Anti-Müllerian hormone as a marker of ovarian reserve in girls before, during and after treatment for childhood cancer

Mörse, Helena

2017

Document Version:
Publisher's PDF, also known as Version of record

Anti-Müllerian hormone as a marker of ovarian reserve in girls before, during and after treatment for childhood cancer

Anti-Müllerian hormone as a marker of ovarian reserve in girls before, during and after treatment for childhood cancer

Helena Mörse

LUND
UNIVERSITY

DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at Belfrage Lecture Hall, BMC, Lund

23 March 2017 at 9.00

Faculty opponent
Professor Smita Bhatia, University of Alabama, Birmingham, USA

Organization LUND UNIVERSITY Department of Paediatrics, Clinical Sciences, Lund Faculty of Medicine Author(s)Helena Mörse	Doctoral thesis Date of issue March 23, 2017 Sponsoring organization

Anti-Müllerian hormone as a marker of ovarian reserve in girls before, during and after treatment for childhood cancer

Abstract

Background: Although tremendous progress in survival after childhood cancer has been achieved with a five-year survival rate just over 80% in the industrialized world, this increase in survival rate is not without cost. Two-thirds of survivors experience a treatment related health condition and the most common late sequelae are the endocrine- and reproductive disturbances. Women are born with a finite number of oocytes (one to two million at birth) a number that declines with age and only 1000 oocytes are left at menopause, at a mean age of 51 years. The depletion of follicles can be hastened by cancer treatments resulting in a compromised ovarian reserve (i.e. a woman´s reproductive potential). Recently, serum-Anti-Müllerian hormone (AMH) has proven to be an effective way to estimate the ovarian reserve.

Aims: Using AMH as a proxy for ovarian reserve, this thesis investigates the effects on ovarian function before, during and after treatment for childhood cancer. Three of the studies (studies I, III, IV) presented here are prospective. These studies are based on a cohort of 104 girls diagnosed and treated for childhood cancer at the department of Paediatric Haematology and Oncology, Skåne University Hospital in Lund, Sweden. The forth study (study II) is an investigation of the effect of an accidental thawing of samples (freezer error) on AMH.

Results: We found a dramatic decline in AMH after three months of treatment, regardless of diagnosis, age and treatment given. We interpreted this result as an acute toxic effect on the ovaries. In patients with acute lymphoblastic leukaemia (ALL) (not high risk) we observed a recovery in AMH as early as during ongoing low dose chemotherapy. However, patients with AML, osteosarcoma, Ewing sarcoma or Wilms tumour (treated with radiation or stem cell transplantation) exhibited no recovery in AMH during treatment. After cessation of treatment (i.e. at follow-up), no recovery in AMH was observed in patients with ALL high risk or patients who had received whole abdomen radiation or stem cell transplantation. In addition, patients with AMH had a later recovery compared with ALL patients (not high risk) while patients treated for Ewing sarcoma showed no or late recovery in AMH.

Reanalysing samples (new aliquots) that were exposed to a freezer error in 2013, and our comparison of two different AMH assays reassured us that our samples were not affected by the freezer error, a conclusion that was confirmed by the thawing experiment we also conducted.

The results in these studies point to the severe impact on ovarian function of girls treated with radiation involving the ovaries and/or stem cell transplantation and probably also those treated for Ewing sarcoma. Ovarian function appears to be preserved in girls with ALL (not high risk) and AML. Surveillance of the long-term impact of cancer therapy and the subsequent risk for premature ovarian insufficiency is needed.

Key words Anti-Müllerian hormone, premature ovarian insufficiency, childhood cancer

Classification system and/or index terms (if any)

Supplementary bibliographical information		Language
ISSN and key title		ISBN
Recipient's notes	Number of pages	Price
	Security classification	

Anti-Müllerian hormone as a marker of ovarian reserve in girls before, during and after treatment for childhood cancer

Helena Mörse

LUND
UNIVERSITY

Cover photo by Anders Bergman (front cover) and Helena Mörse (back cover)

Department of Paediatrics
Clinical Sciences, Lund
Faculty of Medicine
Lund University
Sweden
ISBN 978-91-7619-418-8
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2017

To Theo and Hanna, with love

There is more to life than increasing its speed
Mahatma Gandhi

Contents

Contents 9
Original papers 11
Abbreviations 13
Abstract 15
Introduction 17
Cancer in childhood – epidemiology 17
Improvement of treatment regimens over time 18
Female reproductive system 21
Ovarian reserve 23
Anti-Müllerian hormone 24
AMH assays 27
Late complications after childhood cancer 28
Ovarian dysfunction 29
Oncofertility - fertility preservation in cancer survivors 33
Options for fertility preservation 33
Long-term follow-up 35
Aims 37
The overall objectives 37
The specific objectives 37
Present investigations 38
Study population 38
Methods 38
Statistical analyses 39
Study I 40
Study II 42
Study III 44
Study IV (in manuscript) 46
Discussion 48
AMH pre-treatment 48

AMH during treatment 49
AMH post treatment 50
Limitations of the studies 51
Conclusions 52
Future perspectives 52
Populärvetenskaplig sammanfattning 55
Bakgrund 55
Slutsatser 58
Acknowledgements 60
References 62

Helena Mörse 1
Borttagen: 5

Helena Mörse 1
Borttagen: 5

Helena Mörse 1
Borttagen: 5

Helena Mörse 1
Borttagen: 53

Helena Mörse 1
Borttagen: 53

Helena Mörse 1
Borttagen: 56

Helena Mörse 1
Borttagen: 5

Helena Mörse 1
Borttagen: 5

Helena Mörse 1
Borttagen: 61

Original papers

I. Mörse H, Elfving M, Lindgren A, Wölner-Hanssen P, Yding Andersen C, Øra I.
Acute onset of ovarian dysfunction in young females after start of cancer treatment
Pediatric Blood Cancer 2013 Apr: 60 (4): 676–681(Epub 2012 Sep 26)

II. Mörse H, Øra I, Turkiewicz A, Yding Andersen C, Becker C, Isaksson A, Elfving M.
Reliability of AMH in serum after long-term storage at –80°C and an extended period of thawing
Annals of Clinical and Laboratory Research 2016 Febr 4 (1) 61:1–6

III. Mörse H, Elfving M, Turkiewicz A, Yding Andersen C, Øra I.
Severe gonadotoxic insult manifests early in young girls treated for Ewing sarcoma
Medicine (Baltimore) 2016 Aug 95(33) e4512 (Epub ahead of print)

IV. Mörse H, Turkiewicz A, Yding Andersen C, Elfving M and Øra I.
Prospective analysis of anti-Müllerian hormone in girls during and after treatment for acute leukaemia
In manuscript

Abbreviations

ALL	acute lymphoblastic leukaemia
AMH	anti-Müllerian hormone
AML	acute myeloid leukaemia
$BRCA_2$	breast cancer 2 gene
CCNU	lomustine
CI	confidence interval
$CNKN_2A$	cyclin dependent kinase inhibitor 2A gene
CNS	central nervous system
DSL	diagnostic system laboratories
DOD	death of disease
E2	17 β estradiol
ELISA	enzyme- linked immunosorbent assay
ES	Ewing sarcoma
FSH	follicle stimulating hormone
Gy	gray unit
HSCT	hematopoietic stem cell transplantation
LH	luteinizing hormone
MMR	mismatch repair gene
NOPHO	Nordic Society of Paediatric Haematology and Oncology
OS	osteosarcoma
PNET	primitive neuroectodermal tumour
POI	premature/primary ovarian insufficiency
RT	radiotherapy
SCT	stem cell transplantation
SIOPE	International Society of Paediatric Oncology Europe
SPSS	statistical package for social sciences
TBI	total body irradiation
TRM	treatment

WT Wilms tumour

Abstract

Although tremendous progress in survival after childhood cancer has been achieved with a five-year survival rate just over 80% in the industrialised world, this increase in survival rate is not without cost. Two-thirds of survivors experience a treatment related health condition and the most common late sequelae are the endocrine and reproductive disturbances. Women are born with a finite number of oocytes (one to two million at birth) a number that declines with age and only 1000 oocytes are left at menopause, at a mean age of 51 years. The depletion of follicles can be hastened by cancer treatments resulting in a compromised ovarian reserve (i.e. a woman´s reproductive potential). Recently, serum-Anti-Müllerian hormone (AMH) has proven to be a reliable way to estimate the ovarian reserve. Using AMH as a proxy for ovarian reserve, this thesis investigates the effects on ovarian function before, during and after treatment for childhood cancer. Three of the studies (studies I, III, IV) presented here are prospective. These studies are based on a cohort of 104 girls diagnosed and treated for childhood cancer at the department of Paediatric Haematology and Oncology, Skåne University Hospital in Lund, Sweden. The forth study (study II) is an investigation of the effect of an accidental thawing of samples (freezer error) on AMH.

We found a dramatic decline in AMH after three months of treatment, regardless of diagnosis, age and treatment given. We interpreted this result as an acute toxic effect on the ovaries. In patients with acute lymphoblastic leukaemia (ALL) (not high risk) we observed a recovery in AMH as early as during ongoing low dose chemotherapy. However, patients with acute myeloid leukaemia (AML), osteosarcoma, Ewing sarcoma or Wilms tumour (treated with radiation or stem cell transplantation) exhibited no recovery in AMH during treatment. After cessation of treatment (i.e. at follow-up), no recovery in AMH was observed in patients with ALL high risk or patients who had received whole abdomen radiation or stem cell transplantation. In addition, AML patients had a later recovery when compared with ALL patients (not high risk) while patients treated for Ewing sarcoma showed no or late recovery in AMH.

Reanalysing samples (new aliquots) that were exposed to a freezer error in 2013, and our comparison of two different AMH assays reassured us that our samples were not affected by the freezer error, a conclusion that was confirmed by the thawing experiment we also conducted.

The results in these studies point to the severe impact on ovarian function of girls treated with radiation involving the ovaries and/or stem cell transplantation and probably also those treated for Ewing sarcoma. Ovarian function appears to be preserved in girls with ALL (not high risk) and AML. Surveillance of the long-term impact of cancer therapy and the subsequent risk for premature ovarian insufficiency is needed.

Introduction

The oldest description of cancer is found in Egyptian writings from about 3000 BC.[1] Hippocrates (460–370 BC) was the first person to use the word ¨cancer¨ to describe non-ulcer (*carcinos*) and ulcer forming tumours (*carcinoma*). In the Greek, the words *carcinos* and *carcinoma* refer to a crab, which the Roman physician, Celsus, translated into the Latin word for crab – cancer.

Cancer in childhood – epidemiology

In Sweden, the annual tumour incidence for children younger than 15 is 16/100 000. When including children 15–18 years, approximately 350 children are diagnosed annually.[2] The most common age group at diagnosis is 5 to 6 years. Cancer incidence is slightly higher in boys than girls, (M/F ratio 1.17) but the prognosis for the sexes is same. There is no difference in survival for different age groups except that children >10 years have a worse prognosis.[2]

Cancer in adults and children differs in many ways. Age and lifestyle factors largely contribute to cancer in adults, where cancer is the collection name for 200 different diseases, carcinoma being the most prevalent whereas carcinoma is rare in children.

Cancer in children is largely distributed into three groups; leukaemia (30%), CNS tumours (28%) and solid tumours (42%).[2] In comparison, in adults acute leukaemia stands for only 1% of all cancer cases. Childhood cancer is classified according to the International Classification for Childhood Cancer (ICCC),[3] where the twelve diagnostic groups (based on the International Classification of Diseases for Oncology, ICD-O) are further subdivided into subgroups (Fig. 1).

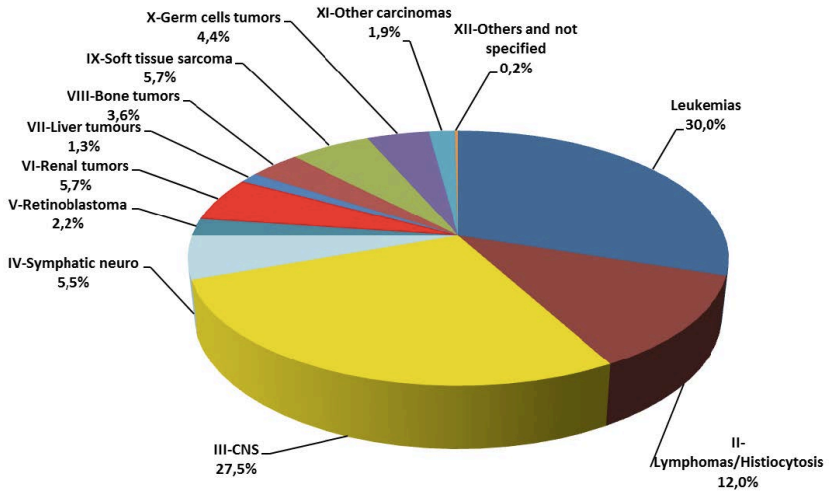

Figure 1. Distribution of childhood cancer malignancies in Sweden diagnosed 1984–2010 < 15 years of age at diagnosis.[4]

As lifestyle factors usually require many years to influence cancer risk they are not thought to play a major role in childhood cancer. Certain DNA changes in the cell caused by random events can transfer them into cancer cells, changes that occur early in a child´s life, possibly even in utero.[5,6] Genetic susceptibility (e.g. mutation in the retinoblastoma gene)[7] has been estimated to account for up to 10% of cases.[8] Inherited mutations associated with familial syndromes (e.g. Li Fraumeni syndrome, von Hippel Lindau syndrome, and Beckwith-Wiedemann syndrome) increase the risk of childhood cancer.[6,9] An increased risk was also reported in families with germline mutations in the $BRCA_2$, MMR and $CDKN_2A$ genes.[10] Previous radiation exposure and certain chemotherapeutic agents such as alkylating agents or topoisomerase II inhibitors can influence the risk of developing a second cancer in childhood or adult life.[11,12]

Improvement of treatment regimens over time

Until relatively recently, the only cancer treatment available to patients was surgery. Later cancer treatment include the use of radiation, a discovery made while attempting to treat breast cancer and only months after the discovery of X-rays by Wilhelm Conrad Röntgen in 1895.[13] But it was not until well into the 1900s that radiotherapy, in addition to surgery was introduced.

In 1940, the average survival after acute lymphoblastic leukaemia (ALL) was no more than three months.[14] In 1948, Dr Sidney Farber, a pathologist from Boston, reported temporary remissions in patients with ALL using aminopterin, a folate antagonist. After World War I, when it was noticed that exposure of nitrogen gas led to lymph node and bone marrow depletion in soldiers, nitrogen mustard was introduced to treat lymphoma; the first results were published in 1946.[15] In 1949, nitrogen mustard (under the name of mustine) became the first licensed chemotherapy agent and in 1948 methotrexate showed efficacy in childhood leukaemia.

According to Dr DeVita´s ¨A history of Cancer chemotherapy¨, in the early1960s attempts to cure cancer with drugs was not considered compatible with sanity.[16] Median survival in acute leukaemia of childhood improved from six months in 1956, when only single agents were used, to remissions measured in years by 1968, when a combination of four different drugs was used.[16,17] During the 1960s ¨The concept of cure¨ was coined; before this, only brief remissions were experienced and it was by means of combined modality treatments – surgery, radiation, and chemotherapy – that cure later would be possible and survival rates increase.[16] In 1973, medical oncology was accepted as a sub-speciality of internal medicine.[14]

Current situation

Tremendous progress in survival after childhood cancer has been achieved; there is now a five-year overall survival rate of just over 80% in the industrialised world[18,19] although survival rates differ depending on cancer type (Fig. 2).

