who may ask for more tailored and detailed information about gonoadotoxity of chemotherapy and its impact on menstrual cycle prior commencing treatment.

In contrast to the breast cancer group, in patients with lymphoma, recovery of ovarian function was good in patients with Hodgkin lymphoma who received only the ABVD regimen. It is, once again, reassuring for Haematology Consultants that the ABVD protocol is relatively 'gonadal-sparing' if used in young women. This study showed that the impact of the ABVD protocol on the ovarian reserve is less pronounced in women aged ≤32. Interestingly, in this particular subgroup of patients, pre-treatment AMH was predictive of higher chances of a return of menses and correlated with post-treatment AMH concentrations. However, this analysis included only a small number of patients and should, therefore, be interpreted with caution.

This study showed that in reproductive age women (18-43 years) diagnosed with cancer, the serum AMH concentration is already diminished prior to commencing chemotherapy, if adjusted for age, parity, type of contraception, BMI and smoking status. It is one of the first studies providing evidence of

reduced ovarian function in this group of patients, where the statistical analysis was corrected for all confounding factors. Additionally, derived from my results' prediction model estimated a 6 year difference in 'fertility age' between healthy volunteers and women newly diagnosed with cancer, which may be used in clinical practice as 'numbers to quote' while counselling patients about fertility preservation. Interestingly, that correlation was stable throughout the whole age span.

From previously published papers, it is known that, among women diagnosed with breast cancer under the age of 40, less than 10% subsequently have children after treatment (Kim *et al.*, 2011). Simultaneously, some studies suggest that 60% of breast cancer survivors express concerns about fertility and early menopause, some 40% strongly wished to have the option of becoming a biological mother still available to them. Only half of those women felt that their concerns about future fertility had been adequately addressed (Partridge *et al.*, 2004).

In my study, more than 35% of women newly diagnosed with cancer still expressed some desire to have children. In a younger subgroup of women below the age of 40, nearly 50% of them still wished to have children even though they were facing a cancer diagnosis. Despite that, amongst all cancer patients recruited into the study, only 9% had been referred to discuss their options with a fertility special fertility prior to chemotherapy.

The implications of my findings are important for both oncologists and fertility specialists. In my opinion, oncologists may need to take into consideration referral of all young women newly diagnosed with cancer to a fertility specialist, especially those wishing for a future pregnancy. One of the first tests that they may receive is a serum AMH assay which would provide information about their remaining ovarian reserve. Together with knowledge of their chemotherapy regimen, this could provide some assessment of their likely chances of subfertility or sterility ensuing after their therapy and inform their decisions regarding fertility preservation. Patients should be aware that they may have a potentially diminished ovarian reserve and that this may be relevant to their stimulation protocol if fertility preservation is offered in the form of egg or embryo freezing. Better coordination between oncology and fertility clinics will be needed if patients are ever to receive a full assessment of their needs and comprehensive information on their prospects.

Additionally, nearly two-thirds of women diagnosed with breast cancer have an oestrogen receptor-positive tumour and would be advised to have adjuvant therapy with Tamoxifen (partial agonist of oestrogen) and/or a GnRH agonist which would further delay and impact their chances of achieving pregnancy (Kim *et al.*, 2011). It would be advisable for women to be advised of this in the light of knowledge about their prospective fertility.

An integrated approach to the wellbeing of reproductive age women diagnosed with cancer should include a fertility consultation. Referral to a Reproductive

Medicine Consultant prior to chemotherapy is still not a routine practice in the UK although, in some Trusts there are already protocols in place (e.g. in Queen Elizabeth Hospital in Birmingham where all newly diagnosed female patients with lymphoma are referred for fertility discussions with a specialist (if patient agrees). Alternatively, regular training or up-dating sessions on fertility preservation could be organised for Oncologists or it could become a regularly discussed topic at oncology conferences to make sure that up-to-date knowledge is being used while discussing these sensitive issues before commencing chemotherapy.

10.2 Limitations of the study design

I planned to recruit exact age matched controls in the ratio of two controls per cancer patient, as advised by Prof. Dunn (Professor of Clinical Trials and Head of Cancer Trials). The final stage of the recruitment process targeted age groups previously underrepresented by controls. Despite that, some cases did not have exact age-matched controls. Given the overall availability of control data, Dr Nick Parsons, Statistician, advised that the data should be analysed as an unmatched case-control study with adjustment for the age imbalance between groups in all analyses.

The breast cancer group was, therefore, significantly older than the group of healthy controls. Of the cancer patients, 42/54 (77%) already had offspring prior to diagnosis, although the risk of early menopause, its consequences and the

need for long term adjuvant hormonal therapy with Tamoxifen in premenopausal patients still makes the question of remaining ovarian function very relevant. Only a small group of patients with lymphoma was recruited. In addition, the statistical analysis was not sufficiently well powered to detect differences in AMH concentrations in sub-groups of patients (e.g. based upon staging of the disease, the dosage of chemotherapy). The extent of analysis possible was not, therefore, as great as anticipated.

Women taking hormonal contraception were not excluded from the study. At the time of recruitment into the study, some of the patients newly diagnosed with breast cancer may have recently stopped hormonal contraception at the request of the oncologist and some residual suppression of AMH was possible. In the control group around 20% of women had taken the combined oral contraception which has been shown to have some effect on AMH concentrations (Bentzen *et al.*, 2012). This study was not published when I devised my protocol. Papers published previously had provided conflicting results (Somunkiran *et al.*, 2007; Streuli *et al.*, 2008; van den Berg *et al.*, 2010; Li *et al.*, 2011). However, all the statistical analyses have been corrected for confounding factors including the use of contraception.

Some of the volunteers opting to take part in the study may have done so because they had concerns about their fertility, or they had, perhaps, been actively trying for a pregnancy. Seven women in the volunteer group became pregnant during the 12 months follow-up period. They have been included in

the final analysis. The overall number of patients who became pregnant during the study remained small and statistical advice indicated that this factor is unlikely to have affected the final analysis. There is conflicting evidence on AMH concentrations in maternal blood samples during pregnancy (La Marca *et al.,* 2005b, Koninger *et al.*, 2013, Koninger *et al.,* 2015). One of the first studies, conducted on 84 participants aged 18-37 examined in the first, second and third trimesters of pregnancy, showed no significant changes in AMH measurements compared with a volunteer group (La Marca *et al.*, 2005b). A larger cross-sectional study presented the results of serum AMH measurements in 450 healthy pregnant women at any gestational age (Koninger *et al.*, 2013). In the first part of this study, AMH results from 15 women were carried out through the first, second and third trimester of pregnancy. The authors observed a statistically significant decline in AMH concentrations during pregnancy, the lowest being in the third trimester. Similarly, during the first 4 days of the postpartum period, levels of AMH remained low. The authors discussed possible causes for lowered AMH including inhibition of folliculogenesis in pregnancy. It can be related to decreased number of growing follicules which are known to be hugly responsible for the production of AMH. Another possible explanation is that high oestrogen levels during pregnancy may suppress AMH promoter activity and decrease AMH concentrations (Chen *et al.*, 2003). Additionally, it is still unclear whether there is any placental production of AMH in humans (La Marca *et al.*, 2005b).

Considering the design of my study, known issues with the AMH assay technology, beyond the control of this study, may indicate that having another reliable test of ovarian reserve, such as AFC, would have been ideal to provide additional validation of my findings but this was never a realistic option for the cancer patient group or a multicentre study such as this.

Finally, this study aims to follow patients up for at least five years, which is beyond the standard duration of an MD degree. I have, therefore, not yet been able to complete the analysis to correlate serum AMH concentrations pre- and post-chemotherapy with clinical end points of early menopause, infertility and live birth at 5 year follow-up.

10.3 Limitations arising from using second generation AMH assay as a marker of ovarian reserve

The company producing the AMH assay kits (Beckman Coulter) issued two field safety notices during the duration of this study. Initially, the methodology was changed from linear to cubic regression. Then, following a publication on the poor reproducibility of AMH results using the second generation assay (Rustamov *et al.*, 2012), in June 2013, another field safety notice was issued

informing users about possible interference of complement in serum samples, lowering the AMH results**.** This led to the decision to re-analyse all my study samples using the new assay method including a pre-mixing step to assure reliability and consistency of all the results presented in my thesis. The pilot study which I conducted on storage of blood samples for AMH assay and strict protocol on sample storage and transport provided the basis for reliable analysis of the results. Additionally, proper preparation and storage of 3-4 aliquots of each collected sample, allowed me to performed unbiased analyses of two different methods of assaying (with and without the pre-dilution step). Statistical analysis, performed by Dr Nick Parsons, showed a consistent difference between old and new data, which would support the view that my research samples were handled appropriately **Appendix 6.** Since then the new method has been shown to give consistent AMH concentrations under different storage conditions (Han *et al.*, 2014).

There is still a lack of international standards for AMH assay measurements, only a limited number of laboratories offer AMH testing and, finally, different methods of blood sample handling and storage may have impacted on all previously reported results (Nelson & La Marca, 2011), especially, if the samples were being sent from distant centres (Fleming *et al.*, 2013a). This situation has exacerbated the amount of conflicting data being published regarding AMH.

The newest, first automated platforms for AMH measurement offer hope for a more robust establishment of this useful test of ovarian reserve (Gassner & Jung, 2014). Such a development would allow availability of this test to be extended beyond the infertile population. Offering it more routinely to cancer patients could help with pre-treatment counselling, fertility preservation discussion and in making decisions about adjuvant hormonal therapy in older reproductive age women with breast cancer (Henry *et al.*, 2014; Lawrenz *et al.*, 2012).

10.4 Recommendations for future research

This study has shown that serum AMH measurement recovers in a minority of patients within one year of initiation of chemotherapy. This study will continue with a view to identifying clinical outcomes in patients. However, there remain several areas of study where further information is needed.

1. The reliability of ovarian reserve tests in predicting early menopause, chances of pregnancy and live birth following cancer therapy needs to be determined in a large cohort longitudinal study with follow-up at 5 -10 years.

2. The best timing for ovarian reserve testing (AMH measurement, AFC) following chemotherapy in order to provide reliable clinically relevant information. This would entail following patients to an end point such as menopause or chemotherapy-induced infertility.

3. Further study is needed to provide a biological explanation for the lowered serum levels of AMH prior to chemotherapy in cancer patients.

5. The stability of AMH assay measurements following storage of samples in different conditions and the potential benefits of using an ultrasensitive AMH assay in the cancer population require further investigation.

6. The desires of cancer patients for greater knowledge of the impacts of chemotherapy upon their fertility and means to avoid infertility where appropriate should be analysed and responded to by both oncologists and fertility specialists.

7. The place of AMH testing in the general population, for example, as means of educating young women regarding declining fertility, diagnosing premature ovarian decline, predicting menopause and advising regarding family planning remain to be explored.

REFERENCES

Adami, H. O., D. Hunter, and D. Trichopoulos, (2008) Textbook of Cancer Epidemiology:669-670.

Agarwal, A. & Said, T. M. (2004) Implications of systemic malignancies on human fertility. Reprod Biomed Online, 9 (6): 673-679.

Ajala, T., Rafi, J., Larsen-Disney, P. & Howell, R. (2010) Fertility preservation for cancer patients: a review. Obstet Gynecol Int, 2010 160386.

Al-Qahtani, A., Muttukrishna, S., Appasamy, M., Johns, J., Cranfield, M., Visser, J. A., Themmen, A. P. N. and Groome, N. P. (2005b) Development of a sensitive enzyme immunoassay for anti-Müllerian hormone and the evaluation of potential clinical applications in males and females. Clinical Endocrinology, 63:267–273

Amorim, C. A., Van Langendonckt, A., David, A., Dolmans, M. M. & Donnez, J. (2009) Survival of human pre-antral follicles after cryopreservation of ovarian tissue, follicular isolation and in vitro culture in a calcium alginate matrix. In: eds. Hum Reprod. England: 92-99.

Anders, C., Marcom, P. K., Peterson, B., Gu, L., Unruhe, S., Welch, R., Lyons, P., Behera, M., Copland, S., Kimmick, G., Shaw, H., Snyder, S., Antenos, M., Woodruff, T. & Blackwell, K. (2008) A pilot study of predictive markers of chemotherapy-related amenorrhea among premenopausal women with early stage breast cancer. Cancer investigation, 26 (3): 286-295.

Andersen, C. Y. & Byskov, A. G. (2006) Estradiol and regulation of anti-Mullerian hormone, inhibin-A, and inhibin-B secretion: analysis of small antral and preovulatory human follicles' fluid. J Clin Endocrinol Metab, 91 (10): 4064-4069.

Andersen, C. Y., Kristensen, S. G., Greve, T. & Schmidt, K. T. (2012) Cryopreservation of ovarian tissue for fertility preservation in young female oncological patients. Future Oncol, 8 (5): 595-608.

Anderson, R. A. & Cameron, D. A. (2011) Pretreatment serum anti-mullerian hormone predicts long-term ovarian function and bone mass after chemotherapy for early breast cancer. The Journal of clinical endocrinology and metabolism, 96 (5): 1336-1343.

Anderson, R. A., Groome, N. P. & Baird, D. T. (1998) Inhibin A and inhibin B in women with polycystic ovarian syndrome during treatment with FSH to induce mono-ovulation. Clinical Endocrinology, 48 (5): 577-584.

Anderson, R. A., Nelson, S. M. & Wallace, W. H. B. (2012) Measuring anti-Mullerian hormone for the assessment of ovarian reserve: when and for whom is it indicated? Maturitas, 71 (1): 28-33.

Anderson, R. A., Rosendahl, M., Kelsey, T. W. & Cameron, D. A. (2013) Pretreatment anti-Mullerian hormone predicts for loss of ovarian function after chemotherapy for early breast cancer. Eur J Cancer, 49 (16): 3404-3411.

Anderson, R. A., Themmen, A. P. N., -Qahtani, A. A., Groome, N. P. & Cameron, D. A. (2006) The effects of chemotherapy and long-term gonadotrophin suppression on the ovarian reserve in premenopausal women with breast cancer. Hum. Reprod., 21 (10): 2583-2592.

Anderson, R. A. & Wallace, W. H. B. (2011) Fertility preservation in girls and young women. Clinical Endocrinology, Volume 75, Issue 4, pages 409–419.

Antoniou, A., Pharoah, P. D., Narod, S., Risch, H. A., Eyfjord, J. E., Hopper, J. L., Loman, N., Olsson, H., Johannsson, O., Borg, A., Pasini, B., Radice, P., Manoukian, S., Eccles, D. M., Tang, N., Olah, E., Anton-Culver, H., Warner, E., Lubinski, J., Gronwald, J., Gorski, B., Tulinius, H., Thorlacius, S., Eerola, H., Nevanlinna, H., Syrjakoski, K., Kallioniemi, O. P., Thompson, D., Evans, C., Peto, J., Lalloo, F., Evans, D. G. & Easton, D. F. (2003) Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies. In: eds. Am J Hum Genet. United States: 1117-1130.

ASRM (2013) Mature oocyte cryopreservation. The Practice Committee of the American Society for Reproductive Medicine and the Society for Assited Reproductive Technology [online] Available from: http://asrm.org/uploadedFiles/ASRM_Content/News_and_Publications/Practice_Guidelines/Committee_Opinions/Ovarian_tissue_and_oocyte%281%29.pdf (Accessed 07/07/2013).

Ataya, K. & Moghissi, K. (1989) Chemotherapy-induced premature ovarian failure: mechanisms and prevention. Steroids, 54 (6): 607-626.

Baarends, W., Uilenbroek, J., Kramer, P., Hoogerbrugge, J., van Leeuwen, E., Themmen, A. & Grootegoed, J. (1995) Anti-mullerian hormone and anti-mullerian hormone type II receptor messenger ribonucleic acid expression in rat

ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth. Endocrinology, 136 (11): 4951-4962.

Bancsi, L. F. J. M. M., Broekmans, F. J. M., Eijkemans, M. J. C., de Jong, F. H., Habbema, J. D. F. & te Velde, E. R. (2002) Predictors of poor ovarian response in in vitro fertilization: a prospective study comparing basal markers of ovarian reserve. Fertility and Sterility, 77 (2): 328-336.