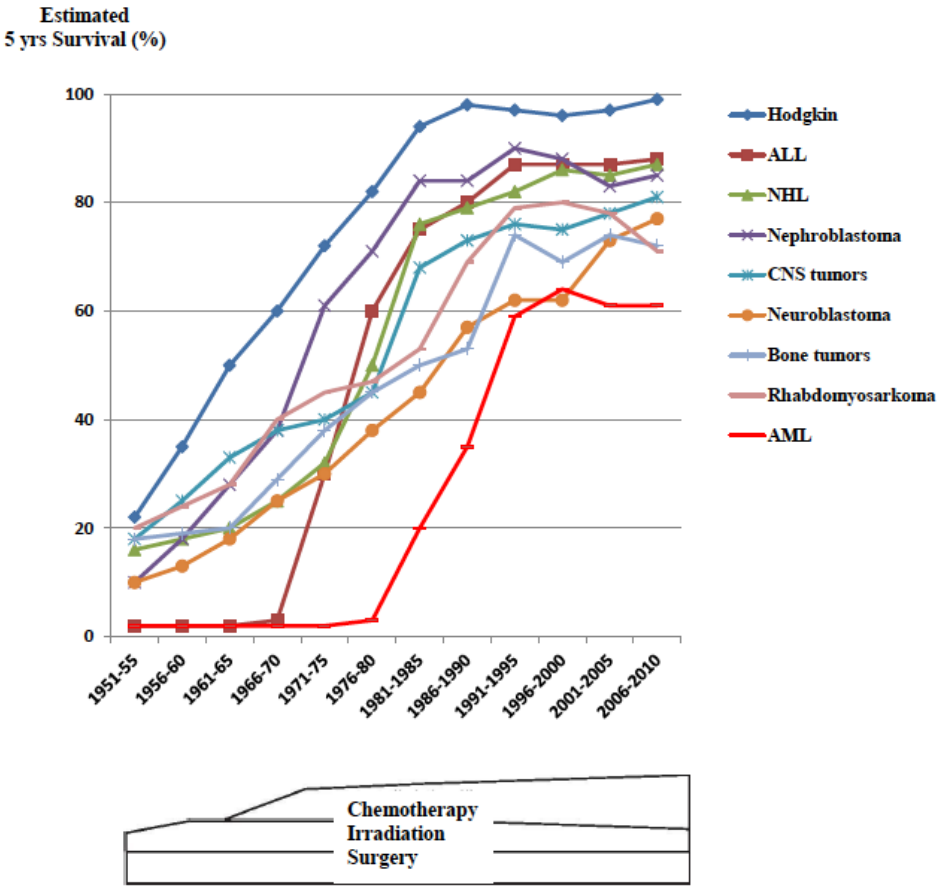

Figure 2. Estimated five-year survival rates over time for selected diagnostic groups in Sweden[4]

This progress is attributed to improved diagnostics, therapeutic advances in combined modality treatment (surgery, radiotherapy, and chemotherapy), multi-agent chemotherapeutic regimens, and improved supportive care (e.g. blood and blood products, treatment of infections, and intensive care). The greatest success was accomplished between the 1980s and the late 1990s, but since the 1990s cure rates have plateaued (Fig. 2).

Female reproductive system

Although challenged,[20] it is a generally accepted fact that girls are born with a finite number of primordial follicles in their ovaries, approximately one to two millions.[21] Primordial follicles decline through atresia to half a million at the start of puberty, when the hypothalamic pituitary gonadal axis becomes active. In every primordial follicle, the immature oocyte is surrounded by somatic granulosa cells. There is a continuous so called ¨initial recruitment¨ of follicles from the dormant primordial follicle pool into the growing follicle pool after which follicles start to express anti-Müllerian hormone (AMH) (Fig. 3) and Inhibin B.[22] Inhibin B is produced by the granulosa cells of the larger early antral and dominant follicles in response to gonadotropin stimulation.

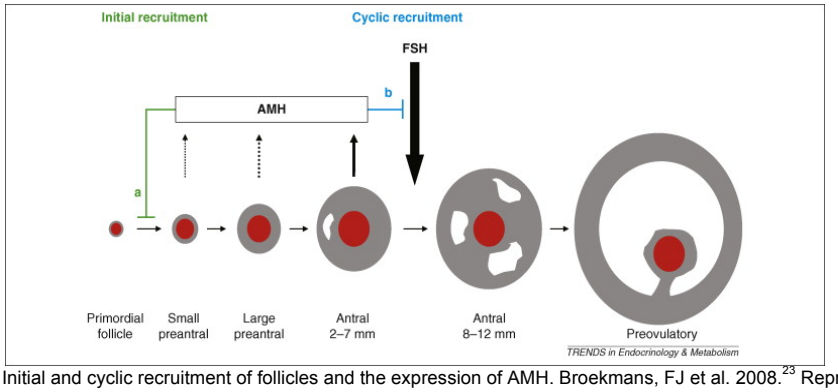

Figure 3 Initial and cyclic recruitment of follicles and the expression of AMH. Broekmans, FJ et al. 2008.[23] Reprinted with permission.

At the onset of puberty, the ¨cyclic recruitment¨ starts with a limited number of antral follicles being rescued from atresia under the influence of the follicle stimulating hormone (FSH) (Fig. 3).[24] One of these follicles will emerge as the dominant follicle and proceed to ovulation, while the remaining follicles undergo atresia (Fig. 4). A woman experiences around 400 monthly ovulatory cycles during a lifetime and at the time of menopause about 1000 follicles are left.[25,26] Thus the majority of the ovarian follicles present at birth will not go on to ovulation but undergo atresia through apoptosis,[27] necrosis,[28] or autophagy.[29]

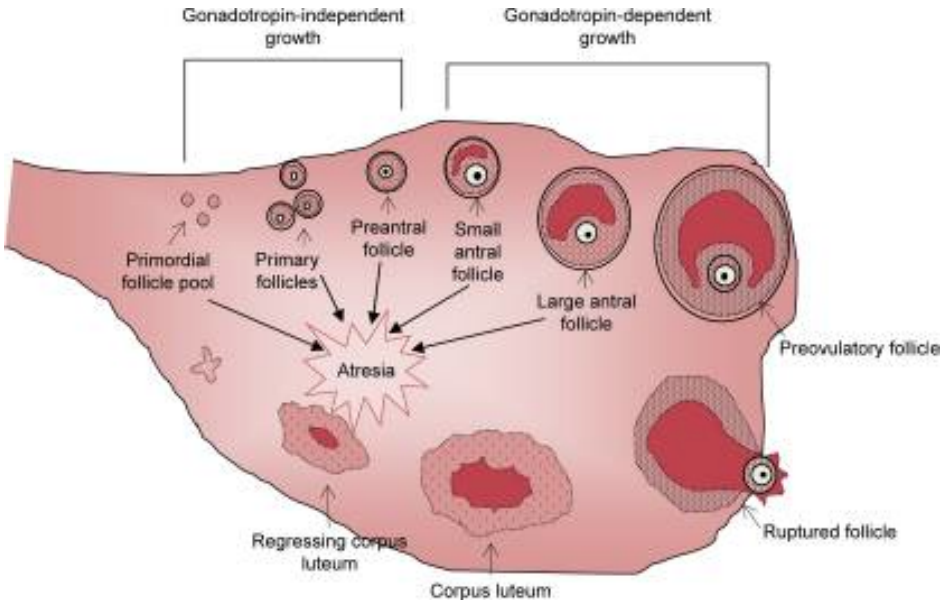

Figure 4. Folliculogenesis from primordial follicles through to follicle ovulation. Chapman C et al. 2015[30] Reprinted with permission.

Menopause

Menopause is defined as the final menstrual period that occurs for a woman and is due to an almost exhausted follicle pool. In the Western world, normal menopause is considered between 40–60 years of age[31] with a mean of 51 years[32] and it is estimated that fertility is decreased approximately ten years before menopause (Fig. 5).[33] The age at which menopause occurs seems to have been unchanged for many generations [34] and has a strong genetic heritability.[35,36]

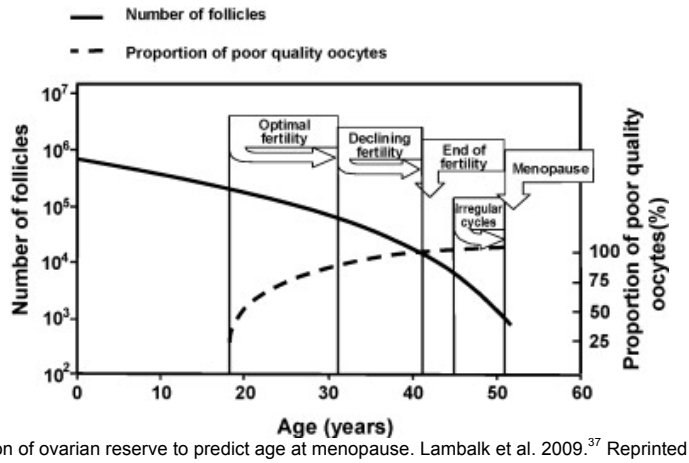

Figure 5 Estimation of ovarian reserve to predict age at menopause. Lambalk et al. 2009.[37] Reprinted with permission.

Ovarian reserve

Ovarian reserve describes both the quantity and the quality of the remaining follicles in the ovaries at any given time and defines a woman´s reproductive potential. It has been debated whether the term ovarian reserve should be used for both the non-growing dormant primordial follicle pool and the growing follicle pool. The size of the growing pool indirectly reflects the size of the non-growing follicles, which are too small to be measured. The term ovulatory potential has been proposed for the growing follicles that are measured by antral follicle count and using anti-Müllerian hormone (AMH).[38]

The gold standard to estimate the ovarian reserve has been through antral follicle count (AFC) via transvaginal ultrasound in combination with FSH and estradiol (E2) sampling in the early follicular phase on cycle day 2–5. AFC measures the number of visible follicles (2–10 mm).[39] FSH is regarded as a late predictor of menopause, with increased levels not until ten years before menopause, which coincides with infertility. As AMH correlates well with AFC[40,41]and unlike FSH is not cycle dependent, it is now routinely used as a marker of ovarian reserve, reflecting the primordial follicle pool.[42-44] In clinical practise, gynaecologists assess the ovarian reserve using all of the above mentioned tests in combination.

Anti-Müllerian hormone

AMH, formerly known as Müllerian inhibiting substance or Müllerian inhibiting factor was first described by Jost in 1947.[45] AMH is produced by the Sertoli cells in the testis[46] and its role during embryogenesis is to inhibit the development of the Müllerian ducts in the male embryo,[45] so it plays a role in male sex differentiation (Fig. 6).

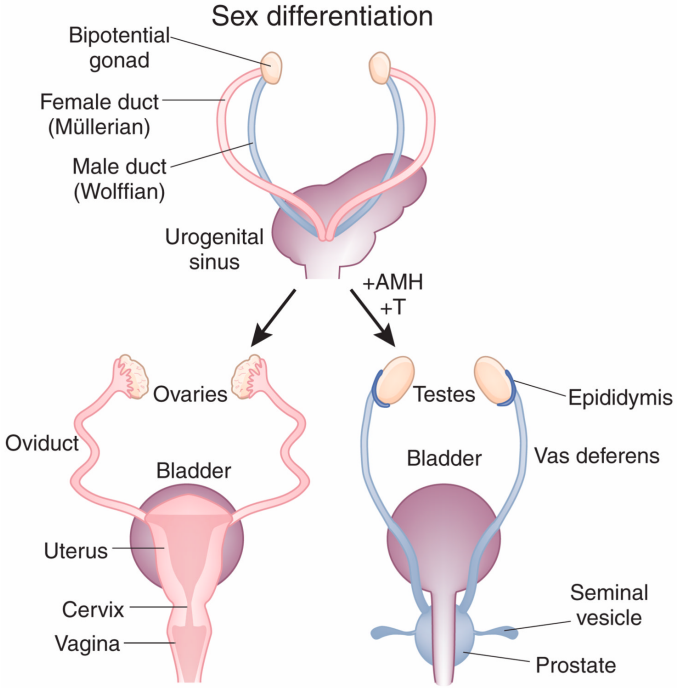

Figure 6. Sex differentiation from Matzuk and Lamb, 2008.[47] Reprinted with permission.

AMH belongs to the group of transforming growth factor-β family,[48] which also includes inhibins and activins. Located on chromosome 19p13.3, the AMH gene codes for a glycoprotein.[23] Also produced by the granulosa cells in the small growing follicles of the ovary, AMH correlates well to AFC [41,49] and might currently be the best biochemical marker for ovarian reserve.[50] AMH is measurable in female follicle fluid from gestational week 36 and in serum from birth.[51,52] Expression is found in granulosa cells of primary follicles as soon as primordial follicles are recruited from the dormant pool.[53] The highest concentration of AMH is found in the ovarian follicles with a size of 4–8 mm.[43] These follicles predominantly contribute to circulating AMH, but smaller follicles

contribute to a lesser extent, probably due to a reduced number of granulosa cells. AMH is only expressed in healthy follicles, not in follicles undergoing atresia.[54,55]

The functional role of AMH in the ovaries was discovered through research on AMH-deficient mice in 1999. In the absence of AMH, the rate of recruitment from the primordial follicle pool is increased and this leads to faster depletion.[22] Thus an important role of AMH is to suppress recruitment of primordial follicles and prevent early exhaustion of the follicle pool. Studies also suggest that AMH acts as a gatekeeper to make sure that antral follicles produce only small amounts of estradiol before selection to enable direct ovarian-pituitary feedback, communication that regulates the selection of a follicle that undergoes ovulation.[43]

The variance in non-growing follicles (the ovarian reserve) is thought to be due to age alone for females up to 25 years old.[56] There is also a large variability of AMH between individuals of the same age due to the variability in the number of antral follicles.[57]

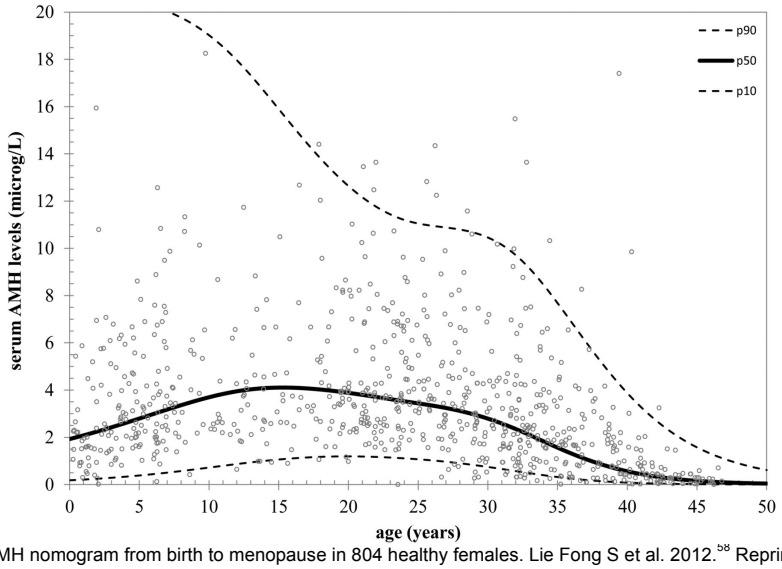

Figure 7. AMH nomogram from birth to menopause in 804 healthy females. Lie Fong S et al. 2012.[58] Reprinted with permission.

There is a steady increase in serum AMH from birth to 15 years of age before a decline is seen, according to one study of 804 females (Fig. 7).[58] A relatively recent study (> 3000 healthy women) found a peak consistent with the mini puberty of the neonate followed by an increase at nine years of age and a decline during puberty before peaking at around 25 years of age. After 25 years of age, there is a steady decline of AMH, reaching undetectable levels at an average age of 50–51 years, corresponding to the menopause (Fig. 8).[59] AMH is found to be very low or undetectable five years before menopause.[60]

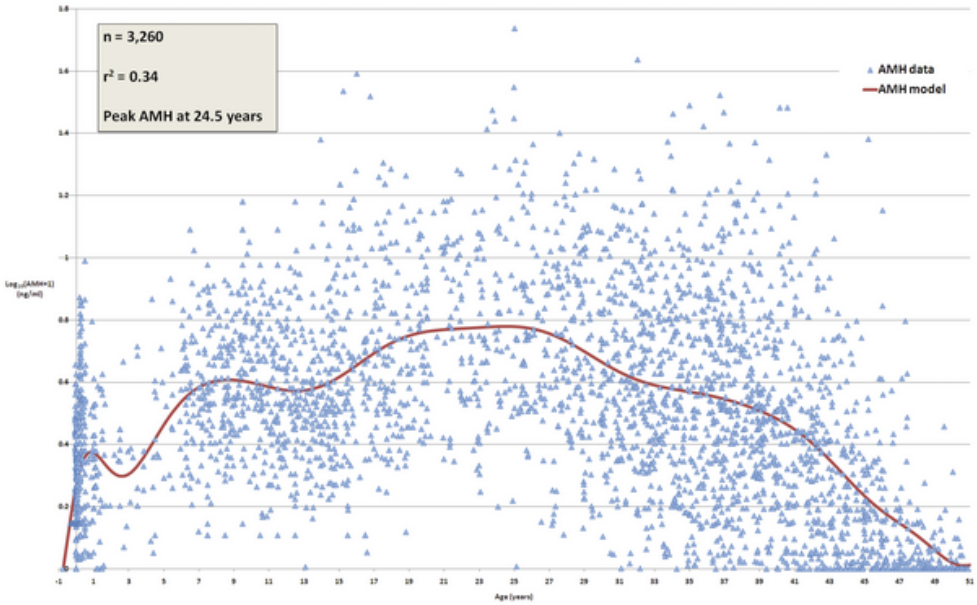

Figure 8. A validated model of AMH from conception to menopause. Kelsey TW et al 2011.[59]

A higher AMH concentration is found in Caucasian women compared with Afro-American and Hispanic women,[61] with multiple genetic and environmental factors at play.[62] There are reports of lower AMH levels in smokers[63] as well as a correlation between AMH and BMI; lower AMH is correlated with obesity.[64,65]

Some studies have shown lower AMH levels in oral contraceptive users[66,67] and AMH should be used with caution as a marker of ovarian reserve in women using hormonal contraception. Low values are also encountered in autoimmune disorders such as lupus and Crohn´s disease.[68,69]

Since AMH is not secreted by the dominant follicle or corpus luteum, it is believed to be relatively stable through the menstrual cycle[70] therefore a measurement does not have to be done on a specific day of the cycle.

There is no definition of a threshold value that suggests reduced fertility potential. Although with lower levels of AMH, there is a probability of a diminished ovarian reserve, with very low levels suggesting pregnancy is less likely to occur.

However, a Danish study did not find reduced fecundability in healthy women in their mid-20s who presented with low AMH levels (< 0.7 ng/ml), levels consistent with high oocyte quality.[71]

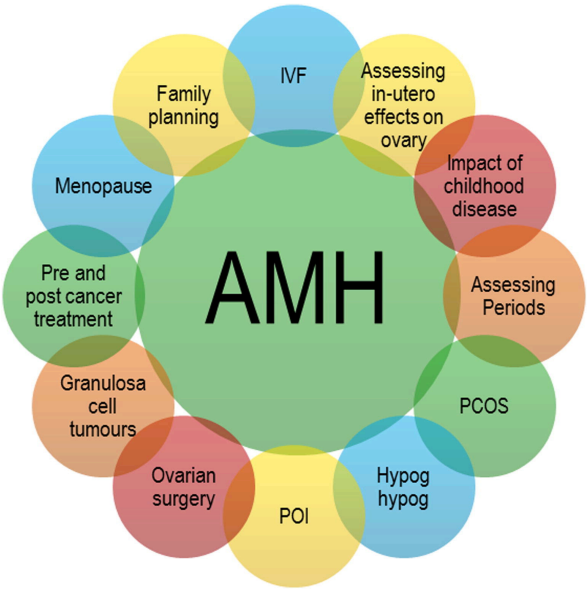

Figure 9. Potential clinical applications of anti-Müllerian hormone by heallth-care providers. Nelson, SM. 2013[72] Reprinted with permission.

Recently, AMH has been used in many applications (Fig. 9), but AMH is mainly used to predict ovarian response to stimulation in assisted reproduction.[73] It is also used to help diagnose and follow-up on granulosa cell tumours,[74] to predict the menopause,[75] and to follow the ovarian reserve after cancer treatment.[76-78] In the future, AMH may be part of the diagnostic criteria for polycystic ovary syndrome, a condition affecting 5–10% of women,[79] with AMH levels two-to three-fold increased. The first published study on AMH in women treated for childhood cancer (published in 2003) showed lower AMH levels in 20 childhood cancer survivors compared with controls.[80]

AMH assays

The development of sensitive assays has made it possible to measure low levels of serum AMH in women. Initially, the assays were mainly used to measure AMH as a marker of testicular function during childhood.[81] Immunotech (IOT, Marseille, France) produced the first commercially available AMH assay in 1999 using a monoclonal antibody pair directed to the pro- and mature region.[82] In 2003, Diagnostic Systems Laboratories (DSL, Webster, Texas) introduced a second assay, where both antibodies were directed to the mature region in an attempt to avoid proteolysis.[83] Both IOT and DSL became part of Beckman Coulter, which

launched a second generation (Gen II) AMH ELISA kit in 2005. This assay used the DSL antibodies and was standardised against the IOT assay.[84]

Since this development, Ansh Lab (Webster, Texas) has introduced another two ELISA assays; the ultrasensitive in 2012 and the pico AMH in 2013.[83] Due to lack of international standard, it has not been possible to compare results from the different assays.[85-87] A comparison of ten laboratories found a 40% variation in AMH values using the same Gen II assay.[88] In 2012 and 2013 Beckman Coulter reported in field safety notices that complement factors could influence the results due to interference (FSN-20434–3). It has also been suggested that AMH assays exhibit pre-analytical variability that could influence the results.[89] AMH sample stability and high assay reproducibility are very important for reliable interpretations of AMH.

Sample handling procedures such as time from collection to freezing, measuring AMH in whole blood or serum, and storage temperatures can affect the results. Storage of whole blood at 20°C gave an increase of 31% in AMH, measured by AMH Gen II, compared with serum. The effect on whole blood was less if stored at 4°C. [90]Another study found an increase of 22.5% in serum samples stored at –20°C over five days compared with fresh samples, whereas samples stored at –80°C were stable.[87] The influence on results after repeated freeze-thaw cycles that are often required in longitudinal studies has also been investigated. One study found a 15% increase in AMH analysed with the Gen II assay after three freeze-thaw cycles,[84] and another reported stable values obtained with the DSL assay after repeated freeze-thaw cycles.[91]

In 2014, two automated assays were made available; Access AMH assay by Beckman Coulter and Elecsys AMH immunoassay by Roche. There is a good correlation between these two assays, which are both standardised against the AMH Gen II assay.

In the future, it might also be possible to measure AMH in urine[72] or in dried blood spots.[92]

Clearly, the increased use of AMH in clinical and research settings suggests that an international AMH standard and highly reproducible AMH assays are needed.

Late complications after childhood cancer

The improvement in survival (five-year survival rate now exceeds 80%)[93] due to diagnostic and therapeutic advances means an increase in the number of long-term survivors. Evaluation of treatment-related late complications in cancer patients diagnosed at younger ages is of increasing importance since these patients have an expected survival of many years. It is estimated that 1 in 1000 adults in the Nordic countries is a childhood cancer survivor.[94] The increase in survival however, is not

without cost. Two-thirds of survivors experience some kind of treatment-related health condition, related to the tumour itself and/or its treatment, and the risk for morbidity and mortality increases with age.[95-97]

Virtually any organ system can be affected, but the most common late sequelae reported are related to the endocrine reproductive disturbances, which affect 40–60% of childhood cancer survivors.[98,99] There are many endocrine disturbances such as pubertal disorders (precocious or delayed puberty), obesity, reduced bone mineral density, dysfunction of the thyroid gland, dysfunction of the hypothalamus-pituitary axis and compromised gonads.[100] Other recognized conditions are cardiovascular,[101] renal impairment,[102] hearing impairment,[103] late neurocognitive effects[104,105] or musculoskeletal complications (e.g., hypoplasia or osteonecrosis).[106,107]

Carcinogenic effects of radiation, chemotherapy, or both carry a risk of developing a second malignant neoplasm, in the breast, brain, bone, and thyroid gland or a second leukaemia, which is the most prevalent.[97] The risk of developing a secondary solid tumour after treatment, including radiation increases with age.[108]

Childbearing is an important factor for the well-being of cancer survivors, and late complications affecting the gonads have a very negative impact on quality of life.[109,110]

Ovarian dysfunction

Premature ovarian insufficiency

The nomenclature for this condition is still under debate. Should the term premature menopause or primary ovarian insufficiency or premature ovarian insufficiency be used? In the USA, the National Institute of Health prefers using primary ovarian insufficiency, whereas the American Society for Reproductive medicine prefers using premature ovarian insufficiency. Since the European Society of Human Reproduction and Embryology also recommends premature ovarian insufficiency, I have chosen to use this term in this thesis.

Premature ovarian insufficiency (POI) is defined as the loss of normal ovarian function before the age of 40 years.[111] POI affects 1% of the general population and 0.1% of women younger than 30 years of age.[112,113] POI is characterized by 4–6 months of amenorrhea, low estrogen levels, and increased follicle stimulating hormone (FSH) levels in the menopausal range, typically > 40 IE/L.[111] POI can occur spontaneously or be iatrogenic. Mother-daughter and sibling studies have shown a family history of POI.[114]

A major cause of POI is depletion of follicles due to low initial numbers or an accelerated loss. Mechanisms behind this are thought to be either a developmental reduction in primordial follicles due to a genetic cause, an accelerated recruitment of primordial follicles, increased atresia, or destruction of follicles.[115,116] The

aetiology of POI is either idiopathic, iatrogenic (chemotherapy and radiation),[117,118] due to X-chromosome abnormalities (e.g., X monosomy in Turner syndrome and fragile X syndrome),[119,120] enzyme deficiencies (galactosemia),[121] viral agents,[122,123] or autoimmunity.[124,125]Other causes of POI are failure of ovarian follicles to grow, dysfunctionality, or follicles not responding to gonadotropins.[126,127,128]

POI may develop over several years and have a continuous spectrum before it manifests; it is described as *incipient* with normal FSH levels and regular menses, as *biochemical* with raised FSH levels and regular menses and as *overt* with raised FSH levels and irregular and finally absent menses.[116,129,130] POI is associated with the loss of fertility, but intermittent ovarian function has been reported. As many as 5 to 10% of women with POI have been reported to conceive spontaneously.[131,132]

Ovarian surgery and cancer treatments are iatrogenic causes of POI. A meta-analysis found that ovarian cystectomy due to endometrioma resulted in a 40% decline in AMH after three months, but the long-term effect was not investigated.[133] Hysterectomy has been reported to result in loss of ovarian function and an earlier menopause,[134] but whether the same is true for unilateral oophorectomy [135,136] is unclear. One study found only a slightly earlier onset of menopause after unilateral oophorectomy.[137]

Loss of ovarian function at a young age may appear as failure of pubertal development or pubertal progression with a need for estrogen substitution. Being diagnosed with POI at a young age has emotional impact as well as major health implications. Hypoestrogenism not only results in menopausal symptoms such as hot flushes, night sweats and vaginal dryness but also increases risk of osteoporosis, cardiovascular disease, and the metabolic syndrome.[34,130,138]

Gonadotoxic treatment in females

The natural ongoing depletion of ovarian follicles can be accelerated by cancer therapy. Knowledge of diminished fertility in women after cancer treatment has been learned from studies of adult female childhood cancer survivors. The extent of gonadal damage depends on the specific treatment administered as well as the cumulative doses received. Often a combination of drugs and treatment modalities are used. Unknown patient–specific factors probably also contribute, since females receiving the same treatment regimen can experience different degrees of gonadotoxicity.

Age at the time of treatment has also been regarded as an important factor for the degree of toxicity and is related to a lower number of follicles remaining in the ovaries with older age. Treatment at a younger age seems favourable in this context[139,140] however, there are studies that report no influence of age on POI after gonadotoxic treatment.[118,141]

Oocytes and the surrounding somatic granulosa cells are not equally vulnerable to chemotherapy, as oocytes, unlike the proliferating granulosa cells are non-dividing.[142] The toxic effect however, is exerted on all sizes of follicles,[143] either directly by an acute vascular insult or via oxidative stress (Fig. 10).[144] Some chemotherapeutic agents such as alkylating agents are considered to be more toxic to the ovaries than others, although no threshold dose could be identified.[78,145]

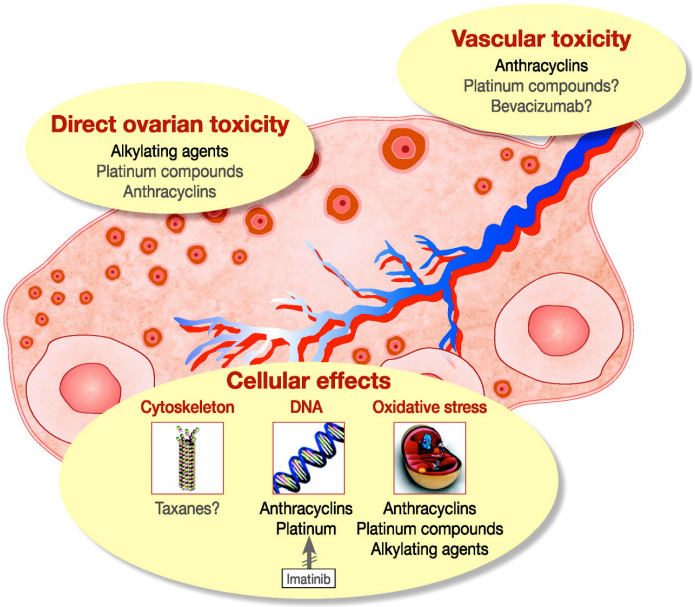

Figure 10. Suggested mechanisms for ovarian toxicity. Ben-Aharon, I et al. Reproduction 2012[144] Reprinted with permission.

Chemotherapy induced damage

Previous studies have identified alkylating agents such as e.g. cyclophosphamide, procarbazine, and busulfan to be the most gonadotoxic of many types of chemotherapeutic agents used.[146-148] Alkylators are non-cycle specific so they can damage both dividing and quiescent cells[142] and also induce cortical fibrosis and vessel damage[149] with a negative impact on the ovary. Other agents such as doxorubicin, an anthracyclin, also appears to be gonadotoxic by inducing granulosa cell dysfunction and a reduction in ovarian blood flow[150] as well as etoposide, a topoisomerase II inhibitor.[151,152] Whether alkylating-like agents/heavy metals (cisplatin and carboplatin) also exert a negative impact on the ovaries warrants further investigations.[148,153]

Methods proposed to calculate cumulative alkylating agent exposure in treated cancer patients are the Alkylating Agent Dose (AAD) and the Cyclophosphamide Equivalent Dose (CED), methods used mainly in the USA.[154]

Table 1. Estimated risk of gonadal dysfunction with chemotherapeutic drugs, modified from Wallace et al.[155]

High risk	**Medium risk**	**Low risk**
Cyclophosphamide	Cisplatin	Vincristine
Ifosfamide	Carboplatin	Methotrexate
Procarbazine	Doxorubicin	Dactinomycin
Busulfan		Bleomycin
Melfalan		Mercaptopurine
		Vinblastine

Radiation induced damage

The adverse effect from radiotherapy depends on the dose, the fractionation schedule, and the age of the patient. Radiotherapy to the whole abdomen or pelvic region can cause permanent ovarian damage,[117,118,156] a consequence also of irradiation of the spine, total body (TBI), total lymphoid system or scattered radiotherapy. The ovaries are very sensitive to radiation with a median lethal dose (LED_{50}) of 2 Gy.[157] Older age at the time of irradiation involves a greater dose-related risk. According to one study, doses of 5 Gy can affect ovarian function in post-pubertal girls,[158] whereas ovaries of pre-pubertal girls tolerate higher doses (≥ 10 Gy). A mathematical model showed that a sterilizing dose to infants and children at birth and at the age of 10 years and 20 years would be 20.3 Gy, 18.4 Gy and 16.5 Gy respectively.[159] In addition, cranial irradiation can cause hypogonadotropic hypogonadism through disruption of the hypothalamus-pituitary axis and thus affects fertility.[160,161] Furthermore, irradiation including the uterus in the involved field entails a risk of miscarriage, premature birth or low birth weight due to reduced elasticity of the musculature and vascular compromise.[162,163]

In summary, cancer patients who receive gonadotoxic treatment to be cured may be deprived of the possibility of retaining their reproductive potential. In addition, today when childbearing often is postponed up to the mid 30s, cancer survivors may have reached premature menopause, due to gonadotoxicity, before they consider family planning and childbearing (Fig. 11).