Bancsi, L. á. F. J. M. M., Broekmans, F. J. M., Mol, B. W. J., Habbema, J. D. F. & te Velde, E. R. (2003) Performance of basal follicle-stimulating hormone in the prediction of poor ovarian response and failure to become pregnant after in vitro fertilization: a meta-analysis. Fertility and Sterility, 79 (5): 1091-1100.

Bath, L. E., Wallace, W. H. B., Shaw, M. P., Fitzpatrick, C. & Anderson, R. A. (2003) Depletion of ovarian reserve in young women after treatment for cancer in childhood: detection by anti-Mullerian hormone, inhibin B and ovarian ultrasound. Hum. Reprod., 18 (11): 2368-2374.

Behringer, K., Breuer, K., Reineke, T., May, M., Nogova, L., Klimm, B., Schmitz, T., Wildt, L., Diehl, V. & Engert, A. (2005) Secondary amenorrhea after Hodgkin's lymphoma is influenced by age at treatment, stage of disease, chemotherapy regimen, and the use of oral contraceptives during therapy: a report from the German Hodgkin's Lymphoma Study Group. J Clin Oncol, 23 (30): 7555-7564.

Behringer, K., Mueller, H., Goergen, H., Thielen, I., Eibl, A. D., Stumpf, V., Wessels, C., Wiehlputz, M., Rosenbrock, J., Halbsguth, T., Reiners, K. S., Schober, T., Renno, J. H., von Wolff, M., van der Ven, K., Kuehr, M., Fuchs, M., Diehl, V., Engert, A. & Borchmann, P. (2013) Gonadal Function and Fertility in Survivors After Hodgkin Lymphoma Treatment Within the German Hodgkin Study Group HD13 to HD15 Trials. Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 31 (2): 231-239.

Ben-Aharon, I., Meizner, I., Granot, T., Uri, S., Hasky, N., Rizel, S., Yerushalmi, R., Sulkes, A. & Stemmer, S. M. (2012) Chemotherapy-Induced Ovarian Failure as a Prototype for Acute Vascular Toxicity. The Oncologist, 17 (11): 1386-1393.

Ben-Aharon I, Granot T, Meizner I, Hasky N, Tobar A, Rizel S, Yerushalmi R, Ben-Haroush A, Fisch B, Stemmer SM (2015). Long-Term Follow-Up of Chemotherapy-Induced Ovarian Failure in Young Breast Cancer Patients: The Role of Vascular Toxicity. Oncologist. 2015 Jun 22. pii: theoncologist.2015-0044.

Bentov, Y., Yavorska, T., Esfandiari, N., Jurisicova, A. & Casper, R. (2011) The contribution of mitochondrial function to reproductive aging. Journal of Assisted Reproduction and Genetics Volume 28, Issue 9, pp 773-783.

Bentzen, J. G., Forman, J. L., Pinborg, A., Lidegaard, Ø., Larsen, E. C., Friis-Hansen, L., Johannsen, T. H. & Nyboe Andersen, A. (2012) Ovarian reserve parameters: a comparison between users and non-users of hormonal contraception. Reproductive BioMedicine Online.

Beral, V. (2003) Breast cancer and hormone-replacement therapy in the Million Women Study. In: eds. Lancet. England: 419-427.

Berliere, M., Dalenc, F., Malingret, N., Vindevogel, A., Piette, P., Roche, H., Donnez, J., Symann, M., Kerger, J. & Machiels, J. P. (2008) Incidence of reversible amenorrhea in women with breast cancer undergoing adjuvant anthracycline-based chemotherapy with or without docetaxel. BMC Cancer, 8 56.

Bines, J., Oleske, D. M. & Cobleigh, M. A. (1996) Ovarian function in premenopausal women treated with adjuvant chemotherapy for breast cancer. J Clin Oncol, 14 (5): 1718-1729.

Blumenfeld, Z., Avivi, I., Eckman, A., Epelbaum, R., Rowe, J. M. & Dann, E. J. (2008) Gonadotropin-releasing hormone agonist decreases chemotherapy-induced gonadotoxicity and premature ovarian failure in young female patients with Hodgkin lymphoma. Fertil Steril, 89 (1): 166-173.

Bokemeyer, C., Schmoll, H. J., van Rhee, J., Kuczyk, M., Schuppert, F. & Poliwoda, H. (1994) Long-term gonadal toxicity after therapy for Hodgkin's and non-Hodgkin's lymphoma. Ann Hematol, 68 (3): 105-110.

Boyle, P. & Levin, B. (2008) World Cancer Report 2008. Available from http://www.iarc.fr/en/media-centre/iarcnews/2008/index.php.

Bozza C, Puglisi F, Lambertini M, Osa EO, Manno M, Del Mastro L. Anti-Mullerian hormone: determination of ovarian reserve in early breast cancer patients.Endocr Relat Cancer. 2014 Jan 21;21(1):R51-65.

Brodin, T., Hadziosmanovic, N., Berglund, L., Olovsson, M. & Holte, J. (2013) Antimüllerian Hormone Levels Are Strongly Associated With Live-Birth Rates After Assisted Reproduction. Journal of Clinical Endocrinology & Metabolism, 98 (3): 1107-1114.

Broekmans, F. J., Kwee, J., Hendriks, D. J., Mol, B. W. & Lambalk, C. B. (2006) A systematic review of tests predicting ovarian reserve and IVF outcome. Human Reproduction Update, 12 (6): 685-718.

Broer, S. L., Dolleman, M., van Disseldorp, J., Broeze, K. A., Opmeer, B. C., Bossuyt, P. M. M., Eijkemans, M. J. C., Mol, B. W., Broekmans, F. J. M. & Group, I.-E. S. (2013) Prediction of an excessive response in invitro fertilization from patient characteristics and ovarian reserve tests and comparison in

subgroups: an individual patient data meta-analysis. Fertility and sterility, 100 (2): 420-429.e427.

Broer, S. L., Eijkemans, M. J. C., Scheffer, G. J., van Rooij, I. A. J., de Vet, A., Themmen, A. P. N., Laven, J. S. E., de Jong, F. H., te Velde, E. R., Fauser, B. C. & Broekmans, F. J. M. (2011) Anti-Müllerian Hormone Predicts Menopause: A Long-Term Follow-Up Study in Normoovulatory Women. Journal of Clinical Endocrinology & Metabolism, 96 (8): 2532-2539.

Broer, S. L., Mol, B. W. J., Hendriks, D. & Broekmans, F. J. M. (2009) The role of antimullerian hormone in prediction of outcome after IVF: comparison with the antral follicle count. Fertility & Sterility, 91 (3): 705-714.

Brougham, M. F., Crofton, P. M., Johnson, E. J., Evans, N., Anderson, R. A. & Wallace, W. H. (2012) Anti-Mullerian hormone is a marker of gonadotoxicity in pre- and postpubertal girls treated for cancer: a prospective study. In: eds. J Clin Endocrinol Metab. United States: 2059-2067.

Brugo Olmedo, S., De Vincentiis, S., De Martino, E., Bedecarras, P., Blanco, A. M., Freire, A., Buffone, M. G. & Rey, R. A. (2013) Prediction of reproductive outcomes according to different serum anti-mullerian hormone levels in females undergoing intracystoplasmic sperm injection. In: eds. PLoS One. United States: e75685.

Brusamolino, E., Lunghi, F., Orlandi, E., Astori, C., Passamonti, F., Barate, C., Pagnucco, G., Baio, A., Franchini, P., Lazzarino, M. & Bernasconi, C. (2000) Treatment of early-stage Hodgkin's disease with four cycles of ABVD followed by adjuvant radio-therapy: analysis of efficacy and long-term toxicity. Haematologica, 85 (10): 1032-1039.

Burstein, H. J., Griggs, J. J., Prestrud, A. A. & Temin, S. (2010) American society of clinical oncology clinical practice guideline update on adjuvant endocrine

therapy for women with hormone receptor-positive breast cancer. J Oncol Pract, 6 (5): 243-246.

Burstein, H. J., Mayer, E., Patridge, A. H., O'Kane, H., Litsas, G., Come, S. E., Hudis, C. A., Goldstein, D. F., Muss, H. B., Winter, E. P. & Garber, J. E. (2006) Inadvertent use of aromatase inhibitors in patients with breast cancer with residual ovarian function: cases and lessons. Clinical Breast Cancer, 7 (2): 158-161.

Burstein, H. J. & Winer, E. P. (2000) Primary care for survivors of breast cancer. N Engl J Med, 343 (15): 1086-1094.

Cancer, C. G. o. H. F. i. B. (2001) Familial breast cancer: collaborative reanalysis of individual data from 52 epidemiological studies including 58,209 women with breast cancer and 101,986 women without the disease. In: eds. Lancet. England: 1389-1399.

Cancer, C. G. o. H. F. i. B. (2002) Breast cancer and breastfeeding: collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50302 women with breast cancer and 96973 women without the disease.

Chang, M.-Y., Chiang, C.-H., Hsieh, T. s.-T. a., Soong, Y.-K. & Hsu, K.-H. (1998) Use of the Antral Follicle Count to Predict the Outcome of Assisted Reproductive Technologies. Fertility and Sterility, 69 (3): 505-510.

Chen, G., Shinka, T., Kinoshita, K., Yan, H. T., Iwamoto, T. & Nakahori, Y. (2003) Roles of estrogen receptor alpha (ER alpha) in the regulation of the human Mullerian inhibitory substance (MIS) promoter. J Med Invest, 50 (3-4): 192-198.

Chen, H., Li, J., Cui, T. & Hu, L. (2011) Adjuvant gonadotropin-releasing hormone analogues for the prevention of chemotherapy induced premature ovarian failure in premenopausal women. Cochrane Database of Systematic Reviews(11):Available:http://onlinelibrary.wiley.com/doi/10.1002/14651858.CD008018.pub2/abstract (Accessed 20/08/2012)

Chisesi, T., Bellei, M., Luminari, S., Montanini, A., Marcheselli, L., Levis, A., Gobbi, P., Vitolo, U., Stelitano, C., Pavone, V., Merli, F., Liberati, M., Baldini, L., Bordonaro, R., Pesce, E. A. & Federico, M. (2011) Long-Term Follow-Up Analysis of HD9601 Trial Comparing ABVD Versus Stanford V Versus MOPP/EBV/CAD in Patients With Newly Diagnosed Advanced-Stage Hodgkin's Lymphoma: A Study From the Intergruppo Italiano Linfomi. Journal of Clinical Oncology, 29 (32): 4227-4233.

Cohenhaguenauer, O., Picard, J. Y., Mattei, M. G., Serero, S., Nguyen, V. C., Detand, M. F., Guerrier, D., Horscayla, M. C., Josso, N. & Frezal, J. (1987) Mapping of the Gene for Anti-Mullerian Hormone to the Short Arm of Human Chromosome-19. Cytogenetics and Cell Genetics, 44 (1): 2-6.

Cook, C. L., Siow, Y., Taylor, S. & Fallat, M. E. (2000a) Serum mullerian-inhibiting substance levels during normal menstrual cycles. Fertility & Sterility, 73 (4): 859-861.

Cook, C. L., Siow, Y., Taylor, S. & Fallat, M. E. (2000b) Serum müllerian-inhibiting substance levels during normal menstrual cycles. Fertility and Sterility, 73 (4): 859-861.

Couzin-Frankel, J. (2013) Reproductive Biology. Faulty DNA repair linked to ovarian aging in mice and humans. In: eds. Science. United States: 749.

Cowchock, F. S., Gibas, Z. & Jackson, L. G. (1993) Chromosome Errors As A Cause Of Spontaneous-Abortion - The Relative Importance Of Maternal Age And Obstetric History. Fertility and Sterility, 59 (5): 1011-1014.

Coxworth, J. E. & Hawkes, K. (2010) Ovarian follicle loss in humans and mice: lessons from statistical model comparison. In: eds. Hum Reprod. England: 1796-1805.

Daan NM, Fauser BC (2015).Menopause prediction and potential implications.Maturitas. 2015 Jul 26. pii: S0378-5122(15)30026-8.

Dafopoulos, A., Dafopoulos, K., Georgoulias, P., Galazios, G., Limberis, V., Tsikouras, P., Koutlaki, N. & Maroulis, G. (2010) Smoking and AMH levels in women with normal reproductive history. Arch Gynecol Obstet, 282 (2): 215-219.

de Vet, A., Laven, J. S. E., de Jong, F. H., Themmen, A. P. N. & Fauser, B. C. J. M. (2002) Antimüllerian hormone serum levels: a putative marker for ovarian aging. Fertility and Sterility, 77 (2): 357-362.

Decanter, C., Morschhauser, F., Pigny, P., Lefebvre, C., Gallo, C. & Dewailly, D. (2010) Anti-Mullerian hormone follow-up in young women treated by chemotherapy for lymphoma: preliminary results. In: eds. Reprod Biomed Online. England: 2009 Reproductive Healthcare Ltd. Published by Elsevier Ltd: 280-285.

Del Mastro, L., Boni, L., Michelotti, A., Gamucci, T., Olmeo, N., Gori, S., Giordano, M., Garrone, O., Pronzato, P., Bighin, C., Levaggi, A., Giraudi, S., Cresti, N., Magnolfi, E., Scotto, T., Vecchio, C. & Venturini, M. (2011) Effect of the gonadotropin-releasing hormone analogue triptorelin on the occurrence of chemotherapy-induced early menopause in premenopausal women with breast cancer: a randomized trial. In: eds. JAMA. United States: 269-276.

Demeestere, I., Basso, O., Moffa, F., Peccatori, F., Poirot, C. & Shalom-Paz, E. (2012) Fertility preservation in female cancer patients. Obstet Gynecol Int, 2012 695041.

Dewailly, D., Andersen, C. Y., Balen, A., Broekmans, F., Dilaver, N., Fanchin, R., Griesinger, G., Kelsey, T. W., La Marca, A., Lambalk, C., Mason, H., Nelson, S. M., Visser, J. A., Wallace, W. H. & Anderson, R. A. (2014) The physiology and clinical utility of anti-Mullerian hormone in women. Hum Reprod Update, 20 (3): 370-385.

Di Paola, R., Costantini, C., Tecchio, C., Salvagno, G. L., Montemezzi, R., Perandini, A., Pizzolo, G., Zaffagnini, S. & Franchi, M. (2013) Anti-Mullerian hormone and antral follicle count reveal a late impairment of ovarian reserve in patients undergoing low-gonadotoxic regimens for hematological malignancies. Oncologist, 18 (12): 1307-1314.

Dieudonne, A. S., Vandenberghe, J., Geerts, I., Billen, J., Paridaens, R., Wildiers, H. & Neven, P. (2011) Undetectable antimullerian hormone levels and recovery of chemotherapy-induced ovarian failure in women with breast cancer on an oral aromatase inhibitor. Menopause, 18 (7): 821-824.

Dillon, K. E., Sammel, M. D., Prewitt, M., Ginsberg, J. P., Walker, D., Mersereau, J. E., Gosiengfiao, Y. & Gracia, C. R. (2013) Pretreatment antimullerian hormone levels determine rate of posttherapy ovarian reserve recovery: acute changes in ovarian reserve during and after chemotherapy. Fertil Steril, 99 (2): 477-483.

Dolmans, M. M., Luyckx, V., Donnez, J., Andersen, C. Y. & Greve, T. (2013) Risk of transferring malignant cells with transplanted frozen-thawed ovarian tissue. Fertil Steril, 99 (6): 1514-1522.

Dólleman M, Verschuren WM, Eijkemans MJ, Broekmans FJ, van der Schouw YT Added value of anti-Müllerian hormone in prediction of menopause: results from a large prospective cohort study.Hum Reprod. 2015 Aug; 30(8):1974-81.

Donnez, J. & Dolmans, M.-M. (2011) Preservation of fertility in females with haematological malignancy. British Journal of Haematology, Volume 154, Issue 2, pages 175–184.