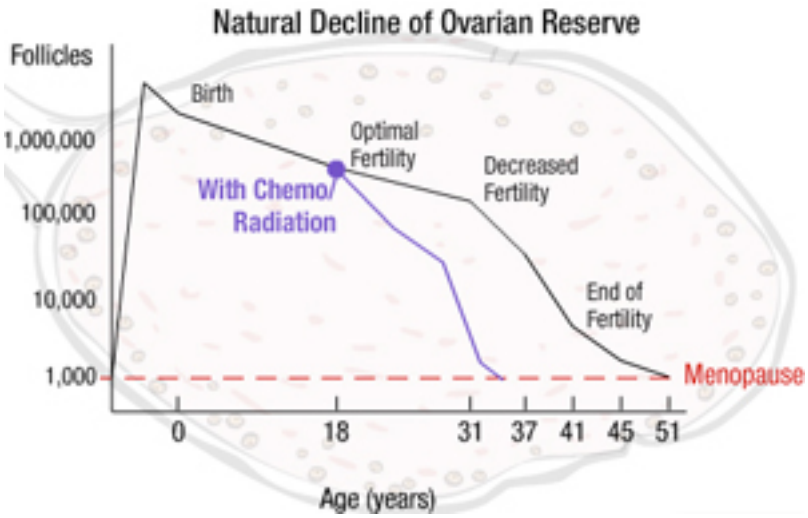

Figure 11. Natural decline of ovarian reserve, developed by J. Mersereau for the Oncofertility Consortium. Reprinted with permission.[164]

Oncofertility - fertility preservation in cancer survivors

It is important to keep in mind that the majority of girls and young women diagnosed and treated for cancer will remain fertile. Counselling our young patients and their parents concerning future fertility and the risk of subfertility is a difficult task, especially at the time of diagnosis when the focus is on survival and not so much on late effects. Some treatment doses and modalities compromise ovarian function more than others and efforts have been made to classify the risk of subfertility according to cancer type and its associated treatment.[155] Treatments that constitute a very high risk include the conditioning treatment for high dose stem cell transplantation and irradiation with the ovaries in the field, whereas treatment e.g. for acute leukaemia or Wilms tumour (without abdominal irradiation) is considered to confer a low risk.[165]

Options for fertility preservation

Who should receive fertility preservation? Is it the decision of the clinician or the patient? Should every girl who is about to start cancer therapy be offered fertility preservation? Should it be offered via a risk-based strategy? Presently, clinicians attempt the risk for future infertility based on available knowledge. Swedish recommendations concerning fertility preservation for children and adolescents

that risk future infertility were formed by a multidisciplinary group and published in 2015.[166]

Ovarian protection techniques

Techniques such as transposition of the ovaries (i.e., oophoropexy), may be undertaken laparoscopically before pelvic irradiation.[167] Pelvic shielding of the ovaries before upper abdominal irradiation is an option under reconsideration.[168] Administration of gonadotropin-releasing hormone (GnRH) analogues may protect the ovaries from the gonadotoxicity of chemotherapy, although the evidence is not obvious and trials performed have not included females under the age of 18 years.[169-171] GnRH analogues are believed to suppress the ovaries via inhibition of the hypothalamic-pituitary-ovarian (HPO) axis. Because the HPO axis is inactive in pre-pubertal females, this option is not used in this patient group.

Cryopreservation of oocytes, embryos, and ovarian tissue

Ovarian cryopreservation is the only preservation option for pre-pubertal girls. Because the technique is still considered experimental, it is recommended only for pre-pubertal girls who have a very high expected risk of infertility or pubertal girls who have a high/very high risk.[166] Excision of an ovary or a part of an ovary is performed laparoscopically. Strips from the ovarian cortex containing primordial follicles are cryopreserved by vitrification (the tissue is placed in liquid nitrogen after cryoprotectant exposure) a process thought to be advantageous to slow freezing.[172] Future re-implantation of the thawed ovarian tissue takes place either orthotopically (at the site of the remaining ovary) or heterotopically (in the subcutaneous tissue of the forearm, the abdominal wall, or rectus muscle).[173]

In 2000, the first report of restoration of ovarian function after transplantation of cryopreserved ovarian tissue was published.[174] In 2004, the first pregnancy from this technique was achieved.[175] Auto-transplantations of ovarian tissue have resulted in 86 live births and nine ongoing pregnancies worldwide.[176] The first successful birth after transplantation of cryopreserved ovarian cortical tissue in Sweden was reported in 2014.[177] To my knowledge, only two children until now have been born after pre-pubertal[178] or premenarchal[179] cryopreservation of ovarian tissue. However, the procedure of re-transplantation of ovarian tissue confers a risk of transferring malignant cells,[180,181] especially in patients treated for leukaemia,[182] but also for Ewing sarcoma.[183] A study of 26 females treated for sarcoma found no sign of minimal disseminated disease in the examined frozen-thawed ovarian tissue.[180]

Oocyte- as well as embryo cryopreservation are well established methods in post-pubertal females, the latter reserved for adult women. The two weeks of hormonal ovarian stimulation needed for oocyte cryopreservation confers a

subsequent unwanted delay of cancer treatment, a delay that makes this technique less feasible for childhood cancer patients.

Long-term follow-up

The need for long-term follow-up after childhood cancer is of utmost importance. In Sweden, approximately 9000 adults are long-term survivors of childhood cancer (D. Petterson The National Board of Health and Welfare, personal communication 2016). Late effect clinics are being formed at the six university hospitals with childhood cancer centres in Sweden. In 1987, the very first ¨late effect clinic¨ was set up in Lund by paediatric oncologist and professor Stanislaw Garwicz together with oncologist and professor Eva Ståhl, followed by Gothenburg in 2012.

Long-term follow-up guidelines from the American Children´s Oncology Group as well as from Sweden, the United Kingdom and Scotland are available for health care professionals.[184-187] Through the collaborative work of the European Network for Cancer research in Children and Adolescents (ENCCA, funded by the European Union) and SIOPE partners a Survivorship Passport has evolved. Professionals and survivors have worked together with the aim to harmonise the follow-up across Europe.[188,189] The passport contains information on the medical history and recommendations regarding follow-up and will hopefully be available across Europe in 2017.
In 2016, both the International Guideline Harmonization Group in collaboration with The PanCareSurfUp Consortium (funded by the European Union) and the Swedish working group for long-term follow-up after childhood cancer (SALUB) published recommendations for surveillance of POI.[185,190]

Girls exposed to alkylating agents and/or radiation with the ovaries in the field should have their ovarian function monitored post treatment. The Swedish and Pan Care guidelines agree with respect to the evaluation of pubertal development and progression in terms of clinical examination and menstrual history. The Swedish guidelines recommend taking annual FSH and LH measurements during puberty and at the age of 18 years, but the Pan Care guidelines make this recommendation only if clinically indicated. Early referral to a paediatric endocrinologist is recommended for pubertal disturbances and transient or manifest ovarian insufficiency for consideration of hormonal replacement therapy. According to Swedish guidelines, girls treated with SCT or radiation should be referred at completion of their cancer treatment, but Pan Care recommends this approach only if the patient exhibits POI symptoms.

In addition, the Swedish guidelines recommend that young females exposed to alkylating agents and/or radiation involving the ovaries should be referred to a

reproduction specialist for information and discussion about fertility preservation as there may be a window of fertility for females at risk of POI.[166]

The European guidelines identify several gaps in knowledge such as the diagnostic and prognostic value of AMH to predict POI in female survivors of childhood, adolescent and young adult cancer. Hence, AMH is not included in the surveillance of females < 25 years treated for cancer, due to the lack of evidence of its use to predict reduced fertility in this cohort. Long-term studies in this field are needed to provide evidence of the efficacy of AMH in younger females.

As today´s recommendations are based on studies on survivors treated using protocols that may no longer be in use, it is important to update guidelines to reflect new knowledge based on evolving national and international research.

Aims

The overall objectives

There are three main objectives in this thesis:

– to study the role of anti-Müllerian hormone (AMH) in serum as a potential marker of ovarian reserve in female childhood cancer patients;

– to study ovarian function in girls with childhood cancer regarding potential and timely adverse effects during and after different treatment regimens; and

– to investigate whether AMH levels during the first years after diagnosis can be used to predict susceptibility for gonadotoxicity, and to use this information to provide appropriate fertility counselling for patients at risk.

The specific objectives

There are four specific objectives in this thesis:

– to prospectively study the acute effect of cytotoxic treatment on AMH levels as a potential marker of ovarian function during cancer treatment;

– to prospectively follow AMH as a marker of the ovarian reserve after treatment and up to three years correlated to treatment given;

– to prospectively investigate ovarian function in young females treated for different cancer types to identify treatment regimens affecting ovarian function; and

– to evaluate the robustness of AMH concentrations in a clinical setting after long-term storage of serum samples at –80°C and to investigate the potential influence of thawing.

Present investigations

Study population

Eligible patients were girls aged 0–18 years diagnosed and treated with cancer since 2007 at the Department of Paediatric Haematology and Oncology, Skåne University Hospital, Lund University, Sweden. A total of 104 girls were included in the cohort in June 2016. Thirteen patients were lost to follow-up, ten denied participation, and nine were excluded for various reasons. Clinical information including menarche was recorded. All patients were treated per national and international treatment protocols in accordance with recommendations from the Swedish national working groups of paediatric oncology.

Methods

Blood was drawn from a central venous catheter or a peripheral vein at the time of diagnosis and approximately every three months during and up to three years after end of treatment. Thereafter, yearly samples were collected. Blood samples were transported at room temperature to the local laboratory where they were centrifuged, aliquoted, and stored at –80°C until analysis.

Serum levels of AMH and inhibin B were measured at the Laboratory of Reproductive Biology, Copenhagen, Denmark using specific ELISA kits according to the manufacturers´ instructions. Samples to be analysed in Denmark were moved from the –80°C freezer, transported on dry ice and on arrival immediately put in –80°C storage.

The first analyses of samples from the cohort included in the study were performed in 2011. AMH was initially measured with DSL–10–14400 from Diagnostic System Laboratories Inc. (Webster, TX). However, this assay became unavailable and a few samples were analysed with a new Gen II assay from Beckman Coulter using the same antibodies as in the DSL assay. There was a strong significant linear correlation between the two assays. The lower limits of

detection for the DSL and Gen II assays were 0.04 ng/ml and 0.16 ng/ml, respectively.

In 2014, the laboratory in Denmark had changed to yet another and improved AMH assay - the ultrasensitive Ansh-AMH. This assay has a lower limit of detection (0.023 ng/ml). For the subsequent studies, new aliquots from the old samples were re-analysed using the Ansh-AMH assay.

Plasma/serum levels of FSH (Roche 11775863) and LH (Roche) were measured at the Department of Clinical Chemistry, Skåne University Hospital, Lund, Sweden. Plasma levels of E2 were measured at the Department of Clinical Chemistry, Skåne University Hospital, Malmö, Sweden. All analyses were performed on thawed serum/plasma samples.

Statistical analyses

Study I: Comparisons between AMH levels from diagnosis to after three months of treatment were analysed using paired samples *t*-test. Pearson´s correlation was used to evaluate the correlation between AMH values and other continuous variables. SPSS was used for statistical analyses. All statistical tests were two-sided.

Study II: We calculated the correlation, agreement, and limits of agreement for the AMH values of the 188 serum samples before and after the thawing episode in the thawing experiment and for the FSH and LH measurements. In the analysis of agreement, we used logarithmic transformation to stabilize variance. We excluded the values under the detection level from the analysis of agreement. The correlation coefficient was calculated using Spearman´s correlation coefficient. All statistical analyses were performed in STATA version 13 and the figures were created in GraphPad prism version 5.01.

Study III: We used linear regression to analyse AMH levels, adjusting for age and diagnosis. All statistical analyses were performed using STATA version 13.

Study IV: For correlation between continuous variables, Spearman's rho was employed. For analysis of AMH values during and after treatment we used a random effects poisson regression model with robust standard errors. To estimate AMH recovery we compared baseline AMH with AMH at last follow-up in the ALL SR, ALL IR and AML groups using a poisson regression model with robust standard errors adjusted for baseline AMH and age.

For all analyses, we considered $p < 0.05$ as statistically significant.

Study I

After three years of prospective collection of samples, we performed an interim analysis to motivate further inclusion of patients in the study. We focused on ovarian function the first months after start of cancer treatment because of the heterogeneity of diagnoses and the variance in follow-up with only a small number of patients with samples after completion of treatment. This study included 34 patients with a mean age of 9½ years (range 4½ months to 16½ years); eleven of these had reached menarche. The included patients were treated for ALL, AML, Hodgkin lymphoma, Wilms tumour, osteosarcoma, Ewing sarcoma, CNS tumours, and other diagnoses. We analysed blood samples for AMH, inhibin B, E2, FSH, and LH.

Results

AMH was detectable in all patients at diagnosis and correlated to age but not to menarche (Fig. 12).

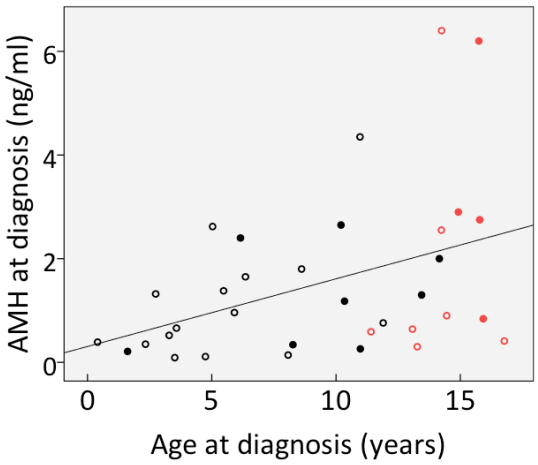

Figure12. AMH in relation to age at diagnosis and whether they had (red dots) or had not (black dots) reached menarche. The filled and open dots represent cases that did and did not have detactable AMH after three months of cancer treatment, respectively. From Study I.

At diagnosis, inhibin B and LH were detected in less than 50% of the patients reflecting the young age of many of the patients. At diagnosis, FSH was detected in all the patients and estradiol in all but two patients.

To our surprise, we found a profound and significant decline in AMH at three months after start of treatment in all patients, regardless of diagnosis, menarche, age at diagnosis, or type of chemotherapeutic drugs the patients received (Fig.13).

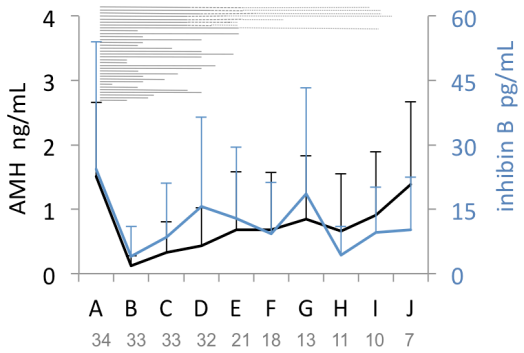

Figure 13. Mean levels of AMH (black) and inhibin B (blue) in 34 patients at diagnosis (time point A) and during treatment and after treatment at intervals of 3–4 months (time points B-J). The lines in the upper left corner illustrate the individual duration of treatment (dotted lines depict maintenance treatment).The grey numbers under the x-axis show the number of patients included at each time point. From Study I.

Both FSH and LH increased correspondingly. Twenty–two patients had undetectable AMH after three months of treatment, the twelve patients with detectable values included four of the eight with ALL, and they were all older than eight years.

Of the 34 patients, 32 were followed for a minimum of one year after diagnosis, and the median time of follow-up after completion of treatment was five months.

Patients with ALL composed the largest group (seven patients), but only a few of them had samples for the whole treatment duration of 2½ years. For those who did, we observed a recovery in AMH as well as in inhibin B as early as during low-dose chemotherapy maintenance treatment. For the nine patients treated with HSCT or radiation below the diaphragm, we found that they did not show any recovery of the ovarian function as measured by AMH after completion of treatment during follow-up (a median follow-up of 18 months).

During the review process of this manuscript, a group in Edinburgh, Scotland published similar results: they also found a decrease in AMH after start of chemotherapy. Their prospective study comprised 22 children with a variety of diagnoses and this was the first prospective study of AMH in girls during cancer treatment; the study was published a few months before our study was available on-line. Both these studies have small cohorts with a large variety of diagnoses, so this meant that there was a low number for each diagnostic group, a limitation that

makes it unsuitable to draw significant conclusions regarding AMH in relation to specific chemotherapeutic agents.

The strength of our study is that we together with the colleagues from Edinburgh for the first time prospectively used AMH as a marker of ovarian function in young females treated for childhood cancer. We confirmed that girls experience the same decrease in AMH after start of chemotherapy as adult females although the dramatic decline in AMH after start of treatment is not yet entirely understood.

Study II

In April 2013, all the samples (initially stored at –80°C) were exposed to an accidental period of thawing: up to 11°C for up to 21 days before they were refrozen. We were unsure whether we could rely on these samples in future analyses. We therefore designed a study (i.e., study II) on stability of AMH after a thawing episode. We also included the question whether a longer storage time would impact the samples and the AMH values.

In 2014, we re-analysed the same samples (new aliquots) that were analysed with the DSL-AMH assay in 2011, i.e. before the freezer error. The DSL assay was no longer available and the laboratory now used a new ultrasensitive assay from Ansh Labs with a good correlation to the former assay.

Results

When comparing AMH results from samples analysed in 2011 with samples analysed in 2014 we found a good correlation; a correlation coefficient of 0.96 with values obtained with the Ansh-AMH assay on average 1.6 times higher (Fig. 14).

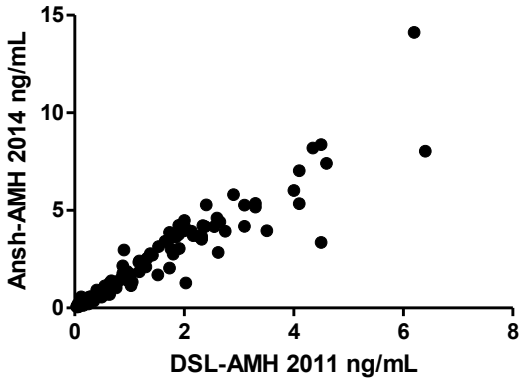

Figure 14. Correlation DSL AMH 2011 and Ansh AMH 2014, n= 188 (in the figure n=104, only detectable values). Ansh AMH showed 1.6 times higher values. From Study II.

We found similar AMH results from analyses of samples from 2007 and 2008 stored two years longer than samples stored in 2009 and 2010; both were 1.6–1.7 times higher compared to the original analysis.

To further strengthen our study, we investigated samples for FSH and LH, analysed at the same time points in 2011 and 2014 using the same analytical methods. We found good overall agreement for FSH and LH values between the two time points, with coefficients of agreement of 0.97 and 0.95, respectively, and a correlation coefficient of r = 0.98 and r = 0.94, respectively. The difference in AMH levels was similar to results in other studies comparing assays using the same antibodies. These results suggest AMH was stable despite the thawing episode and years of storage.

Furthermore, we conducted a thawing experiment on samples from ten pre- or post-menopausal women to investigate whether thawing affected AMH levels. All women had detectable AMH values. Six same-sample aliquots per patient were stored at –80°C. Five aliquots from each patient were then thawed up to 11°C for 1, 3, 7, 14 and 21 days, respectively. Then the aliquots were refrozen and stored for a maximum of three weeks before analysis with the Ansh-AMH assay. Results indicated that AMH in the thawed samples were clearly unaffected in comparison to the frozen reference sample. All these results reassured us that we could rely on our stored samples and the values obtained in spite of the freezer error so we proceeded with our study with further inclusion of patients and collection of samples.

The study was complicated by the use of several AMH assays for which there is no international standard. However, we decided to use coefficients of agreement to calculate how well the actual numbers agree with each other. We provided results as a number indicating how many times higher (or lower) values analysed with

one of the assays were compared to the other assay instead of the actual difference because the distribution of measurements was skewed. In addition, this strategy allowed us to obtain an interpretable measure. We obtained the similar agreement regardless of assay used or length of storage time.

Study III

In Study I, we pointed to the very severe impact of radiation and SCT on the ovaries over time. Treatment with alkylating agents given in high cumulative doses is likewise known to have an adverse outcome on ovarian function.

Due to the intensified treatment, including alkylating agents, of young sarcoma patients over the last several years, we wanted to determine what impact this could have on ovarian function. Patients with Ewing sarcoma and osteosarcoma were included in this study and we compared their outcome with Wilms tumour patients, as the latter group is regarded to have less gonadotoxic treatment.

We analysed 198 blood samples from 21 patients diagnosed with Ewing sarcoma, osteosarcoma, and Wilms tumour. The girls with sarcoma had a mean age of 11.2 years (Ewing sarcoma) and 11.8 years (osteosarcoma), and the patients with Wilms tumour, which typically affects young children, had a mean age of 3.9 years.

Results

The 20 patients with a pre-treatment sample had detectable AMH and FSH. As pre-treatment LH was only detectable in 25% of the patients, we focused on AMH and FSH as LH gave no additional information. Inhibin B was not included as no added information was gained from inhibin B in Study I.

We show that AMH at diagnosis was lower in patients with Ewing sarcoma compared with patients with osteosarcoma after adjusting for age. Similarly, AMH was lower in Wilms tumour patients compared with osteosarcoma patients, although not statistically significant.

In study I, we reported a significant decline in AMH after three months of treatment in all 34 patients. One of the patients with detectable AMH value after three months was a Wilms tumour patient. In this study we show that the only patients without dramatic decline in AMH were Wilms tumour patients (not treated with radiation or SCT), which probably reflects the lower intensity and duration of their treatment (Fig. 15).

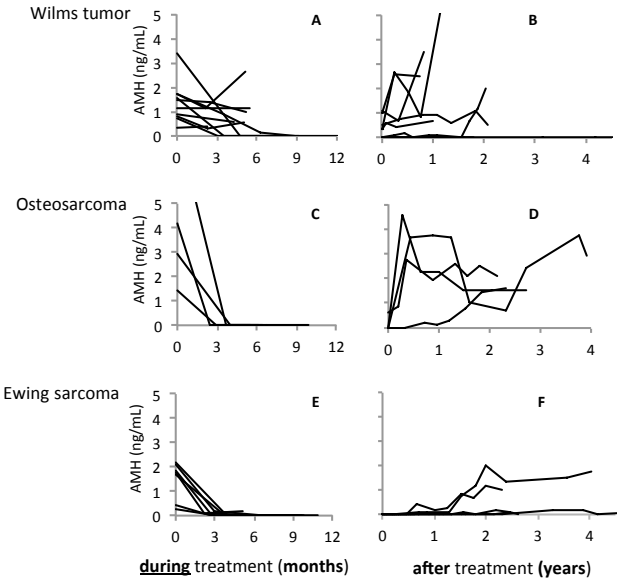

Figure 15. AMH levels before, during and after cancer treatment for Wilms tumour, osteosarcoma and Ewing sarcoma. From Study III.

The three Wilms tumour patients with undetectable AMH at follow-up 2.4 years after completion of treatment were all treated with whole abdominal radiation or SCT. The remaining patients had higher median AMH at the end of follow-up compared with pre-treatment AMH (Fig. 15).

All patients with Ewing sarcoma or osteosarcoma had low or undetectable AMH during treatment. However, AMH increased in all patients with osteosarcoma during follow-up after completion of treatment. In contrast, the Ewing sarcoma patients had very low or undetectable values the first one to two years after treatment (Fig. 15). Two of these patients exhibited a late and slow increase in AMH and interestingly these patients were the two youngest (< 7 years old) sarcoma patients.

To our knowledge, this study is the first to follow girls with the respective diagnoses prospectively regarding AMH during and the first years after treatment. The weakness in this study is the small number of patients. Future studies should include more patients and a substantial length of follow-up to facilitate statistical significance.

Study IV (in manuscript)

Data on fertility after acute leukaemia is based mainly on retrospective studies of female childhood cancer survivors. Only a few studies are published using AMH as a marker for ovarian reserve in female ALL survivors. As all childhood cancer treatments have intensified during the last decades (even for ALL and AML patients) we designed this study to compare patients with ALL and AML regarding ovarian function during and after treatment. In study I, we reported a tendency of a recovery in AMH already during continuous chemotherapy for seven young females with ALL. After gathering data for another three years, we now have data from a larger cohort of girls treated for acute leukaemia. Of the 36 girls included, 29 were diagnosed with ALL and seven with AML. The ALL patients were divided into different risk groups reflecting the treatment intensity; standard risk (SR), intermediate risk (IR), and high risk (HR). The ALL patients were treated for 2½ years and AML patients for approximately ½ year.

Results

All patients, regardless of risk group, had a profound decline in AMH levels after three months of chemotherapy, with an average reduction of 89% of the pre treatment levels. Nevertheless, AMH was detectable but low in 40% of ALL patients. AML patients had AMH levels that were very low or undetectable. During treatment, AMH increased to pre-treatment levels as early as 12 months for ALL SR and ALL IR patients and increased statistically significantly above pre-treatment levels at 18 and 24 months from diagnosis, respectively. ALL HR and all AML patients had low or undetectable values during treatment.

Patients treated for ALL HR or with SCT at relapse had undetectable or very low AMH during the three-year observation period. In patients with AML, AMH reached pre-treatment levels 12 months after treatment. Their AMH levels continued to increase at 24 months, however not statistically significant to pre treatment levels.

Finally, we compared AMH levels at diagnosis with the last obtained sample. The median follow-up time from diagnosis was 3.1 and 3.8 years for patients with ALL SR and ALL IR, respectively, and 3.4 years for AML patients. Patients treated for ALL SR and ALL IR recovered with statistically significantly higher AMH levels when compared with pre-treatment levels. Although AML patients recovered in AMH, they displayed 60% lower AMH levels than those observed in the ALL SR group at last follow-up. Pre treatment AMH was not associated with AMH level at last follow-up.

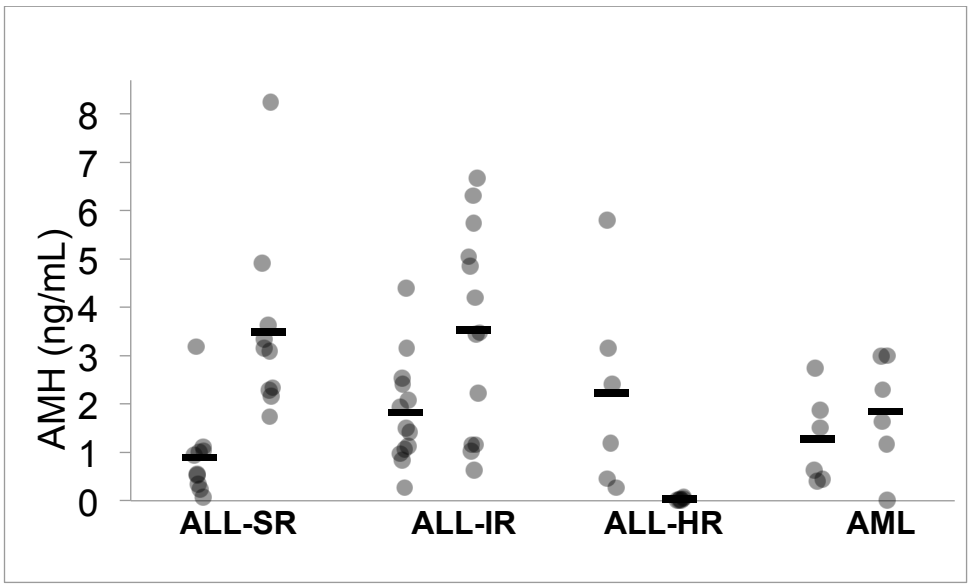

Figure 16. Mean AMH levels at diagnosis and at last follow up for ALL SR, ALL IR, ALL HR, and AML patients. From study IV.

This is the first study to investigate girls with ALL and AML regarding ovarian function during treatment. The data and results allow us to predict that patients with ALL SR and ALL IR probably have intact ovarian function, but more studies are needed to follow them carefully long term. In line with the earlier studies in this thesis, patients treated with HSCT presented AMH values the first years after treatment that indicate severe gonadotoxicity. Regarding AML patients, if their later recovery has any impact on future fertility potential requires long-term follow-up.

Discussion

Knowledge on late complications after childhood cancer, such as impaired fertility, is gained mainly from retrospective studies on adult childhood cancer survivors. When we started our study in 2007, there were no prospective studies published using AMH as a measure of ovarian reserve in either adults or in children. AMH was mainly used by reproduction specialists to evaluate oocyte response after hormonal stimulation in assisted reproduction.

In our daily work as paediatric oncologists or paediatric endocrinologists, we occasionally meet young girls with primary or secondary amenorrhea in the out-patient clinic during follow-up visits. We wanted to know when the negative impact on the ovaries occurs and why (i.e., what treatment protocols were involved). We also wanted to know what to do to avoid complications and how to prevent these negative consequences in future patients. These were the questions that led us to design the studies resulting in this thesis. AMH was not yet investigated in a larger context in children and we therefore designed the prospective study resulting in the data presented here. Simultaneously, we designed a study together with reproduction specialists with the aim to in depth analyse adult women treated for childhood cancer in the southern part of Sweden regarding ovarian function, sexuality, quality of life, osteoporosis, and risk of the metabolic syndrome.

In Lund, as paediatric oncologists, we follow our patients regularly until the age of 18 years before referral to our late effects clinic, with a first appointment three to five years later. There are two questions that need further exploration: 1) Are there any preventive measures that can be undertaken during the supposedly fertile years up until the age of 40 years? 2) Is there a need for intensified surveillance for girls even with low or intermediate risk of premature ovarian insufficiency?

AMH pre-treatment

At diagnosis, AMH was detectable in all the girls included in the studies, which could be expected by the use of modern AMH assays[83,84] and as AMH is measurable in serum from birth.[51,52] We found low pre-treatment AMH levels in girls with Wilms tumour, which most likely reflects their younger age as AMH increases with age up until the age of 25 years (Figs. 7 and 8).[59] After adjustment

for age, pre-treatment AMH values were significantly lower in girls with Ewing sarcoma compared with girls diagnosed with osteosarcoma. A reduced pre-treatment AMH was found in a cohort of childhood cancer patients compared with controls in a study from 2014, a finding that may be related to impaired general health.[191] As Ewing sarcoma is regarded as a systemic disease[192] thus it could affect general health or even exert a direct negative effect on the ovaries via metastatic spread.

It is unclear whether there is a threshold pre-treatment AMH value that protects against toxicity. A study of adult breast cancer patients showed that a pre-treatment AMH > 0.7 ng/ml predicted better ovarian recovery[193] and another study reported AMH > 2.0 ng/ml as a sign of a larger ovarian reserve that could serve as a protection against POI[145]. In our studies pre-treatment AMH did not predict AMH level at last follow-up.

On the other hand, a high AMH value can also indicate polycystic ovary syndrome a condition that impairs fertility and affects 5 to 10% of women.[79] Unlike studies on adults,[194] the large inter-variability in AMH concentration measured in girls, taken together with the fact that AMH increases until the age of 25 years,[59] makes interpretations difficult regarding pre-treatment value and outcome.

AMH during treatment

AMH had a marked decline in the majority of patients after three months of treatment, regardless of pre-treatment AMH, diagnosis, age, menarche, or treatment given. A significant decrease in AMH was found in 17 adult women with breast cancer, lymphoma, or Ewing sarcoma as early as one week after initiation of treatment.[195] Another study reported a significant decline in AMH in 30 young women with lymphoma already after 15 days from start of chemotherapy.[77] In addition, a longitudinal study of 59 women with breast cancer confirmed a marked fall in AMH after two cycles of chemotherapy.[194] All these studies show an acute toxic effect on the ovaries from chemotherapy with depletion of small growing follicles.

Still detectable but low values at this time point were seen in 40% of ALL patients. The values for the majority of the Wilms patients were not as low. The treatment regimen for Wilms is divided into four to six weekly treatments pre-surgery, depending on risk grouping. After pre-surgery treatment, there is usually a period of three weeks waiting for the post surgery pathology report before treatment commences again. ALL treatment starts with weekly induction therapy and continuous glucocorticoids and one could only speculate if a low intensity regimen reflects the AMH values. With a more intense regimen such as the

alkylating containing regimen for Ewing sarcoma, AMH was very low or undetectable during treatment, which was also true for osteosarcoma and ALL HR. Interestingly, AMH started to increase in patients with ALL SR and ALL IR already during the low dose chemotherapy maintenance treatment with a significant increase above baseline at 18 and 24 months, respectively; these results are in line with a recently published study.[196] Treatment for ALL IR is also given during 2½ years, but that treatment is more intense than treatment for ALL SR and includes alkylating agents, which might be reflected in the later recovery.

AMH was very low or undetectable in AML patients during treatment, reflecting the intensive treatment regimens. As expected, AMH was undetectable in patients treated with whole abdominal radiotherapy or SCT during treatment.

AMH post treatment

Ovarian recovery as measured by AMH is highly variable depending on treatment protocols.[77] The recovery observed already during treatment for ALL SR and ALL IR patients was sustained during follow-up, which is line with the prevailing understanding of intact fertility in these patients. When comparing the last sample at follow-up with pre-treatment AMH, there was a significant increase at three years after treatment for both these risk groups of ALL. Patients with ALL HR or treated with HSCT at relapse did not recover in AMH during the three year long observation period. AML patients receive a shorter (six months) intensive pulsed treatment but without alkylating agents, and their recovery in AMH was substantially delayed compared with patients with ALL SR or ALL IR. A recovery to pre-treatment values at 12 months after treatment was seen. Their AMH levels continued to increase at 24 months, however not statistically significant to pre treatment levels. Is the delay in recovery due to a more severe depletion including primordial follicles or does the delay reflect the time in folliculogenesis, the time between primary follicle and larger AMH producing follicles? Earlier studies did not find a significantly reduced fertility in AML patients unless treated with SCT.[197,198]

Cancer treatment affects growing follicles, induces cortical fibrosis and causes vascular toxicity in the ovaries.[144,149] Treatment with alkylating agents such as e.g. cyclophosphamide or procarbazine in high cumulative doses has shown to impact future fertility. Significantly, studies have reported higher AMH levels in patients not receiving alkylating agents[195] as well as low or undetectable levels after treatment with alkylator-containing regimens.[194,199-201] Similarly, no recovery in AMH is seen during follow-up in females treated with radiation involving the ovaries.[78] One study found that undetectable AMH two years post treatment predicted no return of menses.[202]

Alkylating agents such as ifosfamide or cyclophosphamide included in the treatment of Ewing sarcoma act on both quiescent oocytes and the dividing granulosa cells.[142] When the treatment depletes ovaries of small growing follicles and thereby AMH, the inhibiting effect of AMH on recruitment of primordial follicles is lost and recruitment is hastened, resulting in an overall diminished primordial follicle pool.[203]

The studies comprising this thesis show that if the toxic effect from cancer treatment on the ovaries is truly profound, it seems to cause near complete depletion of follicles. This is reflected in the non-apparent recovery in AMH during follow-up after treatment for Ewing sarcoma, ALL HR, and those patients treated with radiation (whole abdominal or TBI) or SCT. These patients are considered to have a high risk of POI.