Durlinger, A. L. L., Gruijters, M. J. G., Kramer, P., Karels, B., Ingraham, H. A., Nachtigal, M. W., Uilenbroek, J. T. J., Grootegoed, J. A. & Themmen, A. P. N. (2002) Anti-Mullerian Hormone Inhibits Initiation of Primordial Follicle Growth in the Mouse Ovary. Endocrinology, 143 (3): 1076-1084.

Durlinger, A. L. L., Gruijters, M. J. G., Kramer, P., Karels, B., Kumar, T. R., Matzuk, M. M., Rose, U. M., de Jong, F. H., Uilenbroek, J. T. J., Grootegoed, J. A. & Themmen, A. P. N. (2001) Anti-Mullerian Hormone Attenuates the Effects of FSH on Follicle Development in the Mouse Ovary. Endocrinology, 142 (11): 4891-4899.

Durlinger, A. L. L., Kramer, P., Karels, B., de Jong, F. H., Uilenbroek, J. T. J., Grootegoed, J. A. & Themmen, A. P. N. (1999) Control of Primordial Follicle Recruitment by Anti-Mullerian Hormone in the Mouse Ovary. Endocrinology, 140 (12): 5789-5796.

El-Shalakany, A. H., Ali, M. S., Abdelmaksoud, A. A., Abd El-Ghany, S. & Hasan, E. A. (2013) Ovarian function in female survivors of childhood malignancies. Pediatr Hematol Oncol, 30 (4): 328-335.

Elgindy, E. A., El-Haieg, D. O. & El-Sebaey, A. (2008) Anti-Müllerian hormone: correlation of early follicular, ovulatory and midluteal levels with ovarian response and cycle outcome in intracytoplasmic sperm injection patients. Fertility and Sterility, 89 (6): 1670-1676.

English, R. (2004) Natallie Evans v Amicus Healthcare Ltd and Others. [online] Available from: http://www.1cor.com/1315/?form_1155.replyids=356 Accessed:15/07/2013

Evers, J. L. H., Slaats, P., Land, J. A., Dumoulin, J. C. M. & Dunselman, G. A. J. (1998) Elevated Levels of Basal Estradiol-17[beta] Predict Poor Response in Patients with Normal Basal Levels of Follicle-Stimulating Hormone Undergoing In Vitro Fertilization. Fertility and Sterility, 69 (6): 1010-1014.

Ewertz, M., Duffy, S. W., Adami, H. O., Kvale, G., Lund, E., Meirik, O., Mellemgaard, A., Soini, I. & Tulinius, H. (1990) Age at first birth, parity and risk of breast cancer: a meta-analysis of 8 studies from the Nordic countries. Int J Cancer, 46 (4): 597-603.

Faddy, M. J. & Gosden, R. G. (1996) Ovary and ovulation: A model conforming the decline in follicle numbers to the age of menopause in women. Human Reproduction, 11 (7): 1484-1486.

Faddy, M. J., Gosden, R. G., Gougeon, A., Richardson, S. J. & Nelson, J. F. (1992) Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. Human Reproduction, 7 (10): 1342-1346.

Familiari, G., Caggiati, A., Nottola, S. A., Ermini, M., Benedetto, M. R. D. & Motta, P. M. (1993) Infertility: Ultrastructure of human ovarian primordial follicles after combination chemotherapy for Hodgkin's disease. Human Reproduction, 8 (12): 2080-2087.

Fanchin, R., Taieb, J., Lozano, D. H., Ducot, B., Frydman, R. & Bouyer, J. (2005) High reproducibility of serum anti-Mullerian hormone measurements suggests a multi-staged follicular secretion and strengthens its role in the assessment of ovarian follicular status. Hum Reprod, 20 (4): 923-927.

Farrag, A. K., Khedr, E. M., Abdel-Aleem, H. & Rageh, T. A. (2002) Effect of surgical menopause on cognitive functions. In: eds. Dement Geriatr Cogn Disord. Switzerland: 2002 S. Karger AG, Basel: 193-198.

Finch, A., Valentini, A., Greenblatt, E., Lynch, H. T., Ghadirian, P., Armel, S., Neuhausen, S. L., Kim-Sing, C., Tung, N., Karlan, B., Foulkes, W. D., Sun, P. & Narod, S. (2013) Frequency of premature menopause in women who carry a BRCA1 or BRCA2 mutation. Fertil Steril, 99 (6): 1724-1728.

Fiçiciog, lu, C., Kutlu, T., Baglam, E. & Bakacak, Z. (2006) Early follicular antimüllerian hormone as an indicator of ovarian reserve. Fertility and Sterility, 85 (3): 592-596.

Fleming, R., Fairbairn, C., Blaney, C., Lucas, D. & Gaudoin, M. (2013a) Stability of AMH measurement in blood and avoidance of proteolytic changes. Reprod Biomed Online, 26 (2): 130-132.

Fleming, R., Fairbairn, C., Lucas, D., Gaudoin, M. & Anderson, R. A. (2013b) Analysis of two assays for the measurement of amh in women with low ovarian reserve. Fertility and sterility, 100 (3): S162.

Fleming R, Seifer DB, Frattarelli JL, Ruman J. (2015)
Assessing ovarian response: antral follicle count versus anti-Müllerian hormone, Reproductive Biomed Online. 2015 Jul 3. pii: S1472-6483(15)00311-9

Fong, S. L., Baart, E. B., Martini, E., Schipper, I., Visser, J. A., Themmen, A. P. N., de Jong, F. H., Fauser, B. & Laven, J. S. E. (2008) Anti-Mullerian hormone: a marker for oocyte quantity, oocyte quality and embryo quality? Reproductive Biomedicine Online, 16 (5): 664-670.

Fraisse, T., Ibecheole, V., Streuli, I., Bischof, P. & de Ziegler, D. (2008) Undetectable serum anti-Mullerian hormone levels and occurrence of ongoing pregnancy. Fertil Steril, 89 (3): 723.e729-711.

Franco, J. G., Jr., Oliveira, J. B. A., Petersen, C. G., Mauri, A. L., Baruffi, R. & Cavagna, M. (2012) Adjuvant therapy with GnRH agonists/tamoxifen in breast cancer should be a good council for patients with hormone receptor-positive tumours and wish to preserve fertility. Medical hypotheses, 78 (4): 442-445.

Frattarelli, J. L., Bergh, P. A., Drews, M. R., Sharara, F. I. & Scott, R. T. (2000) Evaluation of basal estradiol levels in assisted reproductive technology cycles. Fertility and Sterility, 74 (3): 518-524.

Freeman, E. W., Gracia, C. R., Sammel, M. D., Lin, H., Lim, L. C. & Strauss, J. F., 3rd (2007) Association of anti-mullerian hormone levels with obesity in late reproductive-age women. Fertil Steril, 87 (1): 101-106.

Freeman, E. W., Sammel, M. D., Lin, H. & Gracia, C. R. (2012) Anti-Mullerian Hormone as a Predictor of Time to Menopause in Late Reproductive Age Women. Journal of Clinical Endocrinology & Metabolism, 97 (5): 1673-1680.

Fréour, T., Mirallié, S., Bach-Ngohou, K., Denis, M., Barrière, P. & Masson, D. (2007) Measurement of serum Anti-Müllerian Hormone by Beckman Coulter ELISA and DSL ELISA: Comparison and relevance in Assisted Reproduction Technology (ART). Clinica Chimica Acta, 375 (1-2): 162-164.

Gassner, D. & Jung, R. (2014) First fully automated immunoassay for anti-Mullerian hormone. Clin Chem Lab Med. 2014 Aug;52(8):1143-52.

Gerber, B., von Minckwitz, G., Stehle, H., Reimer, T., Felberbaum, R., Maass, N., Fischer, D., Sommer, H. L., Conrad, B., Ortmann, O., Fehm, T., Rezai, M., Mehta, K. & Loibl, S. (2011) Effect of Luteinizing Hormone–Releasing Hormone Agonist on Ovarian Function After Modern Adjuvant Breast Cancer Chemotherapy: The GBG 37 ZORO Study. Journal of Clinical Oncology, 29 (17): 2334-2341.

Gibreel, A., Maheshwari, A., Bhattacharya, S. & Johnson, N. P. (2009) Ultrasound tests of ovarian reserve; a systematic review of accuracy in predicting fertility outcomes. Hum Fertil (Camb), 12 (2): 95-106.

Gilbert, E., Adams, A., Mehanna, H., Harrison, B. & Hartshorne, G. M. (2011) Who should be offered sperm banking for fertility preservation? A survey of UK oncologists and haematologists. In: eds. Ann Oncol. England: 1209-1214.

Giuseppe, L., Attilio, G., Edoardo, D. N., Loredana, G., Cristina, L. & Vincenzo, L. (2007) Ovarian function after cancer treatment in young women affected by Hodgkin disease (HD). Hematology, 12 (2): 141-147.

Gnoth, C., Schuring, A. N., Friol, K., Tigges, J., Mallmann, P. & Godehardt, E. (2008) Relevance of anti-Mullerian hormone measurement in a routine IVF program. Human Reproduction, 23 (6): 1359-1365.

Goodwin, P. J., Ennis, M., Pritchard, K. I., Trudeau, M. & Hood, N. (1999) Risk of menopause during the first year after breast cancer diagnosis. J Clin Oncol, 17 (8): 2365-2370.

Goswami, D. & Conway, G. S. (2005) Premature ovarian failure. Human Reproduction Update, 11 (4): 391-410.

Gougeon, A. (1984) Caracteres qualitatifs et quantitafs de la population folliculaire dans l'ovaire humain adulte. 12: 527–535

Gougeon, A. (1986) Dynamics of follicular growth in the human: a model from preliminary results. Human Reproduction, 1 (2): 81-87.

Gougeon, A. (1996) Regulation of Ovarian Follicular Development in Primates: Facts and Hypotheses. Endocrine Reviews, 17 (2): 121-155.

Gracia, C. R., Sammel, M. D., Freeman, E., Prewitt, M., Carlson, C., Ray, A., Vance, A. & Ginsberg, J. P. (2012) Impact of cancer therapies on ovarian reserve. Fertil Steril, 97 (1): 134-140.e131.

Green, D. M., Nolan, V. G., Kawashima, T., Stovall, M., Donaldson, S. S., Srivastava, D., Leisenring, W., Robison, L. L. & Sklar, C. A. (2011) Decreased fertility among female childhood cancer survivors who received 22-27 Gy hypothalamic/pituitary irradiation: a report from the Childhood Cancer Survivor Study. In: eds. Fertil Steril. United States: 2011 American Society for Reproductive Medicine. Published by Elsevier Inc: 1922-1927, 1927 e1921.

Groome, N. P., Illingworth, P. J., O'Brien, M., Pai, R., Rodger, F. E., Mather, J. P. & McNeilly, A. S. (1996) Measurement of dimeric inhibin B throughout the human menstrual cycle. Journal of Clinical Endocrinology & Metabolism, 81 (4): 1401-1405.

Haadsma, M. L., Bukman, A., Groen, H., Roeloffzen, E. M. A., Groenewoud, E. R., Heineman, M. J. & Hoek, A. (2007) The number of small antral follicles (2-6 mm) determines the outcome of endocrine ovarian reserve tests in a subfertile population. Hum. Reprod., 22 (7): 1925-1931.

Hadji, P., Kauka, A., Bauer, T., Tams, J., Hasenburg, A. & Kieback, D. G. (2012) Effects of exemestane and tamoxifen on hormone levels within the Tamoxifen Exemestane Adjuvant Multicentre (TEAM) trial: results of a German substudy. Climacteric, 15 (5): 460-466.

Hadlow, N., Longhurst, K., McClements, A., Natalwala, J., Brown, S. J. & Matson, P. L. (2013) Variation in antimullerian hormone concentration during the menstrual cycle may change the clinical classification of the ovarian response. Fertil Steril, 99 (6): 1791-1797.

Hagen, C. K. S., Richard A. Anderson & Juul, A. (2012a) Serum levels of antimüllerian hormone in early maturing girls before, during, and after suppression with GnRH agonist. Fertility and Sterility - November 2012 (Vol. 98, Issue 5, Pages 1326-1330, DOI: 10.1016/j.fertnstert.2012.07.1118,

Hagen, C. P., Aksglaede, L., Sørensen, K., Main, K. M., Boas, M., Cleemann, L., Holm, K., Gravholt, C. H., Andersson, A.-M., Pedersen, A. T., Petersen, J. H., Linneberg, A., Kjaergaard, S. & Juul, A. (2010) Serum Levels of Anti-Müllerian Hormone as a Marker of Ovarian Function in 926 Healthy Females from Birth to Adulthood and in 172 Turner Syndrome Patients. Journal of Clinical Endocrinology & Metabolism, 95 (11): 5003-5010.

Hagen, C. P., Vestergaard, S., Juul, A., Skakkebaek, N. E., Andersson, A. M., Main, K. M., Hjollund, N. H., Ernst, E., Bonde, J. P., Anderson, R. A. & Jensen, T. K. (2012b) Low concentration of circulating antimullerian hormone is not predictive of reduced fecundability in young healthy women: a prospective cohort study. Fertil Steril, 98 (6): 1602-1608 e1602.

Halawaty, S., ElKattan, E., Azab, H., ElGhamry, N. & Al-Inany, H. (2010) Effect of obesity on parameters of ovarian reserve in premenopausal women. J Obstet Gynaecol Can, 32 (7): 687-690.

Han, X., McShane, M., Sahertian, R., White, C. & Ledger, W. (2014) Pre-mixing serum samples with assay buffer is a prerequisite for reproducible anti-Mullerian hormone measurement using the Beckman Coulter Gen II assay. Hum Reprod; 29(5):1042-8.

Hansen, K. R., Hodnett, G. M., Knowlton, N. & Craig, L. B. (2011) Correlation of ovarian reserve tests with histologically determined primordial follicle number. Fertil Steril, 95 (1): 170-175.

Hamy AS[1],Porcher R[2],Cuvier C[3],Giacchetti S[3],Schlageter MH[4], Coussieu C[5], Gronier H[3], Feugeas JP[6], Adoui N[6], Lacorte JM[5], Poirot C[7], Habdous M[3], Espié M[3]. Ovarian reserve in breast cancer: assessment with anti-Müllerian hormone.Reprod Biomed Online.2014 Nov;29(5):573-80.

Hansen, K. R., Knowlton, N. S., Thyer, A. C., Charleston, J. S., Soules, M. R. & Klein, N. A. (2008) A new model of reproductive aging: the decline in ovarian non-growing follicle number from birth to menopause. In: eds. Hum Reprod. England: 699-708.

Hazout, A., Bouchard, P., Seifer, D. B., Aussage, P., Junca, A. M. & Cohen-Bacrie, P. (2004) Serum antimüllerian hormone/müllerian-inhibiting substance appears to be a more discriminatory marker of assisted reproductive technology outcome than follicle-stimulating hormone, inhibin B, or estradiol. Fertility and Sterility, 82 (5): 1323-1329.

Hehenkamp, W. J. K., Looman, C. W. N., Themmen, A. P. N., de Jong, F. H., te Velde, E. R. & Broekmans, F. J. M. (2006) Anti-Mullerian Hormone Levels in

the Spontaneous Menstrual Cycle Do Not Show Substantial Fluctuation. J Clin Endocrinol Metab, 91 (10): 4057-4063.

Hendriks, D. J., Kwee, J., Mol, B. W. J., te Velde, E. R. & Broekmans, F. J. M. (2007) Ultrasonography as a tool for the prediction of outcome in IVF patients: a comparative meta-analysis of ovarian volume and antral follicle count. Fertility & Sterility, 87 (4): 764-775.

Hendriks, D. J., Mol, B.-W. J., Bancsi, L. F. J. M. M., te Velde, E. R. & Broekmans, F. J. M. (2005) Antral follicle count in the prediction of poor ovarian response and pregnancy after in vitro fertilization: A meta-analysis and comparison with basal follicle-stimulating hormone level. Fertility and Sterility, 83 (2): 291-301.