However, if the effect of treatment on the ovaries is less or moderate, a recovery in AMH is seen. It is difficult to predict whether a proportion of these patients are still at risk of POI. Girls treated for osteosarcoma, Wilms tumour (without radiation or SCT), ALL SR, ALL IR and AML all fall into this category. Careful long-term follow-up is essential (especially after the use of alkylating-containing treatments), because ovarian reserve can be compromised even when a regular menstrual cycle is evident.

Limitations of the studies

Our paediatric oncology centre is one of six in Sweden and these centres have a relatively low number of annual patients. To gather an appropriate number of patients (and therefore useable data and statistically significant results) requires many years of inclusion of patients. In addition, we were unable to follow-up patients at other departments or at local hospitals and samples from these patients would therefore not be taken.

Not to be regarded as limitations, but rather as complications were the freezer error that possibly could have compromised the samples and also the shift of AMH assays. The use of several assays in this study, for which there is no international standard, reflects the era when technical companies developed AMH assays independently, which can complicate a longitudinal study.

Conclusions

- AMH declines dramatically in all patients after three months of treatment, with the exception of a fraction of Wilms tumour patients, regardless of treatment, age, menarche, and AMH at diagnosis.
- Chemotherapy exerts an acute negative effect on ovarian function.
- Radiation with the ovaries in the field and/or stem cell transplantation have a severe negative impact on the ovaries in the short and long term.
- Young females treated for Ewing sarcoma are at high risk of prolonged ovarian dysfunction up to at least three years after cessation of treatment.
- Ovarian reserve seems less compromised in ALL patients compared with AML patients, unless exposed to high risk treatment or SCT.
- The recovery of ovarian function after treatment is delayed in AML patients; to determine whether this has impact on the ovarian reserve and furure fertility will require careful long-term follow-up.
- Length of storage of samples and accidental thawing of samples up to 11°C for up to 21 days did not negatively impact AMH results.

Future perspectives

The cure of childhood cancer has a cost; two-thirds of survivors experience late-complications, a cost that needs to be reduced. We must address the quality of lives saved and continue to improve treatment protocols with the aim to reduce toxicity and late complications without compromising survival rates. For patients requiring radiotherapy, it is possible to reduce late complications by replacing photons with protons where applicable. Accordingly, alternative replacement of known severe gonadotoxic agents as exemplified in the Hodgkin lymphoma regimen without jeopardizing survival should be considered with a careful and systematic approach.

We should raise awareness of the need and possibilities of fertility preservation and discuss preventive measures for those at risk of premature ovarian insufficiency as early as at diagnosis. Expanded resources for reproduction medicine will be needed to meet the increased demand for fertility preservation for

young females diagnosed and treated for cancer. Advances in research on in vitro maturation of primordial follicles could preclude the need for auto-transplantation of ovarian tissue and thus reduce the risk of transferring malignant cells.

Indeed, we have the opportunity and the responsibility to ensure the quality of lives saved. In general, we need to emphasise the need for long-term follow-up and make it available to every survivor per national and international guidelines, guidelines that need to be continuously updated.

Originating from the studies in this thesis the work could be continued and developed in future studies in the following ways:

- Increase sampling to weekly or monthly during treatment for patient groups at risk to enable a better understanding of the impact of treatment on AMH and thereby the ovaries
- Continue sampling and analyses of AMH in the existing cohort for a long-term follow-up
- Include other centres in Sweden and the Nordic countries to gather a larger cohort that would facilitate significant conclusions regarding e.g. a defined toxic cumulative dose of akylating and alkylating-like agents.
- Analysis of existing samples from our cohort for AMH polymorphism for a better understanding of genetic causes of inter-personal variance in the susceptibility of POI.
- Include prospective investigations of new anti-cancer medications recently introduced e.g., tyrosine kinase inhibitors and different antibody and immunomodulating treatments of which there is no data on potential adverse effects on the ovaries.
- Increased surveillance of young female cancer survivors with FSH and E2 in pre-pubertal children and FSH and AMH in post-pubertal children/young adults annually/biannually up to the age of 35 years with the aim to determine risk of POI in time for fertility interventions.

During the preparation of my research project ahead of my registration as a PhD student, my research plan constituted of two projects: the prospective part, which has now resulted in this thesis and a retrospective part. The second project - a retrospective study of adult female childhood cancer survivors treated in the southern part of Sweden - was initiated by and took place at the Reproductive Medicine Centre in Malmö, Sweden. A total of 167 women agreed to participate by providing blood samples and taking part in a physical exam. The examination gathered data on blood pressure, BMI, waist-hip ratio, and bone density. The participants also provided information about their sexuality and quality of life.

Data from the investigated cohort and an equal number of controls will be analysed soon. My focus will be on evaluating a potential increased risk of POI, osteoporosis, and the metabolic syndrome in relation to diagnosis, age at diagnosis, and treatment received.

Populärvetenskaplig sammanfattning

Behovet av långsiktig uppföljning efter barncancer är stort. Cancerbehandlingens påverkan på fertilitet hos unga flickor är ett område under ständig utveckling sedan det visat sig att vissa som behandlats för cancer senare inte kunnat få barn. Målet med studierna i denna avhandling var att studera äggstockarnas funktion före, under och efter behandling för cancer och korrelera resultaten till diagnos och behandling. När vi påbörjade vår studie hade liknande studier på unga flickor inte gjorts tidigare.

Bakgrund

Idag överlever över 80 procent av de barn och ungdomar under 18 år som insjuknar i cancer. Överlevnaden har ökat dramatiskt sedan 1960-talet. Detta tillskrivs en utvecklad diagnostik och förbättrad behandling där kombinationen av kirurgi, cytostatika och i vissa fall strålning är essentiell, samt även en förbättrad så kallad supportive care. Med supportive care menas omhändertagande av den svårt sjuka patienten med t.ex. behandling mot infektioner, transfusioner av blod och blodprodukter samt möjlighet till intensivvård.

Idag beräknas 1/1000 vuxna i de nordiska länderna ha överlevt cancer i barn och ungdomsåren. Men ökad överlevnad kan ha ett pris i form av biverkningar till följd av cancersjukdomen och dess behandling. Dessa biverkningar som kan uppstå många år efter avslutad behandling, kallas ofta sena effekter eller sena komplikationer. De kan drabba i princip alla organsystem i kroppen och påverka tex njurar, hjärta, hörsel, skelett och kognitiv förmåga. De vanligaste sena effekterna är endokrina (hormonella) eller har med fortplantning att göra och drabbar ca 40% av de som behandlats för cancer som unga. En konsekvens av cancerbehandling i unga år kan således vara påverkan på äggstockarna och deras funktion. Detta kan visa sig på många olika sätt från tex. att puberteten påverkas, till ett för tidigt klimakterium ledande till infertilitet.

Flickor föds med bestämt antal äggceller, ca 1–2 miljoner vid födelsen. Dessa förnyas inte utan minskar gradvis under livet, till ca 500 000 vid första menstruation och 1000 vid klimakteriet som i genomsnitt inträffar i en ålder av 51

år. Fertiliteten är starkt nedsatt ca 10 år innan en kvinna kommer i klimakteriet och menstruationerna upphör. Cancerbehandling med vissa cytostatika och/eller strålning med äggstockarna i strålfältet kan påverka äggstockarna med åtföljande minskning av antalet äggceller i varierande grad. Beroende på graden av påverkan kan det visa sig direkt efter behandlingen genom att äggstockarna slutar att fungera eller senare med att klimakteriet startar många år tidigare än normalt. Detta medför inte bara nedsatt fertilitet tidigare än normalt men också brist på det kvinnliga könshormonet östrogen. Östrogen har många viktiga funktioner och brist kan på sikt leda till benskörhet och ökad risk för hjärt-kärl sjukdom.

Tidigare studier visar att de flesta flickor som behandlas för cancer inte kommer att ha några problem med sin fertilitet. De flickor som framför allt är i riskzonen är de som har fått strålbehandling med äggstockarna i strålfältet eller genomgått högdosbehandling med stamcellsstöd (HSCT).

Antalet och kvaliteten på de kvarvarande äggcellerna i äggstockarna kallas för äggstocksreserv eller ovariell reserv. Den mest använda metoden för att utvärdera äggstocksreserven är att mäta antalet äggceller (av en viss storlek) genom ett vaginalt ultraljud i kombination med blodprov för follikelstimulerande hormon (FSH) och östrogen. På senare år har det visats att AMH, anti-Mülleriskt hormon som bildas i stödjeceller runt äggcellerna och kan mätas i ett blodprov också avspeglar äggstocksreserven på ett tillförlitligt sätt.

Barnonkologin hade inte varit där den är idag utan nationellt och internationellt samarbete med deltagande och registrering i forskningsstudier. Detta gäller inte bara utvecklingen av cancerbehandling utan också uppföljningen efteråt av eventuella sena komplikationer. År 2007 påbörjade vi vår studie av äggstockarnas funktion mätt med AMH, före, under och efter cancerbehandling hos flickor upp till 18 års ålder. Vid den tidpunkten fanns det ingen sådan studie om AMH hos flickor med cancer. De totalt 104 flickor som har inkluderats i studien har lämnat blodprov vid diagnos var tredje månad i tre år och därefter årligen.

Delstudie I

När vi hade samlat prover i drygt 3 år behövde vi göra en första interimsanalys för att utvärdera om resultaten kunde motivera fortsatt insamling. Vi analyserade prover från 34 flickor med olika cancerdiagnoser. Då vi inte hade så lång uppföljningstid fokuserade vi på AMH under första delen av behandlingen.

Resultaten visade att AMH hade sjunkit drastiskt från utgångsvärdet vid diagnos och till 3 månader in i behandlingen hos alla flickorna. Det vill säga att behandlingen gav en akut påverkan på äggstockarna, oavsett vilken cytostatikabehandling som gavs. Vi såg också att flickor med akut lymfatisk leukemi återhämtade sina AMH värden under den mindre intensiva så kallade underhållsbehandlingen. Detta till skillnad från de som fått högdosbehandling med

efterföljande stamcellsstöd (HSCT) eller de som strålats med äggstockarna i strålfältet där vi inte såg någon återhämtning av AMH under uppföljningstiden.

Delstudie II

På våren 2013 fick vi problem med vår frys vilket ledde till ofrivillig upptining av proverna från –80°C till 11°C under upp till 21 dagar. För att ta reda på om vi kunde lita på fortsatta analyser planerade vi denna studie där vi på nytt analyserade de prover som utsatts för fryshaveri och jämförde värdena med mätningar gjorda 2011, före fryshaveri. I mellantiden hade laboratoriet bytt analysmetod för AMH, varför uppnådda värden inte kunde jämföras direkt, men vi fick en god korrelation mellan de båda analysmetoderna. Vi kontrollerade även om prover för FSH och luteiniserande hormon (LH), mätta med samma metod före och efter fryshaveri, vilket visade en utmärkt korrelation. Av detta drog vi slutsaten att skillnaden i AMH före respektive efter fryshaveri berodde på metodbyte och inte upptining av prover. Detta stärktes ytterligare av ett tiningsexperiment som utfördes på prover från 10 vuxna kvinnor. Av sex prover från varje kvinna behölls ett i frysen, de andra fem tinades i 1, 3, 7, 14 respektive 21 dagar innan de frystes ner igen före analys. Proverna visade oförändrade resultat oavsett om, eller hur länge de tinats. Två års längre förvaring i frysen påverkade inte heller provresultaten.

Delstudie III

Delstudie III redovisar äggstocksfunktion mätt med AMH på flickor under och efter behandling för skelettumörerna osteosarkom respektive Ewing sarkom i jämförelse med flickor med njurtumör, så kallad Wilms tumör. Vi ville jämföra dessa diagnosgrupper då det i behandlingen för framför allt Ewing sarkom ingår cytostatika som kan påverka äggstocksfunktion, vilket inte är fallet i standardbehandling för Wilms tumör, såvida inte strålbehandling mot njuren behöver ges.

Vi fann att AMH sjönk eller var omätbart under behandling hos alla med osteosarkom eller Ewing sarkom. Endast de i gruppen med Wilms tumör som fått strålbehandling eller HSCT hade kraftigt påverkade värden under behandling, dessa återhämtade sig inte heller i AMH efter avslutad behandling. AMH-värden för flickor med osteosarkom steg relativt snabbt efter avslutad behandling, i motsats till de med Ewing sarkom där endast två av sju hade en återhämtning två år efter avslutad behandling. Vi redovisar få patienter med en uppföljningstid på 3½ år, men våra resultat pekar på en kraftigt påverkad ovariefunktion även efter behandling för Ewing sarkom i tillägg till de patienter som strålbehandlats eller fått HSCT.

Delstudie IV (i manuskript)

Här redovisar vi äggstocksfunktion mätt med AMH hos 36 flickor under och efter behandling för akut myeloisk (AML) respektive akut lymfatisk leukemi (ALL). Behandlingen för AML är mer intensiv, men är i gengäld kortare, ca sex månader mot 2½ år för ALL. ALL-behandlingen är olika intensiv beroende på riskgrupp: standardrisk (SR), intermediär risk (IR) eller högrisk (HR).

Liksom i studierna ovan sjönk AMH vid kontrollen tre månader in i behandling, dock mer uttalat för dem med AML. Patienter med ALL SR och IR återhämtade sig i AMH, jämfört med utgångsprovet redan vid 18 respektive 24 månader in i den 30 månader långa behandlingen. De som behandlats för ALL HR eller med HSCT återhämtade sig inte i AMH under den tre år långa observationstiden. Patienter med AML hade kraftigt påverkat AMH under behandling som steg först tolv månader efter avslutad behandling, dock inte över sitt utgångsvärde, inte heller efter 24 månader. Vår slutsats är att ovariefunktionen inte påverkas avsevärt, förutom för dem med ALL HR alternativt de som får högdosbehandling med stamcellsstöd. AML patienter uppvisar en senare återhämtning i AMH och uppföljning över längre tid är viktig för att utvärdera om detta har konsekvenser för deras äggstocksfunktion och fertilitet på sikt.

Slutsatser

Flickor och unga kvinnor som har behandlats för cancer har en ökad risk för prematur ovariell insufficiens – ett för tidigt klimakterie. Detta kan leda till infertilitet innan barnafödande har planerats. Tidigare studier har visat att detta framför allt gäller de som har fått strålning mot sina äggstockar eller genomgått högdosbehandling med stamcellsstöd. Våra studier pekar på att denna påverkan på äggstockarna kommer redan tidigt, akut i förloppet. Våra resultat visar också att de som har fått intensiv behandling med höga doser av så kallade alkylerande cytostatika, som vid Ewing sarkom, också verkar ha samma tidiga akuta och i flera fall kvarstående påverkan. Sannolikt leder inte behandling mot akut lymfatisk eller myeloisk leukemi till infertilitet, undantaget de som behandlas med högdosbehandling och efterföljande stamcellsstöd.

Vår slutsats är att det är mycket viktigt med långsiktig noggrann uppföljning efter genomgången cancerbehandling för att hitta tecken på sviktande äggstocksfunktion i tid. De flickor som genom sin behandling anses ha en hög risk för infertilitet bör informeras och erbjudas fertilitetsbevarande åtgärder redan vid diagnos.

Acknowledgements

I would like to express my deepest gratitude to everyone along the way who has contributed to the work that has resulted in this thesis.

First, a very special thank you to all the girls that participated in this study; without your contributions there would not have been any data to analyse and no results to present – to you I am truly and sincerely grateful.

Ingrid, my supervisor. Thank you for your endless support, patience, positive energy and belief in science as the most precious and fantastic activity of all. When I had my doubts, you always persuaded me to move on, forward.