Henry, N. L., Xia, R., Schott, A. F., McConnell, D., Banerjee, M. & Hayes, D. F. (2014) Prediction of postchemotherapy ovarian function using markers of ovarian reserve. Oncologist, 19 (1): 68-74.

Hirobe, S., He, W., Gustafson, M., MacLaughlin, D. & Donahoe, P. (1994) Mullerian inhibiting substance gene expression in the cycling rat ovary correlates with recruited or graafian follicle selection. Biol Reprod, 50 (6): 1238-1243.

Hoshiya, Y., Gupta, V., Segev, D. L., Hoshiya, M., Carey, J. L., Sasur, L. M., Tran, T. T., Ha, T. U. & Maheswaran, S. (2003) Mullerian Inhibiting Substance induces NFkB signaling in breast and prostate cancer cells. Mol Cell Endocrinol, 211 (1-2): 43-49.

Hovatta, O. (2004) Cryopreservation and culture of human ovarian cortical tissue containing early follicles. Eur J Obstet Gynecol Reprod Biol, 113 Suppl 1 S50-54.

Howell, S. & Shalet, S. (1998) Gonadal damage from chemotherapy and radiotherapy. Endocrinol Metab Clin North Am, 27 (4): 927-943.

Hudson, P. L., Dougas, I., Donahoe, P. K., Cate, R. L., Epstein, J., Pepinsky, R. B. & Maclaughlin, D. T. (1990) An Immunoassay to Detect Human Mullerian Inhibiting Substance in Males and Females during Normal Development. Journal of Clinical Endocrinology & Metabolism, 70 (1): 16-22.

I. Demeestere, F. M., F. Peccatori, C. Poirot, and E. Shalom-Paz, (2012) Multiple Approaches for Individualized Fertility Protective Therapy in Cancer Patients. Obstetrics and Gynecology International, 2012.

Iliodromiti, S., Kelsey, T. W., Wu, O., Anderson, R. A. & Nelson, S. M. (2014) The predictive accuracy of anti-Mullerian hormone for live birth after assisted conception: a systematic review and meta-analysis of the literature. *Hum Reprod Update*.

Iliodromiti S[1], Nelson SMCurr Opin Obstet Gynecol. (2015) Ovarian response biomarkers: physiology and performance.2015 Jun;27(3):182-6.

Imai, A., Sugiyama, M., Furui, T., Tamaya, T. & Ohno, T. (2007) Direct protection by a gonadotropin-releasing hormone analog from doxorubicin-induced granulosa cell damage. In: eds. Gynecol Obstet Invest. Switzerland: 102-106.

Iwase, A., Sugita, A., Hirokawa, W., Goto, M., Yamamoto, E., Takikawa, S., Nakahara, T., Nakamura, T., Kondo, M. & Kikkawa, F. Anti-Müllerian hormone as a marker of ovarian reserve following chemotherapy in patients with

gestational trophoblastic neoplasia. European Journal of Obstetrics and Gynecology and Reproductive Biology, 167 (2): 194-198.

Jarvela, I. Y., Sladkevicius, P., Kelly, S., Ojha, K., Campbell, S. & Nargund, G. (2003) Quantification of ovarian power Doppler signal with three-dimensional ultrasonography to predict response during in vitro fertilization. Obstetrics & Gynecology, 102 (4): 816-822.

Jayaprakasan, K., Campbell, B., Hopkisson, J., Clewes, J., Johnson, I. & Raine-Fenning, N. (2008) Establishing the intercycle variability of three-dimensional ultrasonographic predictors of ovarian reserve. Fertility & Sterility, 90 (6): 2126-2132.

Jayaprakasan, K., Hilwah, N., Kendall, N. R., Hopkisson, J. F., Campbell, B. K., Johnson, I. R. & Raine-Fenning, N. J. (2007) Does 3D ultrasound offer any advantage in the pretreatment assessment of ovarian reserve and prediction of outcome after assisted reproduction treatment? Human Reproduction, 22 (7): 1932-1941.

Jeppesen, J. V., Anderson, R. A., Kelsey, T. W., Christiansen, S. L., Kristensen, S. G., Jayaprakasan, K., Raine-Fenning, N., Campbell, B. K. & Yding Andersen, C. (2013) Which follicles make the most anti-Müllerian hormone in humans? Evidence for an abrupt decline in AMH production at the time of follicle selection. Molecular Human Reproduction, 19 (8): 519-527.

Johnson, J., Bagley, J., Skaznik-Wikiel, M., Lee, H.-J., Adams, G. B., Niikura, Y., Tschudy, K. S., Tilly, J. C., Cortes, M. L., Forkert, R., Spitzer, T., Iacomini, J., Scadden, D. T. & Tilly, J. L. (2005) Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. Cell, 122(2):303-15

Johnson, J. & Keefe, D. L. (2013) Ovarian aging: breaking up is hard to fix. In: eds. Sci Transl Med. United States: 172-175.

Johnson, N. P., Bagrie, E. M., Coomarasamy, A., Bhattacharya, S., Shelling, A. N., Jessop, S., Farquhar, C. & Khan, K. S. (2006) Ovarian reserve tests for predicting fertility outcomes for assisted reproductive technology: the International Systematic Collaboration of Ovarian Reserve Evaluation protocol for a systematic review of ovarian reserve test accuracy. BJOG: An International Journal of Obstetrics & Gynaecology, 113 (12): 1472-1480.

Josso, N., Belville, C., di Clemente, N. & Picard, J.-Y. (2005) AMH and AMH receptor defects in persistent Mullerian duct syndrome. Hum Reprod Update, 11 (4): 351-356.

Josso, N., Belville, C. & Picard, J.-Y. (2003) Mutations of AMH and its Receptors. The Endocrinologist, 13 (3): 247-251.

Kallio, S., Puurunen, J., Ruokonen, A., Vaskivuo, T., Piltonen, T. & Tapanainen, J. S. (2013) Antimullerian hormone levels decrease in women using combined contraception independently of administration route. Fertil Steril, 99 (5): 1305-1310.

Kelsey, T. W., Anderson, R. A., Wright, P., Nelson, S. M. & Wallace, W. H. B. (2012) Data-driven assessment of the human ovarian reserve. Molecular Human Reproduction, 18 (2): 79-87.

Kevenaar, M. E., Meerasahib, M. F., Kramer, P., van de Lang-Born, B. M. N., de Jong, F. H., Groome, N. P., Themmen, A. P. N. & Visser, J. A. (2006) Serum Anti-Mullerian Hormone Levels Reflect the Size of the Primordial Follicle Pool in Mice. Endocrinology, 147 (7): 3228-3234.

Kim, J. Y., Yi, G., Kim, Y. R., Chung, J. Y., Ahn, J. H., Uhm, Y. K., Jee, B. C., Suh, C. S. & Kim, S. H. (2013) Association between serum anti-Müllerian hormone level and ovarian response to mild stimulation in normoovulatory women and anovulatory women with polycystic ovary syndrome. Clin Exp Reprod Med, 40 (2): 95-99.

Kim, S. S., Donnez, J., Barri, P., Pellicer, A., Patrizio, P., Rosenwaks, Z., Nagy, P., Falcone, T., Andersen, C., Hovatta, O., Wallace, H., Meirow, D., Gook, D., Kim, S. H., Tzeng, C. R., Suzuki, S., Ishizuka, B. & Dolmans, M. M. (2012) Recommendations for fertility preservation in patients with lymphoma, leukemia, and breast cancer. J Assist Reprod Genet, 29 (6): 465-468.

Kim, S. S., Klemp, J. & Fabian, C. (2011) Breast cancer and fertility preservation. Fertility and Sterility, Volume 95, Issue 5 , Pages 1535-1543.

Kim, S. S., Lee, J. R., Jee, B. C., Suh, C. S., Kim, S. H., Ting, A. & Petroff, B. (2010) Use of hormonal protection for chemotherapy-induced gonadotoxicity. Clin Obstet Gynecol, 53 (4): 740-752.

Kim SS, L. J., Jee BC, Suh CS, Kim SH, Ting A, et al (2010) Use of hormonal protection for chemotherapy-induced gonadotoxicity. Clin Obstet Gynecol., 2010;53:740–752

Knopman, J. M., Noyes, N., Talebian, S., Krey, L. C., Grifo, J. A. & Licciardi, F. (2009) Women with cancer undergoing ART for fertility preservation: a cohort study of their response to exogenous gonadotropins. Fertility and Sterility, 91 (4): 1476-1478.

Kok, H. S., van Asselt, K. M., van der Schouw, Y. T., Peeters, P. H. M. & Wijmenga, C. (2005) Genetic studies to identify genes underlying menopausal age. Human Reproduction Update, 11 (5): 483-493.

Koninger, A., Kauth, A., Schmidt, B., Schmidt, M., Yerlikaya, G., Kasimir-Bauer, S., Kimmig, R. & Birdir, C. (2013) Anti-Mullerian-hormone levels during pregnancy and postpartum. Reprod Biol Endocrinol, 11 60.

Koninger A, Schmidt B, Mach P, Damaske D, Nießen S, Kimmig R, Strowitzki T, Gellhaus A (2015). Anti-Mullerian-Hormone during pregnancy and peripartum using the new Beckman Coulter AMHGen II Assay. Reprod Biol Endocrinol. 2015; 13:86

Krawczuk-Rybak, M., Leszczynska, E., Wysocka, J. & Zelazowska-Rutkowska, B. (2008) Anti-mullerian hormone in young women after chemotherapy and infradiaphragmatic radiotherapy for childhood cancer. Pediatric endocrinology, diabetes, and metabolism, 14 (2): 99-103.

Kumar, A., Kalra, B., Patel, A., McDavid, L. & Roudebush, W. E. (2010) Development of a second generation anti-Mullerian hormone (AMH) ELISA. In: eds. J Immunol Methods. Netherlands: 2010. Published by Elsevier B.V.: 51-59.

La Marca, A., Broekmans, F. J., Volpe, A., Fauser, B. C., Macklon, N. S. & on behalf of the ESHRE Special Interest Group for Reproductive Endocrinology - AMH Round Table (2009) Anti-Mullerian hormone (AMH): what do we still need to know? Hum. Reprod., dep210.

La Marca, A., De Leo, V., Giulini, S., Orvieto, R., Malmusi, S., Giannella, L. & Volpe, A. (2005a) Anti-Mullerian Hormone in Premenopausal Women and After Spontaneous or Surgically Induced Menopause. Journal of the Society for Gynecologic Investigation, 12 (7): 545-548.

La Marca, A., Giulini, S., Orvieto, R., De Leo, V. & Volpe, A. (2005b) Anti-Mullerian hormone concentrations in maternal serum during pregnancy. Hum Reprod, 20 (6): 1569-1572.

La Marca, A., Giulini, S., Tirelli, A., Bertucci, E., Marsella, T., Xella, S. & Volpe, A. (2007) Anti-Mullerian hormone measurement on any day of the menstrual cycle strongly predicts ovarian response in assisted reproductive technology. Hum. Reprod., 22 (3): 766-771.

La Marca, A., Pati, M., Orvieto, R., Stabile, G., Carducci Artenisio, A. & Volpe, A. (2006a) Serum anti-müllerian hormone levels in women with secondary amenorrhea. Fertility and Sterility, 85 (5): 1547-1549.

La Marca, A., Spada, E., Grisendi, V., Argento, C., Papaleo, E., Milani, S. & Volpe, A. (2012) Normal serum anti-Mullerian hormone levels in the general female population and the relationship with reproductive history. Eur J Obstet Gynecol Reprod Biol, 163 (2): 180-184.

La Marca, A., Stabile, G., Artenisio, A. C. & Volpe, A. (2006b) Serum anti-Mullerian hormone throughout the human menstrual cycle. Hum. Reprod., 21 (12): 3103-3107.

Larsen, E. C., Muller, J., Rechnitzer, C., Schmiegelow, K. & Andersen, A. N. (2003a) Diminished ovarian reserve in female childhood cancer survivors with regular menstrual cycles and basal FSH <10 IU/l. Human Reproduction, 18 (2): 417-422.

Larsen, E. C., Muller, J., Schmiegelow, K., Rechnitzer, C. & Andersen, A. N. (2003b) Reduced ovarian function in long-term survivors of radiation- and chemotherapy-treated childhood cancer. Journal of Clinical Endocrinology & Metabolism, 88 (11): 5307-5314.

Lawrenz, B., Fehm, T., von Wolff, M., Soekler, M., Huebner, S., Henes, J., Henes, M. & Centers of Ferti, P. N. (2012) Reduced pretreatment ovarian reserve in premenopausal female patients with Hodgkin lymphoma or non-Hodgkin-lymphoma--evaluation by using antimullerian hormone and retrieved oocytes. Fertility and sterility, 98 (1): 141-144.

Lawrenz, B., Henes, M., Neunhoeffer, E., Kraemer, B. & Fehm, T. (2011) Fertility conservation in breast cancer patients. Womens Health (Lond Engl), 7 (2): 203-212.

Lee, J. R., Kim, S. H., Jee, B. C., Suh, C. S., Kim, K. C. & Moon, S. Y. (2011) Antimüllerian hormone as a predictor of controlled ovarian hyperstimulation outcome: comparison of two commercial immunoassay kits. Fertility and Sterility, 95 (8): 2602-2604.

Lee, M. M., Donahoe, P. K., Hasegawa, T., Silverman, B., Crist, G. B., Best, S., Hasegawa, Y., Noto, R. A., Schoenfeld, D. & MacLaughlin, D. T. (1996) Mullerian inhibiting substance in humans: normal levels from infancy to adulthood. J Clin Endocrinol Metab, 81 (2): 571-576.

Lee, T.-H., Liu, C.-H., Huang, C.-C., Wu, Y.-L., Shih, Y.-T., Ho, H.-N., Yang, Y.-S. & Lee, M.-S. (2008) Serum anti-mullerian hormone and estradiol levels as predictors of ovarian hyperstimulation syndrome in assisted reproduction technology cycles. Hum. Reprod., 23 (1): 160-167.

Lekamge, D. N., Barry, M., Kolo, M., Lane, M., Gilchrist, R. B. & Tremellen, K. P. (2007) Anti-Mullerian hormone as a predictor of IVF outcome. Reproductive Biomedicine Online, 14 (5): 602-610.

Letourneau, J., Chan, S. W. & Rosen, M. P. (2013) Accelerating ovarian age: cancer treatment in the premenopausal woman. Semin Reprod Med, 31 (6): 462-468.

Letourneau, J. M., Ebbel, E. E., Katz, P. P., Oktay, K. H., McCulloch, C. E., Ai, W. Z., Chien, A. J., Melisko, M. E., Cedars, M. I. & Rosen, M. P. (2012) Acute ovarian failure underestimates age-specific reproductive impairment for young women undergoing chemotherapy for cancer. Cancer, 118 (7): 1933-1939.

Li, H. W., Wong, C. Y., Yeung, W. S., Ho, P. C. & Ng, E. H. (2011) Serum anti-mullerian hormone level is not altered in women using hormonal contraceptives. Contraception, 83 (6): 582-585.

Licciardi, F. L., Liu, H. C. & Rosenwaks, Z. (1995) Day 3 estradiol serum concentrations as prognosticators of ovarian stimulation response and pregnancy outcome in patients undergoing in vitro fertilization.[see comment]. Fertility & Sterility, 64 (5): 991-994.

Lie Fong, S., Laven, J. S., Hakvoort-Cammel, F. G., Schipper, I., Visser, J. A., Themmen, A. P., de Jong, F. H. & van den Heuvel-Eibrink, M. M. (2009) Assessment of ovarian reserve in adult childhood cancer survivors using anti-Mullerian hormone. In: eds. Hum Reprod. England: 982-990.

Lie Fong, S., Lugtenburg, P. J., Schipper, I., Themmen, A. P. N., de Jong, F. H., Sonneveld, P. & Laven, J. S. E. (2008) Anti-mullerian hormone as a marker of ovarian function in women after chemotherapy and radiotherapy for haematological malignancies. Human Reproduction, 23 (3): 674-678.