Maria, my co-supervisor.Thanks to you (and Pål Wölner-Hanssen) this project was launched in 2007, without you there would not have been any studies to perform or publish. I thank you for your support over these many years. You have a very gentle personality- much appreciated at difficult times.

Jacek Toporski, Katarina Johnsson and Johan Svahn current and former directors of Department of Paediatrics. Thank you for facilitating the opportunity to carry out research.

Thanks also to all my colleagues in the Paediatric Haematology and Oncology team who made it possible for me to take time off from the clinical work to do research and also to finally finish this thesis. And thank you for creating an environment, a team spirit, I fully enjoy.

A very warm thank you also goes to the following people:

Ingrid Hagelin for supervising the study and keeping track of sampling and samples.

Katarina Lindahl for being *the* most helpful and serviceminded person I know.

Patrik, my room mate, I thank you for all the laughter and also much-needed technical support (also from Georg).

The CNS team for our teamwork! Charlotte and Marie also for being dear friends of mine.

Karin N for friendship and joining me in cultural events.

Petra, Vickan, Eva my very dear supportive friends i ¨vått och torrt¨ - 24/7

Magnus & Charlotte for providing friendship, endless dinner invitations, interesting discussions on small and big issues and last but not least; tee times at Falsterbo GK.

Ann for a long-lasting friendship since forty years.

Anna and Cecilia for good times on and off the golf course

Marianne for our friendship and introducing me to the world of the Lusitanos. I hope there will be many more trips to Portugal.

Anne-Marie and Christian, my dear sister and brother (with families), for your support and encouragement.

All my old *Stockholm buddies* for being there, even after my 24 years in Skåne (I still count myself as a Stockholmare).

My extended family: Leif, David, Josefin och Christina, we go back for decades. David and Josefin, I am really looking forward to follow what lies ahead of you. I am convinced it will be a fabulous journey!

Theo och Hanna -the most precious of all, my wonderful children of whom I am very proud. For you I only wish the very best!

Financial support

The work presented in this thesis was supported by grants from the Swedish Childhood Cancer Foundation, the Swedish Cancer Society, Skåne University Hospital Donation Fund, the Skåne County Council Research and Development Foundation, the Danish Cancer Society, and the Danish Medical Research Council.

References

1. https://www.cancer.org/cancer/cancer-basics/history-of-cancer/what-is-cancer.html.
2. https://www.cceg.ki.se/index.php?file=research&tab=research.
3. Birch JM, Marsden HB. A classification scheme for childhood cancer. *Int J Cancer.* 1987;40(5):620-624.
4. https://www.barncancerfonden.se/globalassets/global/forskning/pagaende-satsningar/childhoodcancerincidenceandsurvivalinsweden1984_2010.pdf.
5. https://www.cancer.org/cancer/cancer-in-children/risk-factors-and-causes.html.
6. Stiller CA. Epidemiology and genetics of childhood cancer. *Oncogene.* 2004;23(38):6429-6444.
7. Moore SW. Developmental genes and cancer in children. *Pediatric blood & cancer.* 2009;52(7):755-760.
8. Narod SA, Stiller C, Lenoir GM. An estimate of the heritable fraction of childhood cancer. *Br J Cancer.* 1991;63(6):993-999.
9. DeBaun MR, Tucker MA. Risk of cancer during the first four years of life in children from The Beckwith-Wiedemann Syndrome Registry. *J Pediatr.* 1998;132(3 Pt 1):398-400.
10. Magnusson S, Borg A, Kristoffersson U, Nilbert M, Wiebe T, Olsson H. Higher occurrence of childhood cancer in families with germline mutations in BRCA2, MMR and CDKN2A genes. *Fam Cancer.* 2008;7(4):331-337.
11. Bhatia S, Sklar C. Second cancers in survivors of childhood cancer. *Nat Rev Cancer.* 2002;2(2):124-132.
12. Bhatia S. Therapy-related myelodysplasia and acute myeloid leukemia. *Semin Oncol.* 2013;40(6):666-675.
13. http://www.pbs.org/newshour/updates/emil-grubbe-first-use-radiation-treat-breast-cancer/.
14. Pearson HA. History of pediatric hematology oncology. *Pediatr Res.* 2002;52(6):979-992.

15. Goodman LS, Wintrobe MM, et al. Nitrogen mustard therapy; use of methyl-bis (beta-chloroethyl) amine hydrochloride and tris (beta-chloroethyl) amine hydrochloride for Hodgkin's disease, lymphosarcoma, leukemia and certain allied and miscellaneous disorders. *J Am Med Assoc.* 1946;132:126-132.
16. DeVita VT, Jr., Chu E. A history of cancer chemotherapy. *Cancer Res.* 2008;68(21):8643-8653.
17. Willoughby ML, Laurie HC. Cyclic chemotherapy in childhood acute leukaemia. *Arch Dis Child.* 1968;43(228):187-194.
18. Gatta G, Zigon G, Capocaccia R, et al. Survival of European children and young adults with cancer diagnosed 1995-2002. *European journal of cancer.* 2009;45(6):992-1005.
19. Smith MA, Seibel NL, Altekruse SF, et al. Outcomes for children and adolescents with cancer: challenges for the twenty-first century. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* 2010;28(15):2625-2634.
20. Johnson J, Canning J, Kaneko T, Pru JK, Tilly JL. Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature.* 2004;428(6979):145-150.
21. Block E. A quantitative morphological investigation of the follicular system in newborn female infants. *Acta Anat (Basel).* 1953;17(3):201-206.
22. Durlinger AL, Kramer P, Karels B, et al. Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse ovary. *Endocrinology.* 1999;140(12):5789-5796.
23. Broekmans FJ, Visser JA, Laven JS, Broer SL, Themmen AP, Fauser BC. Anti-Mullerian hormone and ovarian dysfunction. *Trends Endocrinol Metab.* 2008;19(9):340-347.
24. McGee EA, Hsueh AJ. Initial and cyclic recruitment of ovarian follicles. *Endocrine reviews.* 2000;21(2):200-214.
25. Faddy MJ, Gosden RG, Gougeon A, Richardson SJ, Nelson JF. Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. *Human reproduction.* 1992;7(10):1342-1346.
26. Wallace WH, Kelsey TW. Human ovarian reserve from conception to the menopause. *PloS one.* 2010;5(1):e8772.
27. Durlinger AL, Kramer P, Karels B, Grootegoed JA, Uilenbroek JT, Themmen AP. Apoptotic and proliferative changes during

induced atresia of pre-ovulatory follicles in the rat. *Human reproduction.* 2000;15(12):2504-2511.
28. de Bruin JP, Dorland M, Spek ER, et al. Ultrastructure of the resting ovarian follicle pool in healthy young women. *Biol Reprod.* 2002;66(4):1151-1160.
29. Choi JY, Jo MW, Lee EY, Yoon BK, Choi DS. The role of autophagy in follicular development and atresia in rat granulosa cells. *Fertility and sterility.* 2010;93(8):2532-2537.
30. Chapman C, Cree L, Shelling AN. The genetics of premature ovarian failure: current perspectives. *Int J Womens Health.* 2015;7:799-810.
31. Broekmans FJ, Faddy MJ, Scheffer G, te Velde ER. Antral follicle counts are related to age at natural fertility loss and age at menopause. *Menopause.* 2004;11(6 Pt 1):607-614.
32. Treloar AE. Menstrual cyclicity and the pre-menopause. *Maturitas.* 1981;3(3-4):249-264.
33. te Velde ER, Pearson PL. The variability of female reproductive ageing. *Human reproduction update.* 2002;8(2):141-154.
34. Shelling AN. Premature ovarian failure. *Reproduction.* 2010;140(5):633-641.
35. Snieder H, MacGregor AJ, Spector TD. Genes control the cessation of a woman's reproductive life: a twin study of hysterectomy and age at menopause. *The Journal of clinical endocrinology and metabolism.* 1998;83(6):1875-1880.
36. de Bruin JP, Bovenhuis H, van Noord PA, et al. The role of genetic factors in age at natural menopause. *Human reproduction.* 2001;16(9):2014-2018.
37. Lambalk CB, van Disseldorp J, de Koning CH, Broekmans FJ. Testing ovarian reserve to predict age at menopause. *Maturitas.* 2009;63(4):280-291.
38. Findlay JK, Hutt KJ, Hickey M, Anderson RA. What is the "ovarian reserve"? *Fertility and sterility.* 2015;103(3):628-630.
39. Scheffer GJ, Broekmans FJ, Looman CW, et al. The number of antral follicles in normal women with proven fertility is the best reflection of reproductive age. *Human reproduction.* 2003;18(4):700-706.
40. van Rooij IA, Broekmans FJ, te Velde ER, et al. Serum anti-Mullerian hormone levels: a novel measure of ovarian reserve. *Human reproduction.* 2002;17(12):3065-3071.

41. Hansen KR, Hodnett GM, Knowlton N, Craig LB. Correlation of ovarian reserve tests with histologically determined primordial follicle number. *Fertility and sterility.* 2011;95(1):170-175.
42. Andersen CY, Schmidt KT, Kristensen SG, Rosendahl M, Byskov AG, Ernst E. Concentrations of AMH and inhibin-B in relation to follicular diameter in normal human small antral follicles. *Human reproduction.* 2010;25(5):1282-1287.
43. Jeppesen JV, Anderson RA, Kelsey TW, et al. Which follicles make the most anti-Mullerian hormone in humans? Evidence for an abrupt decline in AMH production at the time of follicle selection. *Molecular human reproduction.* 2013;19(8):519-527.
44. Dunlop CE, Anderson RA. Uses of anti-Mullerian hormone (AMH) measurement before and after cancer treatment in women. *Maturitas.* 2015;80(3):245-250.
45. Jost A. The age factor in the castration of male rabbit fetuses. *Proc Soc Exp Biol Med.* 1947;66(2):302.
46. Josso N, Cate RL, Picard JY, et al. Anti-mullerian hormone: the Jost factor. *Recent Prog Horm Res.* 1993;48:1-59.
47. Matzuk MM, Lamb DJ. The biology of infertility: research advances and clinical challenges. *Nat Med.* 2008;14(11):1197-1213.
48. Cate RL, Mattaliano RJ, Hession C, et al. Isolation of the bovine and human genes for Mullerian inhibiting substance and expression of the human gene in animal cells. *Cell.* 1986;45(5):685-698.
49. Fanchin R, Schonauer LM, Righini C, Guibourdenche J, Frydman R, Taieb J. Serum anti-Mullerian hormone is more strongly related to ovarian follicular status than serum inhibin B, estradiol, FSH and LH on day 3. *Human reproduction.* 2003;18(2):323-327.
50. van Rooij IA, Broekmans FJ, Scheffer GJ, et al. Serum antimullerian hormone levels best reflect the reproductive decline with age in normal women with proven fertility: a longitudinal study. *Fertility and sterility.* 2005;83(4):979-987.
51. Rajpert-De Meyts E, Jorgensen N, Graem N, Muller J, Cate RL, Skakkebaek NE. Expression of anti-Mullerian hormone during normal and pathological gonadal development: association with differentiation of Sertoli and granulosa cells. *The Journal of clinical endocrinology and metabolism.* 1999;84(10):3836-3844.

52. Hagen CP, Aksglaede L, Sorensen K, et al. Serum levels of anti-Mullerian hormone as a marker of ovarian function in 926 healthy females from birth to adulthood and in 172 Turner syndrome patients. *The Journal of clinical endocrinology and metabolism.* 2010;95(11):5003-5010.
53. Visser JA, Schipper I, Laven JS, Themmen AP. Anti-Mullerian hormone: an ovarian reserve marker in primary ovarian insufficiency. *Nat Rev Endocrinol.* 2012;8(6):331-341.
54. Durlinger AL, Gruijters MJ, Kramer P, et al. Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. *Endocrinology.* 2002;143(3):1076-1084.
55. Weenen C, Laven JS, Von Bergh AR, et al. Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. *Molecular human reproduction.* 2004;10(2):77-83.
56. Anderson RA, Wallace WH. Antimullerian hormone, the assessment of the ovarian reserve, and the reproductive outcome of the young patient with cancer. *Fertility and sterility.* 2013;99(6):1469-1475.
57. La Marca A, Spada E, Sighinolfi G, et al. Age-specific nomogram for the decline in antral follicle count throughout the reproductive period. *Fertility and sterility.* 2011;95(2):684-688.
58. Lie Fong S, Visser JA, Welt CK, et al. Serum anti-mullerian hormone levels in healthy females: a nomogram ranging from infancy to adulthood. *The Journal of clinical endocrinology and metabolism.* 2012;97(12):4650-4655.
59. Kelsey TW, Wright P, Nelson SM, Anderson RA, Wallace WH. A validated model of serum anti-mullerian hormone from conception to menopause. *PloS one.* 2011;6(7):e22024.
60. Sowers MR, Eyvazzadeh AD, McConnell D, et al. Anti-mullerian hormone and inhibin B in the definition of ovarian aging and the menopause transition. *The Journal of clinical endocrinology and metabolism.* 2008;93(9):3478-3483.
61. Seifer DB, Golub ET, Lambert-Messerlian G, et al. Variations in serum mullerian inhibiting substance between white, black, and Hispanic women. *Fertility and sterility.* 2009;92(5):1674-1678.
62. Tal R, Seifer DB. Potential mechanisms for racial and ethnic differences in antimullerian hormone and ovarian reserve. *International journal of endocrinology.* 2013;2013:818912.

63. Freour T, Masson D, Dessolle L, et al. Ovarian reserve and in vitro fertilization cycles outcome according to women smoking status and stimulation regimen. *Arch Gynecol Obstet.* 2012;285(4):1177-1182.
64. Steiner AZ, Stanczyk FZ, Patel S, Edelman A. Antimullerian hormone and obesity: insights in oral contraceptive users. *Contraception.* 2010;81(3):245-248.
65. Freeman EW, Gracia CR, Sammel MD, Lin H, Lim LC, Strauss JF, 3rd. Association of anti-mullerian hormone levels with obesity in late reproductive-age women. *Fertility and sterility.* 2007;87(1):101-106.
66. Bentzen JG, Forman JL, Pinborg A, et al. Ovarian reserve parameters: a comparison between users and non-users of hormonal contraception. *Reproductive biomedicine online.* 2012;25(6):612-619.
67. Kristensen SL, Ramlau-Hansen CH, Andersen CY, et al. The association between circulating levels of antimullerian hormone and follicle number, androgens, and menstrual cycle characteristics in young women. *Fertility and sterility.* 2012;97(3):779-785.
68. Senates E, Colak Y, Erdem ED, et al. Serum anti-Mullerian hormone levels are lower in reproductive-age women with Crohn's disease compared to healthy control women. *J Crohns Colitis.* 2013;7(2):e29-34.
69. Ma W, Zhan Z, Liang X, Chen J, Huang X, Liao C. Subclinical impairment of ovarian reserve in systemic lupus erythematosus patients with normal menstruation not using alkylating therapy. *J Womens Health (Larchmt).* 2013;22(12):1023-1027.
70. La Marca A, Stabile G, Artenisio AC, Volpe A. Serum anti-Mullerian hormone throughout the human menstrual cycle. *Human reproduction.* 2006;21(12):3103-3107.
71. Hagen CP, Vestergaard S, Juul A, et al. Low concentration of circulating antimullerian hormone is not predictive of reduced fecundability in young healthy women: a prospective cohort study. *Fertility and sterility.* 2012;98(6):1602-1608 e1602.
72. Nelson SM. Biomarkers of ovarian response: current and future applications. *Fertility and sterility.* 2013;99(4):963-969.
73. Broer SL, van Disseldorp J, Broeze KA, et al. Added value of ovarian reserve testing on patient characteristics in the

prediction of ovarian response and ongoing pregnancy: an individual patient data approach. *Human reproduction update.* 2013;19(1):26-36.
74. Rey R, Sabourin JC, Venara M, et al. Anti-Mullerian hormone is a specific marker of sertoli- and granulosa-cell origin in gonadal tumors. *Hum Pathol.* 2000;31(10):1202-1208.
75. Freeman EW, Sammel MD, Lin H, Gracia CR. Anti-mullerian hormone as a predictor of time to menopause in late reproductive age women. *The Journal of clinical endocrinology and metabolism.* 2012;97(5):1673-1680.
76. Brougham MF, Crofton PM, Johnson EJ, Evans N, Anderson RA, Wallace WH. Anti-Mullerian hormone is a marker of gonadotoxicity in pre- and postpubertal girls treated for cancer: a prospective study. *The Journal of clinical endocrinology and metabolism.* 2012;97(6):2059-2067.
77. Decanter C, Morschhauser F, Pigny P, Lefebvre C, Gallo C, Dewailly D. Anti-Mullerian hormone follow-up in young women treated by chemotherapy for lymphoma: preliminary results. *Reproductive biomedicine online.* 2010;20(2):280-285.
78. Lie Fong S, Laven JS, Hakvoort-Cammel FG, et al. Assessment of ovarian reserve in adult childhood cancer survivors using anti-Mullerian hormone. *Human reproduction.* 2009;24(4):982-990.
79. Franks S. Polycystic ovary syndrome in adolescents. *Int J Obes (Lond).* 2008;32(7):1035-1041.
80. Bath LE, Wallace WH, Shaw MP, Fitzpatrick C, Anderson RA. Depletion of ovarian reserve in young women after treatment for cancer in childhood: detection by anti-Mullerian hormone, inhibin B and ovarian ultrasound. *Human reproduction.* 2003;18(11):2368-2374.
81. Baker ML, Metcalfe SA, Hutson JM. Serum levels of mullerian inhibiting substance in boys from birth to 18 years, as determined by enzyme immunoassay. *The Journal of clinical endocrinology and metabolism.* 1990;70(1):11-15.
82. Long WQ, Ranchin V, Pautier P, et al. Detection of minimal levels of serum anti-Mullerian hormone during follow-up of patients with ovarian granulosa cell tumor by means of a highly sensitive enzyme-linked immunosorbent assay. *The Journal of clinical endocrinology and metabolism.* 2000;85(2):540-544.