Lin, W. T., Beattie, M., Chen, L. M., Oktay, K., Crawford, S. L., Gold, E. B., Cedars, M. & Rosen, M. (2013) Comparison of age at natural menopause in BRCA1/2 mutation carriers with a non-clinic-based sample of women in northern California. Cancer, 119 (9): 1652-1659.

Lockwood, G. (2004) The diagnostic value of inhibin in infertility evaluation. Semin Reprod Med, 22 (3): 195-208.

Long, W.-Q., Ranchin, V., Pautier, P., Belville, C., Denizot, P., Cailla, H., Lhommé, C., Picard, J.-Y., Bidart, J.-M. & Rey, R. (2000) Detection of Minimal Levels of Serum Anti-Müllerian Hormone during Follow-Up of Patients with Ovarian Granulosa Cell Tumor by Means of a Highly Sensitive Enzyme-Linked Immunosorbent Assay. Journal of Clinical Endocrinology & Metabolism, 85 (2): 540-544.

Lower, E. E., Blau, R., Gazder, P. & Tummala, R. (1999) The risk of premature menopause induced by chemotherapy for early breast cancer. J Womens Health Gend Based Med, 8 (7): 949-954.

Luesley, D. & Baker, P. (2004) Obstetrics and Gynaecology. London: Arnold.(2):533-538

Lum, S. S., Woltering, E. A., Fletcher, W. S. & Pommier, R. F. (1997) Changes in serum estrogen levels in women during tamoxifen therapy. Am J Surg, 173 (5): 399-402.

Lutchman Singh, K., Davies, M. & Chatterjee, R. (2005) Fertility in female cancer survivors: pathophysiology, preservation and the role of ovarian reserve testing. Human Reproduction Update, 11 (1): 69-89.

Lutchman Singh, K., Muttukrishna, S., Stein, R. C., McGarrigle, H. H., Patel, A., Parikh, B., Groome, N. P., Davies, M. C. & Chatterjee, R. (2007) Predictors of ovarian reserve in young women with breast cancer. Br J Cancer, 96 (12): 1808-1816.

Maheshwari, A., Gibreel, A., Bhattacharya, S. & Johnson, N. P. (2009) Dynamic tests of ovarian reserve: A systematic review of diagnostic accuracy. Reproductive BioMedicine Online, 18 (5): 717-734.

McGee, E. A. & Hsueh, A. J. W. (2000) Initial and Cyclic Recruitment of Ovarian Follicles. Endocrine Reviews, 21 (2): 200-214.

McIlveen, M., Skull, J. D. & Ledger, W. L. (2007) Evaluation of the utility of multiple endocrine and ultrasound measures of ovarian reserve in the prediction of cycle cancellation in a high-risk IVF population. Hum. Reprod., 22 (3): 778-785.

Megdal, S. P., Kroenke, C. H., Laden, F., Pukkala, E. & Schernhammer, E. S. (2005) Night work and breast cancer risk: a systematic review and meta-analysis. In: eds. Eur J Cancer. England: 2023-2032.

Meirow, D. (1999) Ovarian injury and modern options to preserve fertility in female cancer patients treated with high dose radio-chemotherapy for hemato-oncological neoplasias and other cancers. Leuk Lymphoma, 33 (1-2): 65-76.

Meirow, D., Biederman, H., Anderson, R. A. & Wallace, W. H. B. (2010) Toxicity of Chemotherapy and Radiation on Female Reproduction. Clinical Obstetrics andGynecology, 53 (4): 727-739 710.1097/GRF.1090b1013e3181f1096b1054.

Meirow, D., Dor, J., Kaufman, B., Shrim, A., Rabinovici, J., Schiff, E., Raanani, H., Levron, J. & Fridman, E. (2007) Cortical fibrosis and blood-vessels damage in human ovaries exposed to chemotherapy. Potential mechanisms of ovarian injury. Human Reproduction, 22 (6): 1626-1633.

Meirow, D., Lewis, H., Nugent, D. & Epstein, M. (1999) Subclinical depletion of primordial follicular reserve in mice treated with cyclophosphamide: clinical importance and proposed accurate investigative tool. Human Reproduction, 14 (7): 1903-1907.

Meirow, D. & Nugent, D. (2001) The effects of radiotherapy and chemotherapy on female reproduction. Hum Reprod Update, 7 (6): 535-543.

Mendez Lozano, D. H., Taieb, J., Feyereisen, E., Alby, C., Frydman, R. & Fanchin, R. (2006) O-4: Detailed dynamics of serum anti-mullerian hormone (AMH) levels during the menstrual cycle and its antral follicle correlates. Fertility and Sterility, 86 (3, Supplement 1): S2-S2.

Meng, K., Tian, W., Zhou, M., Chen, H. & Deng, Y. (2013) Impact of chemotherapy-induced amenorrhea in breast cancer patients: the evaluation of ovarian function by menstrual history and hormonal levels. World J Surg Oncol, 11 101.

Michaelson-Cohen, R., Mor, P., Srebnik, N., Beller, U., Levy-Lahad, E. & Eldar-Geva, T. (2014) BRCA mutation carriers do not have compromised ovarian reserve. Int J Gynecol Cancer, 24 (2): 233-237.

Mohamed, K. A., Davies, W. A. R. & Lashen, H. (2006) Antimüllerian hormone and pituitary gland activity after prolonged down-regulation with goserelin acetate. Fertility and Sterility, 86 (5): 1515-1517.

Morgan, S., Anderson, R. A., Gourley, C., Wallace, W. H. & Spears, N. (2012) How do chemotherapeutic agents damage the ovary? Human Reproduction Update, 18 (5): 525-535.

Moy V[1], Jindal S, Lieman H, Buyuk E (2015) Obesity adversely affects serum anti-müllerian hormone (AMH) levels in Caucasian women. J Assist Reprod Genet. 2015

Murabito, J. M., Yang, Q., Fox, C., Wilson, P. W. F. & Cupples, L. A. (2005) Heritability of Age at Natural Menopause in the Framingham Heart Study. Journal of Clinical Endocrinology & Metabolism, 90 (6): 3427-3430.

Muttukrishna, S., Suharjono, H., McGarrigle, H. & Sathanandan, M. (2004) Inhibin B and anti-Mullerian hormone: markers of ovarian response in IVF/ICSI patients? Bjog-an International Journal of Obstetrics and Gynaecology, 111 (11): 1248-1253.

Nair S, Slaughter JC, Terry JG, Appiah D, Ebong I, Wang E, Siscovick DS, Sternfeld B, Schreiner PJ, Lewis CE, Kabagambe EK, Wellons MF (2015). Anti-mullerian hormone (AMH) is associated with natural menopause in a population-based sample: The CARDIA Women's Study.Maturitas. 2015 Aug; 81(4):

Nakhuda, G. S., Sauer, M. V., Wang, J. G., Ferin, M. & Lobo, R. A. (2007) Mullerian inhibiting substance is an accurate marker of ovarian response in women of advanced reproductive age undergoing IVF. Reproductive Biomedicine Online, 14 (4): 450-454.

Nardo, L. G., Gelbaya, T. A., Wilkinson, H., Roberts, S. A., Yates, A., Pemberton, P. & Laing, I. (2009) Circulating basal anti-Müllerian hormone levels as predictor of ovarian response in women undergoing ovarian stimulation for in vitro fertilization. Fertil Steril, 92 (5): 1586-1593.

NCIN (2009) Haematological cancers. [online] Available from: http://www.ncin.org.uk/cancer_type_and_topic_specific_work/cancer_type_specific_work/haematological_cancers/ (Accessed 11/11/2012).

NCIN (2011) The Second all breast cancer report. [online] Available from: http://www.ncin.org.uk/cancer_type_and_topic_specific_work/cancer_type_specific_work/breast_cancer (Accessed 11/11/2012).

Nelson, L. M. (2009) Primary Ovarian Insufficiency. New England Journal of Medicine, 360 (6): 606-614.

Nelson, S. M. & La Marca, A. (2011) The journey from the old to the new AMH assay: how to avoid getting lost in the values. Reproductive BioMedicine Online, 23 (4): 411-420.

Nelson, S. M. & Lawlor, D. A. (2011) Predicting live birth, preterm delivery, and low birth weight in infants born from in vitro fertilisation: a prospective study of 144,018 treatment cycles. PLoS Med, 8 (1): e1000386.

Nelson, S. M., Telfer, E. E. & Anderson, R. A. (2013) The ageing ovary and uterus: new biological insights. Human Reproduction Update, 19 (1): 67-83.

Nelson, S. M., Yates, R. W. & Fleming, R. (2007) Serum anti-Mullerian hormone and FSH: prediction of live birth and extremes of response in stimulated cycles implications for individualization of therapy. Hum. Reprod., 22 (9): 2414-2421.

Nelson, S. M., Yates, R. W., Lyall, H., Jamieson, M., Traynor, I., Gaudoin, M., Mitchell, P., Ambrose, P. & Fleming, R. (2009) Anti-Mullerian hormone-based approach to controlled ovarian stimulation for assisted conception. Hum. Reprod., 24 (4): 867-875.

NICE (2013a) CG156 Fertility: NICE guideline. Available online from http://guidance.nice.org.uk/CG156/NICEGuidance/pdf/English

NICE, N. I. f. H. a. C. E. (2006) TA112 Breast cancer (early) - hormonal treatments:guidance.
Available online from:http://guidance.nice.org.uk/TA112/Guidance/pdf/English

NICE, N. I. f. H. a. C. E. (2009a) Breast cancer (early and locally advanced) CG80. Available online from: http://guidance.nice.org.uk/CG80

NICE, N. I. f. H. a. C. E. (2009b) Advanced breast cancer. Available online from: http://www.nice.org.uk/nicemedia/pdf/CG81NICEGuideline.pdf

NICE, N. I. f. H. a. C. E. (2013b) Gene expression profiling and expanded immunohistochemistry tests for guiding adjuvant chemotherapy decisions in early breast cancer management: MammaPrint, Oncotype DX, IHC4 and Mammostrat. Available online from: http://publications.nice.org.uk/gene-expression-profiling-and-expanded-immunohistochemistry-tests-for-guiding-adjuvant-chemotherapy-dg10.

Nielsen, S. N., Andersen, A. N., Schmidt, K. T., Rechnitzer, C., Schmiegelow, K., Bentzen, J. G. & Larsen, E. C. (2013) A 10-year follow up of reproductive function in women treated for childhood cancer. Reprod Biomed Online, 27 (2): 192-200.

Noyes, N., Porcu, E. & Borini, A. (2009) Over 900 oocyte cryopreservation babies born with no apparent increase in congenital anomalies. Reprod Biomed Online, 18 (6): 769-776.

Oktay, K. (2005) Further evidence on the safety and success of ovarian stimulation with letrozole and tamoxifen in breast cancer patients undergoing in vitro fertilization to cryopreserve their embryos for fertility preservation. Journal of Clinical Oncology, 23 (16): 3858-3859.

Oktay, K., Bedoschi, G., Dickler, M., Goldfarb, S., Turan, V. & Moy, F. (2013) The impact of long-term tamoxifen treatment on ovarian reserve markers in women with breast cancer: A prospective-longitudinal study. Fertility and Sterility, 1) S64.

Oktay, K., Buyuk, E., Libertella, N., Akar, M. & Rosenwaks, Z. (2005) Fertility preservation in breast cancer patients: A prospective controlled comparison of ovarian stimulation with tamoxifen and letrozole for embryo cryopreservation. Journal of Clinical Oncology, 23 (19): 4347-4353.

ONS (2011) Cancer Survival in England - Patients Diagnosed 2005-2009 and Followed up.
Available from: http://www.ons.gov.uk/ons/dcp171778_240942.pdf (Accessed 11/11/2012).

ONS (2012a) Breast Cancer: Incidence, Mortality and Survival, 2010. [online] Available from: http://www.ons.gov.uk/ons/rel/cancer-unit/breast-cancer-in-england/2010/sum-1.html (Accessed 11/11/2012).

ONS (2012b) Cancer Incidence and Mortality in the United Kingdom, 2008-10. [online] Available from: http://www.ons.gov.uk/ons/dcp171778_240942.pdf (Accessed 11/11/2012).

Park, H. J., Koo, Y. A., Im, Y. H., Yoon, B. K. & Choi, D. (2010) GnRH agonist therapy to protect ovarian function in young Korean breast cancer patients. J Korean Med Sci, 25 (1): 110-116.

Parkin, D. M. (2011) 3. Cancers attributable to consumption of alcohol in the UK in 2010. Br J Cancer, 105 (S2): S14-S18.

Parkin, D. M. & Boyd, L. (2011) 8. Cancers attributable to overweight and obesity in the UK in 2010. Br J Cancer, 105 (S2): S34-S37.

Parkin, D. M. & Darby, S. C. (2011) 12. Cancers in 2010 attributable to ionising radiation exposure in the UK. Br J Cancer, 105 (S2): S57-S65.

Partridge, A. H., Gelber, S., Peppercorn, J., Sampson, E., Knudsen, K., Laufer, M., Rosenberg, R., Przypyszny, M., Rein, A. & Winer, E. P. (2004) Web-Based Survey of Fertility Issues in Young Women With Breast Cancer. Journal of Clinical Oncology, 22 (20): 4174-4183.

Partridge, A. H., Ruddy, K. J., Gelber, S., Schapira, L., Abusief, M., Meyer, M. & Ginsburg, E. (2010b) Ovarian reserve in women who remain premenopausal after chemotherapy for early stage breast cancer. Fertility and sterility, 94 (2): 638-644.

Peigné M1, Decanter C.Serum AMH level as a marker of acute and long-term effects of chemotherapy on the ovarian follicular content: a systematic review. Reprod Biol Endocrinol. 2014 Mar 26;12:26.

Pellicer, A., Ardiles, G., Neuspiller, F., Remohi, J., Simon, C. & Bonilla-Musoles, F. (1998) Evaluation of the ovarian reserve in young low responders with normal

basal levels of follicle-stimulating hormone using three-dimensional ultrasonography. Fertility & Sterility, 70 (4): 671-675.

Pepinsky, R., Sinclair, L., Chow, E., Mattaliano, R., Manganaro, T., Donahoe, P. & Cate, R. (1988) Proteolytic processing of mullerian inhibiting substance produces a transforming growth factor-beta-like fragment. J. Biol. Chem., 263 (35): 18961-18964.

Petrek, J. A., Naughton, M. J., Case, L. D., Paskett, E. D., Naftalis, E. Z., Singletary, S. E. & Sukumvanich, P. (2006) Incidence, Time Course, and Determinants of Menstrual Bleeding After Breast Cancer Treatment: A Prospective Study. Journal of Clinical Oncology, 24 (7): 1045-1051.

PHE (2013) NHS Screening programme. [online] Available from: http://www.cancerscreening.nhs.uk/breastscreen/breastcancer.html (Accessed 05/06/2013).

Phophong, P., Ranieri, D. M., Khadum, I., Meo, F. & Serhal, P. (2000) Basal 17[beta]-estradiol did not correlate with ovarian response and in vitro fertilization treatment outcome. Fertility and Sterility, 74 (6): 1133-1136.

Plante, B. J., Cooper, G. S., Baird, D. D. & Steiner, A. Z. (2010) The impact of smoking on antimullerian hormone levels in women aged 38 to 50 years. Menopause, 17 (3): 571-576.

Quinn, G. P., Vadaparampil, S. T., Lee, J.-H., Jacobsen, P. B., Bepler, G., Lancaster, J., Keefe, D. L. & Albrecht, T. L. (2009) Physician Referral for Fertility Preservation in Oncology Patients: A National Study of Practice Behaviors. Journal of Clinical Oncology, 27 (35): 5952-5957.

Quintero, R. B., Helmer, A., Huang, J. Q. & Westphal, L. M. (2010) Ovarian stimulation for fertility preservation in patients with cancer. Fertility and Sterility, 93 (3): 865-868.

Rajpert-De Meyts, E., Jorgensen, N., Gram, N., Muller, J., Cate, R. L. & Skakkebak, N. E. (1999) Expression of Anti-Mullerian Hormone during Normal and Pathological Gonadal Development: Association with Differentiation of Sertoli and Granulosa Cells. J Clin Endocrinol Metab, 84 (10): 3836-3844.

Recchia F, S. G., Amiconi G, Di Blasio A, Cesta A, Candeloro G, Rea S (2006) Gonadotropin-releasing hormone analogues added to adjuvant chemotherapy protect ovarian function and improve clinical outcomes in young women with early breast carcinoma. Cancer., 106 (3): 514-523.

Reh, A., Oktem, O. & Oktay, K. (2008) Impact of breast cancer chemotherapy on ovarian reserve: a prospective observational analysis by menstrual history and ovarian reserve markers. Fertility & Sterility, 90 (5): 1635-1639.

Reulen, R. C., Zeegers, M. P., Wallace, W. H., Frobisher, C., Taylor, A. J., Lancashire, E. R., Winter, D. L. & Hawkins, M. M. (2009) Pregnancy outcomes among adult survivors of childhood cancer in the British Childhood Cancer Survivor Study. In: eds. Cancer Epidemiol Biomarkers Prev. United States: 2239-2247.

Riggs, R. M., Duran, E. H., Baker, M. W., Kimble, T. D., Hobeika, E., Yin, L., Matos-Bodden, L., Leader, B. & Stadtmauer, L. (2008) Assessment of ovarian reserve with anti-Müllerian hormone: a comparison of the predictive value of anti-Müllerian hormone, follicle-stimulating hormone, inhibin B, and age. American Journal of Obstetrics and Gynecology, 199 (2): 202.e201-202.e208.

Rocca, W. A., Grossardt, B. R., de Andrade, M., Malkasian, G. D. & Melton, L. J., 3rd (2006) Survival patterns after oophorectomy in premenopausal women: a population-based cohort study. In: eds. Lancet Oncol. England: 821-828.

Rocca, W. A., Shuster, L. T., Grossardt, B. R., Maraganore, D. M., Gostout, B. S., Geda, Y. E. & Melton, L. J., 3rd (2009) Long-term effects of bilateral oophorectomy on brain aging: unanswered questions from the Mayo Clinic Cohort Study of Oophorectomy and Aging. Womens Health (Lond Engl), 5 (1): 39-48.

Rose, D. P. & Davis, T. E. (1997) Ovarian Function In Patients Receiving Adjuvant Chemotherapy For Breast Cancer. Lancet, Volume 309, Issue 8023, Pages 1174 - 1176 (8023):

Rosendahl, M., Andersen, C., la Cour Freiesleben, N., Juul, A., Løssl, K. & Andersen, A. (2009) Dynamics and mechanisms of chemotherapy-induced ovarian follicular depletion in women of fertile age. Fertil Steril,

Rosendahl, M., Greve, T. & Andersen, C. Y. (2013) The safety of transplanting cryopreserved ovarian tissue in cancer patients: a review of the literature. J Assist Reprod Genet, 30 (1): 11-24.

Salmassi A[1], Mettler L[1], Hedderich J[2],Jonat W[1], Deenadayal A[1],von Otte S[1],Eckmann-Scholz C[1], Schmutzler AG[1] (2015) Int J Fertil Steril. Cut-Off Levels of Anti-Mullerian Hormone for The Prediction of Ovarian Response, In Vitro Fertilization Outcome and Ovarian Hyperstimulation Syndrome.2015 Jul-Sep;9 (2):157-67.

Scheffer, G. J., Broekmans, F. J. M., Looman, C. W. N., Blankenstein, M., Fauser, B. C. J. M., de Jong, F. H. & te Velde, E. R. (2003) The number of antral follicles in normal women with proven fertility is the best reflection of reproductive age. Hum. Reprod., 18 (4): 700-706.

Scott, R. T., Jr. & Hofmann, G. E. (1995) Prognostic assessment of ovarian reserve. Fertil Steril, 63 (1): 1-11.

Seifer, D. B., MacLaughlin, D. T., Christian, B. P., Feng, B. & Shelden, R. M. (2002) Early follicular serum mullerian-inhibiting substance levels are associated with ovarian response during assisted reproductive technology cycles. Fertility & Sterility, 77 (3): 468-471.

Seifer, D. B., Scott, R. T., Bergh, P. A., Abrogast, L. K., Friedman, C. I., Mack, C. K. & Danforth, D. R. (1999) Women with declining ovarian reserve may demonstrate a decrease in day 3 serum inhibin B before a rise in day 3 follicle-stimulating hormone. Fertil Steril, 72 (1): 63-65.

Shaw, R., Soutter, P. & Stanton, S. (2003) Gynaecology 3rd edition. Edinburgh : Churchill Livingstone/Elsevier:203-213

Sherbahn, R. & Deutch, T. (2009) Follicular measurements using a computerized 3D ultrasound system (SonoAVC) for monitoring ovarian stimulation for IVF is effective and efficient.

Shuster, L. T. [a.] D. J. R., [b] Bobbie S. Gostout,[c] Brandon R. Grossardt,[d] and Walter A. Rocca[e,f] (2010) Premature menopause or early menopause: long-term healthconsequences. Maturitas. 2010 February; 65(2): 161.

Smith, G. C., Cordeaux, Y., White, I. R., Pasupathy, D., Missfelder-Lobos, H., Pell, J. P., Charnock-Jones, D. S. & Fleming, M. (2008) The effect of delaying childbirth on primary cesarean section rates. PLoS Med, 5 (7): e144.

Smith, I. E., Dowsett, M., Yap, Y. S., Walsh, G., Lonning, P. E., Santen, R. J. & Hayes, D. (2006) Adjuvant aromatase inhibitors for early breast cancer after chemotherapy-induced amenorrhoea: caution and suggested guidelines. J Clin Oncol, 24 (16): 2444-2447.

Somunkiran, A., Yavuz, T., Yucel, O. & Ozdemir, I. (2007) Anti-Müllerian hormone levels during hormonal contraception in women with polycystic ovary syndrome. European Journal of Obstetrics & Gynecology and Reproductive Biology, 134 (2): 196-201.

Sowers, M., McConnell, D., Gast, K., Zheng, H., Nan, B., McCarthy, J. D. & Randolph, J. F. (2010b) Anti-Müllerian hormone and inhibin B variability during normal menstrual cycles. Fertility and sterility, 94 (4): 1482-1486.

Sowers, M. R., Eyvazzadeh, A. D., McConnell, D., Yosef, M., Jannausch, M. L., Zhang, D., Harlow, S. & Randolph, J. F., Jr. (2008) Anti-Mullerian Hormone and Inhibin B in the Definition of Ovarian Aging and the Menopause Transition. J Clin Endocrinol Metab, 93 (9): 3478-3483.

Steiner, A. Z., Herring, A. H., Kesner, J. S., Meadows, J. W., Stanczyk, F. Z., Hoberman, S. & Baird, D. D. (2011) Antimullerian hormone as a predictor of natural fecundability in women aged 30-42 years. Obstet Gynecol, 117 (4): 798-804.

Strauss Iii, J. F. & Williams, C. J. (2009) CHAPTER 8 - The Ovarian Life Cycle. In: eds. Yen & Jaffe's Reproductive Endocrinology (Sixth Edition). Philadelphia: W.B. Saunders: 155-190.

Streuli, I., Fraisse, T., Chapron, C., Bijaoui, G., Bischof, P. & de Ziegler, D. (2009) Clinical uses of anti-Müllerian hormone assays: pitfalls and promises. Fertility and Sterility, 91 (1): 226-230.

Streuli, I., Fraisse, T., Pillet, C., Ibecheole, V., Bischof, P. & de Ziegler, D. (2008) Serum antimullerian hormone levels remain stable throughout the menstrual cycle and after oral or vaginal administration of synthetic sex steroids. Fertility and sterility, 90 (2): 395-400.

Su, H. I., Flatt, S. W., Natarajan, L., Demichele, A. & Steiner, A. Z. (2013) Impact of breast cancer on anti-mullerian hormone levels in young women. Breast cancer research and treatment, 137 (2): 571-577.

Su, H. I., Sammel, M. D., Green, J., Velders, L., Stankiewicz, C., Matro, J., Freeman, E. W., Gracia, C. R. & DeMichele, A. (2010) Antimullerian hormone and inhibin B are hormone measures of ovarian function in late reproductive-aged breast cancer survivors. Cancer, 116 (3): 592-599.

Sun, L., Tan, L., Yang, F., Luo, Y., Li, X., Deng, H. W. & Dvornyk, V. (2012) Meta-analysis suggests that smoking is associated with an increased risk of early natural menopause. Menopause, 19 (2): 126-132.

Tehrani, F. R., Solaymani-Dodaran, M., Tohidi, M., Gohari, M. R. & Azizi, F. (2013) Modeling Age at Menopause Using Serum Concentration of Anti-Mullerian Hormone. Journal of Clinical Endocrinology & Metabolism, 98 (2): 729-735.

Telfer, E. E. & McLaughlin, M. (2007) Natural history of the mammalian oocyte. Reproductive BioMedicine Online, 15 (3): 288-295.

Tinkanen, H., Bläuer, M., Laippala, P., Tuohimaa, P. & Kujansuu, E. (2001) Correlation between serum inhibin B and other indicators of the ovarian function. European Journal of Obstetrics & Gynecology and Reproductive Biology, 94 (1): 109-113.

Titus, S., Li, F., Stobezki, R., Akula, K., Unsal, E., Jeong, K., Dickler, M., Robson, M., Moy, F., Goswami, S. & Oktay, K. (2013) Impairment of BRCA1-Related DNA Double-Strand Break Repair Leads to Ovarian Aging in Mice and Humans. Science Translational Medicine, 5 (172): 172ra121.

Tremellen, K. P., Kolo, M., Gilmore, A. & Lekamge, D. N. (2005) Anti-mullerian hormone as a marker of ovarian reserve. Australian & New Zealand Journal of Obstetrics & Gynaecology, 45 (1): 20-24.

Tsepelidis, S., Devreker, F., Demeestere, I., Flahaut, A., Gervy, C. & Englert, Y. (2007) Stable serum levels of anti-Mullerian hormone during the menstrual cycle: a prospective study in normo-ovulatory women. Hum. Reprod., 22 (7): 1837-1840.

Turnbull, C. & Rahman, N. (2008) Genetic predisposition to breast cancer: past, present, and future. Annu Rev Genomics Hum Genet, 9 321-345.

Ueno, S., Kuroda, T., Maclaughlin, D. T., Ragin, R. C., Manganaro, T. F. & Donahoe, P. K. (1989) Mullerian Inhibiting Substance In The Adult Rat Ovary During Various Stages Of The Estrous Cycle. Endocrinology, 125 (2): 1060-1066.

van Beek, R. D., van den Heuvel-Eibrink, M. M., Laven, J. S. E., de Jong, F. H., Themmen, A. P. N., Hakvoort-Cammel, F. G., van den Bos, C., van den Berg, H., Pieters, R. & de Muinck Keizer-Schrama, S. M. P. F. (2007) Anti-Mullerian hormone is a sensitive serum marker for gonadal function in women treated for

Hodgkin's lymphoma during childhood. Journal of Clinical Endocrinology & Metabolism, 92 (10): 3869-3874.

van den Berg, M. H., van Dulmen-den Broeder, E., Overbeek, A., Twisk, J. W. R., Schats, R., van Leeuwen, F. E., Kaspers, G. J. & Lambalk, C. B. (2010) Comparison of ovarian function markers in users of hormonal contraceptives during the hormone-free interval and subsequent natural early follicular phases. Human Reproduction, 25 (6): 1520-1527.

van Disseldorp, J., Lambalk, C. B., Kwee, J., Looman, C. W., Eijkemans, M. J., Fauser, B. C. & Broekmans, F. J. (2010) Comparison of inter- and intra-cycle variability of anti-Mullerian hormone and antral follicle counts. Hum Reprod, 25 (1): 221-227.

van Dorp, W., van den Heuvel-Eibrink, M. M., de Vries, A. C., Pluijm, S. M., Visser, J. A., Pieters, R. & Laven, J. S. (2014) Decreased serum anti-Mullerian hormone levels in girls with newly diagnosed cancer. Hum Reprod, 29 (2): 337-342.

van Montfrans, J. M., van Hooff, M. H. A., Huirne, J. A., Tanahatoe, S. J., Sadrezadeh, S., Martens, F., van Vugt, J. M. G. & Lambalk, C. B. (2004) Basal FSH concentrations as a marker of ovarian ageing are not related to pregnancy outcome in a general population of women over 30 years.[see comment]. Human Reproduction, 19 (2): 430-434.

van Noord, P. A. H., Dubas, J. S., Dorland, M., Boersma, H. & Velde, E. t. (1997) Age at natural menopause in a population-based screening cohort: the role of menarche, fecundity, and lifestyle factors. Fertility and sterility, 68 (1): 95-102.

van Rooij, I. A., Tonkelaar, I., Broekmans, F. J., Looman, C. W., Scheffer, G. J., de Jong, F. H., Themmen, A. P. & te Velde, E. R. (2004) Anti-mullerian hormone

is a promising predictor for the occurrence of the menopausal transition. Menopause, 11 (6 Pt 1): 601-606.

Vogel,V.(2008)MerckManual.http://www.merckmanuals.com/home/womens_health_issues/breast_disorders/breast_cancer.html:

Vázquez, M. E., Verez, J. R., Stem, J. J., Najar, A. G. & Asch, R. H. (1998) Elevated basal estradiol levels have no negative prognosis in young women undergoing ART cycles. Gynecological Endocrinology, 12 (3): 155-159.

Wallace, A. M., Faye, S. A., Fleming, R. & Nelson, S. M. (2011) A multicentre evaluation of the new Beckman Coulter anti-Müllerian hormone immunoassay (AMH Gen II). Annals of Clinical Biochemistry, 48 (4): 370-373.

Wallace, W. H. & Kelsey, T. W. (2010) Human ovarian reserve from conception to the menopause. PLoS One, 5 (1): e8772.

Wallace, W. H. B., Thomson, A. B. & Kelsey, T. W. (2003) The radiosensitivity of the human oocyte. Human Reproduction, 18 (1): 117-121.

Weekes, C. D., Vose, J. M., Lynch, J. C., Weisenburger, D. D., Bierman, P. J., Greiner, T., Bociek, G., Enke, C., Bast, M., Chan, W. C. & Armitage, J. O. (2002) Hodgkin's disease in the elderly: improved treatment outcome with a doxorubicin-containing regimen. J Clin Oncol, 20 (4): 1087-1093.

Weenen, C., Laven, J. S. E., von Bergh, A. R. M., Cranfield, M., Groome, N. P., Visser, J. A., Kramer, P., Fauser, B. C. J. M. & Themmen, A. P. N. (2004) Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. Mol. Hum. Reprod., 10 (2): 77-83.

Winther, J. F., Boice, J. D., Jr., Mulvihill, J. J., Stovall, M., Frederiksen, K., Tawn, E. J. & Olsen, J. H. (2004) Chromosomal abnormalities among offspring of childhood-cancer survivors in Denmark: a population-based study. In: eds. Am J Hum Genet. United States: 1282-1285.

Wunder, D. M., Bersinger, N. A., Yared, M., Kretschmer, R. & Birkhauser, M. H. (2008a) Statistically significant changes of antimullerian hormone and inhibin levels during the physiologic menstrual cycle in reproductive age women. In: eds. Fertil Steril. United States: 927-933.

Wunder, D. M., Guibourdenche, J., Birkhauser, M. H. & Bersinger, N. A. (2008b) Anti-Mullerian hormone and inhibin B as predictors of pregnancy after treatment by in vitro fertilization/intracytoplasmic sperm injection. Fertility & Sterility, 90 (6): 2203-2210.

Xu, H. B., Liu, Y. J. & Li, L. (2011) Aromatase inhibitor versus tamoxifen in postmenopausal woman with advanced breast cancer: a literature-based meta-analysis. Clin Breast Cancer, 11 (4): 246-251.

Yeomanson, D. J., Morgan, S. & Pacey, A. A. (2013) Discussing fertility preservation at the time of cancer diagnosis: Dissatisfaction of young females. Pediatr Blood Cancer, 60 (12): 1996-2000.

Yu, B., Douglas, N., Ferin, M. J., Nakhuda, G. S., Crew, K., Lobo, R. A. & Hershman, D. L. (2010) Changes in markers of ovarian reserve and endocrine function in young women with breast cancer undergoing adjuvant chemotherapy. Cancer, 116 (9): 2099-2105.

ABBREVIATION

ABVD- doxorubicin, bleomycin, vinblastine and darcarbacine

AC- cyclophosphamide, doxorubicin

AFC- antral follicle count

AMH- anti-Müllerian Hormone

BEACOPP- bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine and prednisolone

BMI-body mass index

BRCA mutation- mutation of gene BRCA1 or BRCA2

Ca- cancer

ChlVPP-chlorambucil, vinblastine, procarbazine and prednisolone

CI- Chief Investigator

CMF- cyclophosphamide, methotrexate and fluorouracil

DNA- deoxyribonucleic acid

EC-epirubicin and cyclophosphamide

ELISA- The enzyme-linked immunosorbent assay

E2-Estradiol

FAC- 5-fluorouracil, doxorubicin, cyclophosphamide

FEC- 5-fluorouracil, epirubicin and cyclophosphamide

FSH-F Follicle stimulating hormone

GnRH-Gonadotropin-releasing hormone

GnRHa- GnRH agonist

HCG-human chorionic gonadotropin

HRT-hormone replacement therapy

IVF- In vitro fertilisation

LH- Luteinizing hormone

MALT lymphoma- form of lymphoma involving the mucosa-associated lymphoid tissue

MFS/MF- Midland Fertility Services/Midland Fertility from end of 2014

MIS-Müllerian inhibiting substance

NIHR- The National Institute for Health Research

ORT- ovarian reserve test

PCOS- Polycystic ovary syndrome

PI-Principle Investigator

POI-premature ovarian failure

REC-research ethics committee

R&D- Research and Development

TGF-transforming growth factor

TMB- tetramethylbenzidine

UKCRN-UK Clinical Research Network

USS –ultrasound

APPENDICES

Appendix 1 Proforma- data extraction- literature review

Proforma for study inclusion and data extraction

Date **Reviewer**

Paper number **1st author** **Language**

Selection criteria

Population: reproductive age women (adult) with cancer who received chemotherapy

YES NO

Ref standard ovarian reserve test including AMH

Select this diagnostic test study YES NO

If no reject ad specify reason

……

……

……

……

Data retrieval

Population:

N=

Mean age

Study design……..Cohort/Cross-sectional/Case control/other

Data collection…….Prospective/Retrospective/Can't tell/Other

Patient enrolment……Consectutive/Arbitary/Can't tell/Other

Blind comparison with ref standard Yes/No/Can't tell

Differential use of ref standard……. Yes/No/Can't tell

Inclusion criteria clearly stated Yes/No/Can't tell

Criterion: 1

2

3

4

5

Exclusion…… YES/NO/Can't tell

Sample population: infertility menstrual disorder regular menses

Type of cancer:

Stage of cancer YES/NO/Can't tell what stage:

A prior calculation of sample size YES/NO/Can't tell

Intervention

Type of intervention CT RT CTplus RT

Regimen described YES/NO/Can't tell

Dosage given YES/NO/Can't tell

Duration of therapy YES/NO/Can't tell

Relapse…… YES/NO/Can't tell

Follow-up

Duration of follow up

Loss to follow up:

Cancer group N=

Control group N=

Recruitment

Original population

Pre-enrolment exclusion

Post enrolment exclusion

Analysed data

Ovarian reserve test characteristics

Test performed:

……

……

What was measured at baseline?

…….

Reference standard

……

……

Type of AMH assay used (1st, 2nd generation/other)

Time interval between first and second measurments

……

Time interval between second and third measurments

……

Time between the last dose of chemotherapy and ORT

……

Blinding of the test results

Results:

-comparison of mean/median

-ROC

-LR

Sensitivity/Specificity

-NPV

-PPV

Others:

Outcome measures

Changes in hormone levels

Amenorrhea

Pregnancy

Live birth

Recurrence of menses

Early menopause

Other……

Completeness of follow up ……%

Bias:

selection bias – if same selection criteria have been used for all participants

loss to follow-up –if it differs between exposed and not exposed group

information bias –if same quality and extent of information is available from exposed and not exposed groups

Appendix 2 Study protocol

Full title: Effects of chemotherapy upon fertility amongst women of reproductive age, using AMH as a marker of ovarian reserve

Short title: Measuring effects of cancer therapy upon female fertility

Version 5, 29th of September 2010

Title

Effects of chemotherapy upon fertility amongst women of reproductive age, using AMH as a marker of ovarian reserve.

Introduction

Reproductive function in women after cancer treatment is increasing in importance because improvements in the survival rates mean that many more women live longer after cancer (Sonmezer and Oktay, 2004; Wallace *et al*., 2005). Some of the newer, more effective therapies are also more aggressive and impact substantially upon fertility. In parallel, new methods of fertility preservation have been developed, such as oocyte and embryo cryopreservation. Women may wish to access these, and to understand the impact of their treatment upon their future chances of fertility. For many women,

fertility, or the prospect of it, is a major factor influencing self-esteem, personal identity and wellbeing.

Chemotherapy frequently leads to subfertility, principally through direct effects that reduce the number of oocytes remaining in the ovary. Oocytes are laid down before birth and then enclosed into small follicles that persist into adult life. This supply of follicles, which is finite and pre-determined at birth, is called the 'ovarian reserve'. The follicles are gradually used up over the woman's reproductive life. Once used (growth towards 'ovulation') or damaged, they are irreplaceable. Total or near complete loss of oocytes results in menopause and sterility, while partial loss has a correspondingly lesser impact.

The exact, long-term effect of the different types of chemotherapeutic agent and regimens upon ovarian reserve amongst women of reproductive age is still unknown. The degree of chemotherapy-induced ovarian follicle loss depends on the type of chemotherapy, its dosage, duration and the age of patients, ie how large their ovarian reserve is before treatment begins (Whitehead *et al*., 1983; Meirow and Nugent, 2001, Lower *et al*., 1999). An accurate assessment of remaining ovarian function and therefore fertility potential after chemotherapy is not straightforward. Women who regain spontaneous menstrual cycles following chemotherapy may yet have evidence of occult ovarian dysfunction and be at risk of subsequent premature menopause (Bath *et al*., 2003; Larsen

et al., 2003). These effects may be particularly dependent upon the chemotherapy regimens.

Counselling prior to chemotherapy should include discussion regarding family planning, potential for and implications of premature menopause and options of fertility preservation treatments, all of which should be considered as an important, integral part of care for young women with cancer. Yet, it is clear that many women do not have the opportunity for detailed assessment of their fertility potential or to avail themselves of cryopreservation measures, since there is often little time between diagnosis and the initiation of treatment. Additionally, in this tumultuous period, they may be unable to assimilate the complex issues that may result from fertility-related interventions. Therefore, an accurate and rapid method of testing ovarian reserve would be extremely useful to both clinicians and to women to inform subsequent fertility management in such situations.

Recent studies have shown that anti-mullerian hormone (AMH) is a valuable marker of ovarian reserve. It is produced by granulosa cells of the growing follicles (Baarends *et al*., 1995; Durlinger *et al*., 2002b; Weenen*et al*., 2004) with expression initiated in the smallest growing primary follicles and only declining in the early antral stages or as follicles become atretic. AMH concentration thus is independent of the menstrual cycle (Cook *et al*., 2000) (cycle dependence

was a problem with all previous markers) and accurate even amongst women with irregular menstrual cycles. It is promising as a useful routine marker for potential reproductive capacity in women of childbearing age pre- and post-chemotherapy (Bath *et al*., 2003), however its application to this situation has not been fully assessed in a prospective study.

We therefore aim to undertake a prospective study to evaluate the adverse effects upon fertility of some of the chemotherapeutic regimens used for cancers affecting young women, and the accuracy of serum AMH measurement in predicting women's potential reproductive capacity following chemotherapy treatments. The results of this study would be crucial in informing decision-makers and various stakeholders regarding fertility treatment prognosis, and in informing oncologists' and women's choices about chemotherapeutic options.

Objectives

The objectives of this prospective study are (i) to assess the effect of different types, dosage, and duration of chemotherapy and/or radiotherapy regimens on ovarian reserve using AMH as a marker, (ii) to assess the accuracy of AMH in predicting future reproductive capacity, and (iii) to achieve a reliable marker of ovarian function for women undergoing chemotherapy, which would not be menstrual cycle dependent.

Methods

We plan to recruit between 50-100 reproductive age women newly diagnosed with cancers, prior to chemotherapy/radiotherapy within the West Midlands Cancer Network. A prospective cohort study design will allow us to assess the effect of exposure to chemotherapy agents on ovarian function over time. Serum AMH levels will be measured in a group of patients undergoing treatment with potentially gonadotoxic drugs before commencing chemotherapy and with a follow up at 6, 9 months and 1 year and will be compared with an age-matched control group cohort without known fertility problems.

A 5-year follow-up questionnaire including a detailed history of number of pregnancies and live births is planned to assess the accuracy of AMH measurement in predicting longer-term fertility.

Women aged 18-43 with newly diagnosed cancer, prior to chemotherapy/radiotherapy would be eligible. Women will be excluded if they have a history of previous exposure to gonadotoxic agents and/or radiotherapy; been diagnosed with end stage cancer having a very poor prognosis (less than 10% chances of 1 year survival); or significant ovarian pathology. Usage of hormonal contraception methods will not be an exclusion criterion.

An age-matched control group will be recruited to compare the changes in AMH in women receiving no cancer treatment.

AMH in blood samples will be measured using a newly developed AMH Immunoassay. This assay has been established in a research setting at the PI's clinic by Al-Qahtani A (Al-Qahtani, 2005b). Patients, clinicians and laboratory personnel would be blinded to either patient clinical progress or the AMH concentration, which will not be used to manage the patient.

Recruitment and data collection:

Cancer patients:

Female patients meeting our criteria who are about to proceed with chemotherapy will be identified through the direct healthcare team - oncology teams within PAN Birmingham Cancer Network, Wolverhampton and Coventry Hospitals and will be recruited consecutively where possible.

An information leaflet describing the study and its objectives will be circulated to all prospective patients (inviting them to participate) by oncology teams.

Having received the leaflet, any patient considering joining the study will be informed by an oncology team member about the research program and the blood tests involved. The researchers will provide further details direct to patients, if necessary.

Blood samples will be obtained in the oncology clinic together with a medical questionnaire including obstetric and gynaecological history (e.g. menstrual history) and consent form.

Additional information will be provided to the researchers by oncology teams from the medical records (regarding the type of cancer and chemotherapy regimen)**.**

All forms together with the blood sample will be sent to the MFS laboratory to be tested.

Healthy volunteers:

Healthy, reproductive age volunteers (aged 18-43) will be recruited by two methods: advertisement in local newspapers, locally displayed posters and local GP surgeries, near to MFS. These volunteers will be invited to attend MFS for procedures associated with the study.

Notices displayed at hospitals participating in the study (specifically UHCW), aimed at recruiting female staff to participate as volunteer members of the control group. These volunteers will be able to take part in the study from their workplace, subject to local PI agreement.

Volunteers will be given a telephone number and an email address to contact the study coordinator (Dr Karolina Palinska-Rudzka) and local PI/research

nurse. More information about the study and the risks involved will be given verbally and/or via email or post. The information sheet and consent form will be made available to the prospective volunteer.

Volunteers will have time to decide if they want to participate (up to 12 months- see study time table).

In the case of a positive reply, a suitable date and time will be arranged at the most appropriate location for the volunteer. During the appointment, written consent will be sought, and if consent is forthcoming, a blood sample will be taken and the medical questionnaire will be completed.

The volunteer will be given a copy of the information sheet and consent form to keep. This provides the contact details of the researchers and the opportunity for follow up in the event of any concerns.

Both groups:

Identifiable data (name, DOB, address, contact telephone number) will be recorded on a separate (paper based) database and a unique research reference number applied. Identifiable data will be stored in lockable filling system in medical record area at Midlands Fertility Services with limited access to documentation by researchers only. At the time of processing a serum sample unidentifiable data will be saved on excel file to provide a point of central references for research. A label with the same unique reference number will be

applied on each blood sample on its arrival at the Laboratory.The computer database will only contain the unique research reference number without any personal details. Any information stored electronically will be encrypted.

Data analysis

Anonymised clinical data will be stored in a Warwick University database and statistical support from statistician at Warwick University is available.

In consultation with our statistical colleagues, we will use the appropriate statistical tools to perform our data analysis. Changes in hormone concentrations with time will be analysed by analysis of variance for repeated measures; to investigate at what time points significant treatment effects are evident. The impact of the chemotherapy will be measured by testing the deviation of the cohort from the age-matched control group.

All statistical analysis will be carried out using SPSS or similar software.

Project management

This project will be undertaken by Dr Karolina Palinska-Rudzka, an MD candidate at the University of Warwick, under the academic supervision of Prof Geraldine Hartshorne. Progress of the study and its compliance with protocol will be assessed on a regular basis during meetings with the academic supervisor.

The University of Warwick will act as sponsor of this study.

Expert opinion on clinical implications and understanding of ovarian reserve test will be provided by Dr Gill Lockwood- Fertility Specialist.

The funding for the project has been secured from Midland Fertility Services.

Cancer patients:

The oncology teams agreeing to take part in our project will be offered an initial 1-hour training session in obtaining research consents including presentation on effect of chemotherapy on ovarian reserve and AMH marker. All the consent forms, medical questionnaire, information leaflets, pre-paid envelopes, blood bottles will be distributed by the Study Coordinator on a regular basis to all oncology clinics involved in the project.

A member of the NHS healthcare team will make the initial approach to potential participants. During the treatment planning appointment in oncology clinics all women aged 18-43 will be given verbal information and/or leaflets on ovarian reserve tests and details of our project.

Patients will have time to decide if they want to participate until their next appointment, when a written consent will be requested.

Initially, all information will be given to patients by the oncology teams, but patients may also seek further information from the researchers by telephone or email.

Whole blood samples will be obtained by adequately trained oncology teams (handled according to standard hospital practice when sending samples for analysis off site) and send via post to be processed at MFS within 24 hours. Detailed instruction on blood samples transport and equipment required for packing will be provided according to UK Transport Legislation 602 and/or 605/UN Packaging Instruction P650. Accompanying forms will be send together with blood samples in previously pre-paid (first class) envelope.

Whole blood in serum tubes, received at MFS will be separated on the day of delivery. Serum samples will be rendered acellular by centrifugation and removal of supernatant. The resulting serum samples will be stored frozen at MFS. The cellular fraction will be disposed of immediately the serum has been removed.

On arrival to Midlands Fertility Laboratory unique reference number will be applied and sample process according to MFS protocol L077 AMH.

Volunteers:

Once volunteers have been recruited to the study, after the first visit, appointments for follow up blood samples will be given in advance or sent via email/txt or letter, according to the volunteer's preference.

Information about publication arrangements are included in the participant information sheet.

Volunteers, in contrast to cancer patients, will be sent a letter with their own AMH results and its interpretation. On request, verbal explanation of the results and its implications will be given by fertility specialist appointed locally and/or by Dr Karolina Palinska-Rudzka and Dr Gillian Lockwood (contact details will be provided with result letter).A thank you letter with the full study results will be sent to participants (if they wish to receive it).

Appendix 3 Patient/volunteers information leaflets and consent

Version 4, 29th September 2010

Study number: 09/H1211/87

VOLUNTEER INFORMATION SHEET

Measuring effects of cancer therapy upon female fertility

Dear Volunteer,

You are being invited to take part in a research study.

Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether you wish to take part. If you do choose to participate in the research, you remain free to withdraw your consent at any time before your material has been used in the research. Such withdrawal of consent will have no effect on any aspect of how you are treated by us.

Thank you for reading this.

WHAT IS THE PURPOSE OF THE STUDY**?**

We would like you to take part in our research to enable us to compare the results obtained for patients treated for cancer with those of a healthy population, the ‘control’ group. We are asking if you would be willing to be a member of the ‘control’ group for this study.

Treatment of cancer, and some related conditions, produces many side effects. In particular, we are interested in the effects upon fertility in young women undergoing chemotherapy. For many women, the possible effects of cancer treatments upon their future chances of having children are a very important issue. Unfortunately, there is currently no easy way to assess ovarian function and predict future fertility.

The exact, long-term effects of different types of cancer treatment upon the ovaries of women of reproductive age are still uncertain. A reliable and quick method of testing the ovaries would be extremely useful. Such a test would help women to know their fertility potential, and likely reproductive time-span, allowing them to plan for their future. It would also help doctors to choose the most appropriate therapies for their patients, by providing new information on the likely impact on fertility.

Recent studies have shown that anti-mullerian hormone (AMH) is a valuable marker of ovarian reserve; however, it has not been used clinically for patients undergoing chemotherapy. Further information is essential before this can take

place. We therefore aim to conduct a prospective study to assess the effect of chemotherapy, used for cancers affecting young women, upon ovarian reserve, as measured using AMH. We shall also determine the accuracy of AMH measurement in blood samples, in predicting women's potential reproductive capacity following chemotherapy treatments. In order for us to analyse the data properly, we need to be able to compare the results with those in a control group of women of similar ages, who do not have cancer and who are not known to have any particular problems with fertility.

Why have I been chosen?

We are aiming to recruit around 50-100 volunteer women of age 18-43. We would like to ask if you would be willing to donate blood samples taken 4 times during a year, to measure your AMH levels for comparison with the group of cancer patients.

Do I have to take part?

Participation is voluntary.

What will happen to me if I take part?

If you agree to participate, you are requested to provide four blood samples within a one-year period - the first at the start of your involvement in the study and the others approximately 6 months, 9 months and 1 year later. AMH will be

measured in the blood samples provided by you. You are also requested to fill in a medical questionnaire at the time of your blood tests and another similar one after about 5 years. The questionnaires will take around 5-10 minutes to complete and you are asked to return them at the time of the blood tests, or post them back in the prepaid envelope supplied. The first questionnaire is attached herewith.

If you wish to participate, we will take blood samples at a time convenient for you. Please call us or send an email and we will make the arrangements. All dates of the appointment will be given in advance and a reminder will be send to you via email, txt message or a letter, according to your preference.

What do I have to do?

Please take time to read the information and discuss any questions that you may have with a member of the research team (our contact details are provided in this information). If you wish to take part, please complete and sign three copies of the consent form.

What are the possible benefits and risks of taking part?

You will receive no direct benefit from participating in this study. In the future, we hope that this research may bring benefits to young women going through cancer treatment. There are no risks of this study over and above those of taking

the blood samples. There is a very small (less than 1 in 100) risk of skin infection following any blood test.

Psychological discomfort is possible for the following reasons: you may feel uncomfortable with some questions in the medical questionnaire, which will include your past medical history, questions about your periods, fertility, and surrounding issues. None of those questions will be asked directly (no face- to-face interview).

Please note, AMH is commonly measured in women commencing fertility treatment because it helps to predict response of the ovaries to stimulation medicines. For you, the AMH test will assess the 'biological age' of your ovaries and the four measurements together could perhaps show the natural decline of your ovarian function over time. However, please be aware that AMH measurements cannot predict pregnancies because many other factors are involved, as well as AMH.

It is up to you whether you want to be told about the results of your tests. If you wish to know your AMH result, please be aware that the occurrence of an abnormal result might cause you anxiety. If you decide that you do want to know, we will provide you with the results and a full explanation. If your results cause you particular anxiety, we can offer the possibility to discuss further with a fertility doctor.

What happens when the research study stops?

At the end of this study, any blood samples that have been used for research are normally disposed. However, if you agree, it is possible that they can be kept in case they could be useful for other research projects in future. We would like to keep them for this purpose; however, you are under to obligation to agree to this, regardless of whether you agree to participate in the present study. Please would you indicate on the consent form whether you agree for this to be done. Rest assured that a Research Ethics Committee would have approved any such future research in advance.

What happens if I lose the capacity to consent during the project?

In the unlikely event of your losing capacity to consent during the course of the project, we propose that any samples and data that had already been collected would continue to be used confidentially for the study, but no further samples or data would be taken from you. This could also include further research after the current project has ended. You are asked to indicate your consent to this on the consent form.

What if something goes wrong?

If you experience any concerns about the research, you are very welcome to contact the researchers in the first instance. We do not envisage any problems arising during the course of this research. However, if an untoward event were to occur, please be assured that we will do everything we can to put matters right.

Will my taking part in this study be kept confidential?

Yes, your participation will be confidential. Any samples you provide and non-identifying information about your medical history will be stored in an anonymous form. Please note that such anonymised information may be stored on a computer database. Your personal details would only be accessible under strictly controlled conditions and then only to the researchers involved in the study.

What will happen to the results of the research study?

We understand that your AMH results could be of personal interest to you. It is important for you to know that AMH is a new marker of ovarian reserve, only recently been registered for diagnostic use and has mostly been validated in patients undergoing IVF treatment. Since you are not undergoing such treatment, and are a normal healthy volunteer, the results may not be particularly useful or relevant to you. However, we are happy to let you know the results if you are interested in them.

If you wish to know your results, please show this on your consent form. The letter with explanations of the results will be sent to the address given by you. If you have any queries arising from the results, please contact us and we will make arrangements to discuss the results further with you.

The collected results from all participants will be published in a medical journal and presented to learned societies at conferences, but you would not be identified in any publication. If you would like a copy of the final publication, please write to Professor Hartshorne at the address below. Please note, it usually takes several years for results to be fully analysed and published.

Who is organising and funding the research?

This study is receiving funding from the Midlands Fertility Services, Third Floor, Centre House, Aldridge, WS9 8LT, which employs Dr Karolina Palinska-Rudzka, and provides the facilities and funds needed to run the specialised AMH assay. Through this research, Dr Palinska Rudzka is studying for a Doctor of Medicine degree at the University of Warwick, under the academic supervision of Professor Hartshorne. Staff involved in NHS treatment, who agree to provide information about the study or take blood samples, are not remunerated for their involvement. No inducement or financial reward will be given to any of the staff involved in this study. If you would like to take part, please complete all three copies of the consent form. You will be given the information sheet and a signed consent form to keep. If you have any questions

or queries, please ask one of the staff, or contact (provided). Thank you for your consideration.

PATIENT/VOLUNTEER CONSENT FORM, Study Number: 09/H1211/87
Measuring effects of cancer therapy upon female fertility (Name of Researchers: Dr Karolina Palinska-Rudzka, Prof Geraldine Hartshorne)

Please, write yes or no and initial each box.

1. I confirm that I have read and understand the Information

Sheet **dated 29th September 2010 (version 4**) for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time until any samples are used, without giving any reason. My care or legal rights will not be affected.

3. I agree to have blood samples taken for this research and to be contacted to make arrangements for this.

I am willing to fill in a medical questionnaire now and I am happyto be contacted again in 5 year time.

4. I understand that responsible individuals involved in the research may look at my medical questionnaire. I give permission for them to use anonymised medical history data from my medical questionnaire for analysis of this study.

5. At the end of the study, I agree that my anonymised blood sample can be kept.

6. In the event of my losing the capacity to consent during research, I agreethat any samples or data already collected will be retained for use in the study and in any future research.

7. I wish to receive a letter with explanation about my AMH results at the address given by me overleaf

Name of Person taking consent	Date	Signature
Researcher	Date	Signature

Appendix 4 Medical questionnaire

Version 2, 23rd of September 2009

Study number: 09/H1211/87

MEDICAL QUESTIONNAIRE

MEASURING EFFECTS OF CANCER THERAPY UPON FEMALE FERTILITY

This is your *first medical questionnaire* for purpose of this study. It should take no longer than 5 to 10 minutes to fill it in. The questions are mainly about your age and past medical history including previous pregnancies.

All this information is important for our study in order to provide the most accurate results. Please do your best to provide full information. If you are unsure, you are welcome to contact us to ask, or to provide any additional information that you feel is relevant. You will be asked in the future to fill in a similar questionnaire called the follow-up medical questionnaire. A prepaid addressed envelope is enclosed for your convenience. ***Thank you for taking the time to do this.***

Please fill in the gaps and/or circle your answers

Which day of your menstrual cycle are you today?(day one of menstrual cycle=1first day of bleeding)

for example last bleeding started around 10 days ago

.....

Are your periods normally regular/irregular?

Normal length of your menstrual cycles (*for example every 28 days*) every.....days

How old were you when you had your first period..... years

Have your periods recently become more irregular or have they stopped for a long time?

No/Yes If yes, please provide details?

.....

Have you ever been investigated or treated for problems with getting pregnant?

No/Yes If yes, please provide details

Number of pregnancies.....

Number of births.....

Age of children…….....

Do you have any known gynaecological problems (e.g polycystic ovaries, endometriosis)?

No/Yes If yes, please provide details or give name of your condition

.....

Do you have any other significant medical problems?

No/Yes If yes, please provide details or give name of your condition

.....

Do you have a family history of early menopause (menopause starting before age of 45)?

No/Yes *If yes please give details (e.g my mum/sister/cousin had menopause at age of 40)*

.....

Are you on any medications (not including chemotherapy)?

No/Yes *If yes*, please write the name of any medications that you take or state what it is for (e.g I am taking some tablets for my blood pressure)

.....

Are you currently taking the contraceptive pill or any other hormonal treatment?

No/Yes *If yes please give details*

.....

How would you describe your desire to have children?

None/Neutral/Maybe/Strong

Others/comments:.....

Your height …… metres/feet

Your weight ……. Kg/stone

Are you a smoker/ non-smoker/ ex-smoker/ passive smoker?

Any other comments you may wish to make.

Version 3, 8 October 2010

Study number: 09/H1211/87

10.4.1 MEDICAL QUESTIONNAIRE

MEASURING EFFECTS OF CANCER THERAPY UPON FEMALE FERTILITY

This is your *follow-up medical questionnaire*. It should take no longer than 5 to 10 minutes to fill it in.

The questions are mainly about your age and past medical history including previous pregnancies.

All this information is important for our study in order to provide the most accurate results. Please do your best to provide full information. If you are unsure, you are welcome to contact us to ask, or to provide any additional information that you feel is relevant (see contact details on your consent form).

Thank you for taking the time to do this.

A prepaid addressed envelope is enclosed for your convenience.

Please fill in the gaps and/or circle your answers

Which day of your menstrual cycle are you today?**(day one of menstrual cycle=1first day of bleeding)** ***for example last bleeding started around 10 days ago***

.....

.....

Are your periods regular/irregular?

Have your periods recently become more irregular or have they stopped for a long time**? No/Yes If yes, please provide details?**

.....

.....

Do you have any significant medical problems**?**

No/Yes **If yes, please provide details or give name of your condition**

Are you on any medications?

No/ Yes ***If yes*, please write the name of any medications that you**

.....

Are you currently taking the contraceptive pill or any other hormonal treatment?

No/Yes *If yes please give details*

.....

How would you describe your desire to have children?

None/Neutral/Maybe/Strong

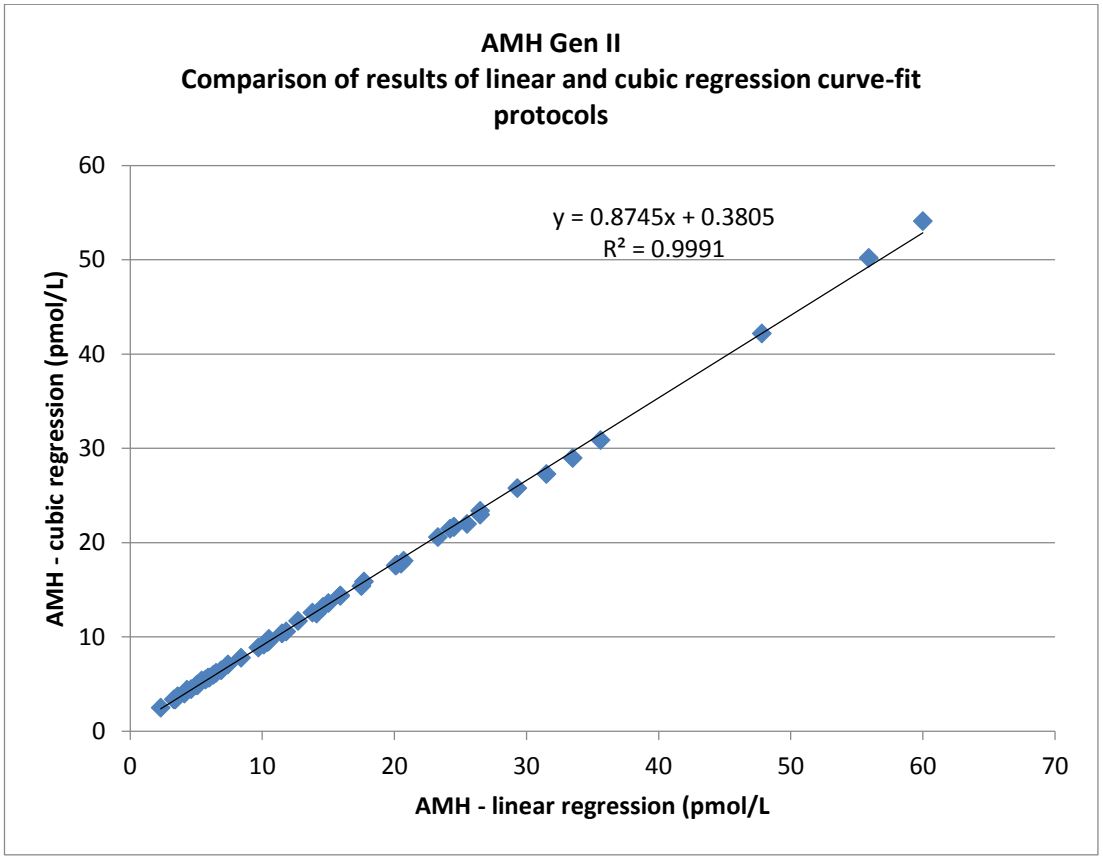
Others/comments:.....

Your weight ……. Kg/stone

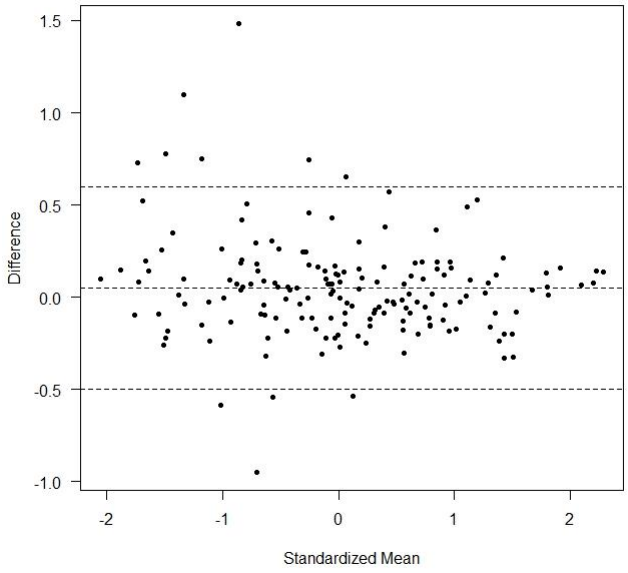
Are you a smoker/non-smoker/ex-smoker/passive smoker?

Any other comments you may wish to make.

Appendix 5 Linear versus cubic regression model (second generation AMH assay).



Appendix 6 Comparison of two methods of second generation assay AMH assay.



Bland-Altman plot suggests small but consistent difference between old and new data. Supports view that fixed additive corrective adjustment is sufficient.