83. Su HI, Sammel MD, Homer MV, Bui K, Haunschild C, Stanczyk FZ. Comparability of antimullerian hormone levels among commercially available immunoassays. *Fertility and sterility.* 2014;101(6):1766-1772 e1761.
84. Kumar A, Kalra B, Patel A, McDavid L, Roudebush WE. Development of a second generation anti-Mullerian hormone (AMH) ELISA. *Journal of immunological methods.* 2010;362(1-2):51-59.
85. Wallace AM, Faye SA, Fleming R, Nelson SM. A multicentre evaluation of the new Beckman Coulter anti-Mullerian hormone immunoassay (AMH Gen II). *Annals of clinical biochemistry.* 2011;48(Pt 4):370-373.
86. Li HW, Ng EH, Wong BP, Anderson RA, Ho PC, Yeung WS. Correlation between three assay systems for anti-Mullerian hormone (AMH) determination. *Journal of assisted reproduction and genetics.* 2012;29(12):1443-1446.
87. Rustamov O, Smith A, Roberts SA, et al. Anti-Mullerian hormone: poor assay reproducibility in a large cohort of subjects suggests sample instability. *Human reproduction.* 2012;27(10):3085-3091.
88. Zuvela E, Walls M, Matson P. Within-laboratory and between-laboratory variability in the measurement of anti-mullerian hormone determined within an external quality assurance scheme. *Reprod Biol.* 2013;13(3):255-257.
89. Rustamov O, Smith A, Roberts SA, et al. The measurement of anti-Mullerian hormone: a critical appraisal. *The Journal of clinical endocrinology and metabolism.* 2014;99(3):723-732.
90. Fleming R, Fairbairn C, Blaney C, Lucas D, Gaudoin M. Stability of AMH measurement in blood and avoidance of proteolytic changes. *Reproductive biomedicine online.* 2013;26(2):130-132.
91. Kevenaar ME, Meerasahib MF, Kramer P, et al. Serum anti-mullerian hormone levels reflect the size of the primordial follicle pool in mice. *Endocrinology.* 2006;147(7):3228-3234.
92. McDade TW, Woodruff TK, Huang YY, et al. Quantification of anti-Mullerian hormone (AMH) in dried blood spots: validation of a minimally invasive method for assessing ovarian reserve. *Human reproduction.* 2012;27(8):2503-2508.
93. Gatta G, Capocaccia R, Stiller C, et al. Childhood cancer survival trends in Europe: a EUROCARE Working Group study. *Journal of*

clinical oncology : official journal of the American Society of Clinical Oncology. 2005;23(16):3742-3751.
94. Olsen JH, Moller T, Anderson H, et al. Lifelong cancer incidence in 47,697 patients treated for childhood cancer in the Nordic countries. *Journal of the National Cancer Institute.* 2009;101(11):806-813.
95. Oeffinger KC, Mertens AC, Sklar CA, et al. Chronic health conditions in adult survivors of childhood cancer. *The New England journal of medicine.* 2006;355(15):1572-1582.
96. Geenen MM, Cardous-Ubbink MC, Kremer LC, et al. Medical assessment of adverse health outcomes in long-term survivors of childhood cancer. *JAMA : the journal of the American Medical Association.* 2007;297(24):2705-2715.
97. Armstrong GT, Kawashima T, Leisenring W, et al. Aging and risk of severe, disabling, life-threatening, and fatal events in the childhood cancer survivor study. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* 2014;32(12):1218-1227.
98. Hudson MM, Ness KK, Gurney JG, et al. Clinical ascertainment of health outcomes among adults treated for childhood cancer. *JAMA : the journal of the American Medical Association.* 2013;309(22):2371-2381.
99. de Fine Licht S, Winther JF, Gudmundsdottir T, et al. Hospital contacts for endocrine disorders in Adult Life after Childhood Cancer in Scandinavia (ALiCCS): a population-based cohort study. *Lancet.* 2014;383(9933):1981-1989.
100. Chemaitilly W, Sklar CA. Endocrine complications in long-term survivors of childhood cancers. *Endocr Relat Cancer.* 2010;17(3):R141-159.
101. Gudmundsdottir T, Winther JF, de Fine Licht S, et al. Cardiovascular disease in Adult Life after Childhood Cancer in Scandinavia: A population-based cohort study of 32,308 one-year survivors. *Int J Cancer.* 2015;137(5):1176-1186.
102. Knijnenburg SL, Mulder RL, Schouten-Van Meeteren AY, et al. Early and late renal adverse effects after potentially nephrotoxic treatment for childhood cancer. *Cochrane Database Syst Rev.* 2013(10):CD008944.
103. Whelan K, Stratton K, Kawashima T, et al. Auditory complications in childhood cancer survivors: a report from the

childhood cancer survivor study. *Pediatric blood & cancer.* 2011;57(1):126-134.
104. Mulhern RK, Merchant TE, Gajjar A, Reddick WE, Kun LE. Late neurocognitive sequelae in survivors of brain tumours in childhood. *The Lancet. Oncology.* 2004;5(7):399-408.
105. Krull KR, Zhang N, Santucci A, et al. Long-term decline in intelligence among adult survivors of childhood acute lymphoblastic leukemia treated with cranial radiation. *Blood.* 2013;122(4):550-553.
106. Gawade PL, Hudson MM, Kaste SC, et al. A systematic review of selected musculoskeletal late effects in survivors of childhood cancer. *Curr Pediatr Rev.* 2014;10(4):249-262.
107. Kaste SC, Karimova EJ, Neel MD. Osteonecrosis in children after therapy for malignancy. *AJR Am J Roentgenol.* 2011;196(5):1011-1018.
108. Friedman DL, Whitton J, Leisenring W, et al. Subsequent neoplasms in 5-year survivors of childhood cancer: the Childhood Cancer Survivor Study. *Journal of the National Cancer Institute.* 2010;102(14):1083-1095.
109. Kondapalli LA, Dillon KE, Sammel MD, et al. Quality of life in female cancer survivors: is it related to ovarian reserve? *Qual Life Res.* 2014;23(2):585-592.
110. Zebrack BJ, Casillas J, Nohr L, Adams H, Zeltzer LK. Fertility issues for young adult survivors of childhood cancer. *Psychooncology.* 2004;13(10):689-699.
111. Conway GS. Premature ovarian failure. *Br Med Bull.* 2000;56(3):643-649.
112. Coulam CB, Adamson SC, Annegers JF. Incidence of premature ovarian failure. *Obstet Gynecol.* 1986;67(4):604-606.
113. Anasti JN. Premature ovarian failure: an update. *Fertility and sterility.* 1998;70(1):1-15.
114. Cramer DW, Xu H, Harlow BL. Family history as a predictor of early menopause. *Fertility and sterility.* 1995;64(4):740-745.
115. Persani L, Rossetti R, Cacciatore C. Genes involved in human premature ovarian failure. *J Mol Endocrinol.* 2010;45(5):257-279.
116. Nelson LM. Clinical practice. Primary ovarian insufficiency. *The New England journal of medicine.* 2009;360(6):606-614.

117. Green DM, Sklar CA, Boice JD, Jr., et al. Ovarian failure and reproductive outcomes after childhood cancer treatment: results from the Childhood Cancer Survivor Study. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* 2009;27(14):2374-2381.
118. Sklar CA, Mertens AC, Mitby P, et al. Premature menopause in survivors of childhood cancer: a report from the childhood cancer survivor study. *Journal of the National Cancer Institute.* 2006;98(13):890-896.
119. Bondy CA, Turner Syndrome Study G. Care of girls and women with Turner syndrome: a guideline of the Turner Syndrome Study Group. *The Journal of clinical endocrinology and metabolism.* 2007;92(1):10-25.
120. Sherman SL. Premature ovarian failure in the fragile X syndrome. *Am J Med Genet.* 2000;97(3):189-194.
121. Guerrero NV, Singh RH, Manatunga A, Berry GT, Steiner RD, Elsas LJ, 2nd. Risk factors for premature ovarian failure in females with galactosemia. *J Pediatr.* 2000;137(6):833-841.
122. Panay N, Kalu E. Management of premature ovarian failure. *Best Pract Res Clin Obstet Gynaecol.* 2009;23(1):129-140.
123. Morrison JC, Givens JR, Wiser WL, Fish SA. Mumps oophoritis: a cause of premature menopause. *Fertility and sterility.* 1975;26(7):655-659.
124. Conway GS, Kaltsas G, Patel A, Davies MC, Jacobs HS. Characterization of idiopathic premature ovarian failure. *Fertility and sterility.* 1996;65(2):337-341.
125. La Marca A, Brozzetti A, Sighinolfi G, Marzotti S, Volpe A, Falorni A. Primary ovarian insufficiency: autoimmune causes. *Curr Opin Obstet Gynecol.* 2010;22(4):277-282.
126. Araki S, Chikazawa K, Sekiguchi I, Yamauchi H, Motoyama M, Tamada T. Arrest of follicular development in a patient with 17 alpha-hydroxylase deficiency: folliculogenesis in association with a lack of estrogen synthesis in the ovaries. *Fertility and sterility.* 1987;47(1):169-172.
127. Latronico AC, Anasti J, Arnhold IJ, et al. Brief report: testicular and ovarian resistance to luteinizing hormone caused by inactivating mutations of the luteinizing hormone-receptor gene. *The New England journal of medicine.* 1996;334(8):507-512.

128. Aittomaki K, Lucena JL, Pakarinen P, et al. Mutation in the follicle-stimulating hormone receptor gene causes hereditary hypergonadotropic ovarian failure. *Cell.* 1995;82(6):959-968.
129. Welt CK. Primary ovarian insufficiency: a more accurate term for premature ovarian failure. *Clinical endocrinology.* 2008;68(4):499-509.
130. De Vos M, Devroey P, Fauser BC. Primary ovarian insufficiency. *Lancet.* 2010;376(9744):911-921.
131. Nelson LM, Covington SN, Rebar RW. An update: spontaneous premature ovarian failure is not an early menopause. *Fertility and sterility.* 2005;83(5):1327-1332.
132. Tsuji I, Ami K, Fujinami N. Pregnancy following ovarian induction in a patient with premature ovarian failure and undetectable serum anti-Mullerian hormone. *J Obstet Gynaecol Res.* 2013;39(5):1070-1072.
133. Raffi F, Metwally M, Amer S. The impact of excision of ovarian endometrioma on ovarian reserve: a systematic review and meta-analysis. *The Journal of clinical endocrinology and metabolism.* 2012;97(9):3146-3154.
134. Farquhar CM, Sadler L, Harvey SA, Stewart AW. The association of hysterectomy and menopause: a prospective cohort study. *BJOG.* 2005;112(7):956-962.
135. Cramer DW, Xu H, Harlow BL. Does "incessant" ovulation increase risk for early menopause? *Am J Obstet Gynecol.* 1995;172(2 Pt 1):568-573.
136. Lass A. The fertility potential of women with a single ovary. *Human reproduction update.* 1999;5(5):546-550.
137. Bjelland EK, Wilkosz P, Tanbo TG, Eskild A. Is unilateral oophorectomy associated with age at menopause? A population study (the HUNT2 Survey). *Human reproduction.* 2014;29(4):835-841.
138. Podfigurna-Stopa A, Czyzyk A, Grymowicz M, et al. Premature ovarian insufficiency: the context of long-term effects. *J Endocrinol Invest.* 2016;39(9):983-990.
139. De Vos M, Smitz J, Woodruff TK. Fertility preservation in women with cancer. *Lancet.* 2014;384(9950):1302-1310.
140. Meirow D, Nugent D. The effects of radiotherapy and chemotherapy on female reproduction. *Human reproduction update.* 2001;7(6):535-543.

141. Rendtorff R, Hohmann C, Reinmuth S, et al. Hormone and Sperm Analyses after Chemo- and Radiotherapy in Childhood and Adolescence. *Klin Padiatr.* 2010;222(3):145-149.
142. Morgan S, Anderson RA, Gourley C, Wallace WH, Spears N. How do chemotherapeutic agents damage the ovary? *Human reproduction update.* 2012;18(5):525-535.
143. Oktem O, Oktay K. Quantitative assessment of the impact of chemotherapy on ovarian follicle reserve and stromal function. *Cancer.* 2007;110(10):2222-2229.
144. Ben-Aharon I, Shalgi R. What lies behind chemotherapy-induced ovarian toxicity? *Reproduction.* 2012;144(2):153-163.
145. Dillon KE, Sammel MD, Prewitt M, et al. Pretreatment antimullerian hormone levels determine rate of posttherapy ovarian reserve recovery: acute changes in ovarian reserve during and after chemotherapy. *Fertility and sterility.* 2013;99(2):477-483.
146. Metzger ML, Meacham LR, Patterson B, et al. Female reproductive health after childhood, adolescent, and young adult cancers: guidelines for the assessment and management of female reproductive complications. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* 2013;31(9):1239-1247.
147. Thomas-Teinturier C, Allodji RS, Svetlova E, et al. Ovarian reserve after treatment with alkylating agents during childhood. *Human reproduction.* 2015;30(6):1437-1446.
148. Chow EJ, Stratton KL, Leisenring WM, et al. Pregnancy after chemotherapy in male and female survivors of childhood cancer treated between 1970 and 1999: a report from the Childhood Cancer Survivor Study cohort. *The Lancet. Oncology.* 2016;17(5):567-576.
149. Meirow D, Dor J, Kaufman B, et al. Cortical fibrosis and blood-vessels damage in human ovaries exposed to chemotherapy. Potential mechanisms of ovarian injury. *Human reproduction.* 2007;22(6):1626-1633.
150. Ben-Aharon I, Bar-Joseph H, Tzarfaty G, et al. Doxorubicin-induced ovarian toxicity. *Reproductive biology and endocrinology : RB&E.* 2010;8:20.
151. Choo YC, Chan SY, Wong LC, Ma HK. Ovarian dysfunction in patients with gestational trophoblastic neoplasia treated with

short intensive courses of etoposide (VP-16-213). *Cancer.* 1985;55(10):2348-2352.
152. Swerdlow AJ, Cooke R, Bates A, et al. Risk of premature menopause after treatment for Hodgkin's lymphoma. *Journal of the National Cancer Institute.* 2014;106(9).
153. Meirow D. Reproduction post-chemotherapy in young cancer patients. *Molecular and cellular endocrinology.* 2000;169(1-2):123-131.
154. Green DM, Nolan VG, Goodman PJ, et al. The cyclophosphamide equivalent dose as an approach for quantifying alkylating agent exposure: a report from the Childhood Cancer Survivor Study. *Pediatric blood & cancer.* 2014;61(1):53-67.
155. Wallace WH, Anderson RA, Irvine DS. Fertility preservation for young patients with cancer: who is at risk and what can be offered? *The Lancet. Oncology.* 2005;6(4):209-218.
156. Larsen EC, Muller J, Schmiegelow K, Rechnitzer C, Andersen AN. Reduced ovarian function in long-term survivors of radiation- and chemotherapy-treated childhood cancer. *The Journal of clinical endocrinology and metabolism.* 2003;88(11):5307-5314.
157. Wallace WH, Thomson AB, Kelsey TW. The radiosensitivity of the human oocyte. *Human reproduction.* 2003;18(1):117-121.
158. Damewood MD, Grochow LB. Prospects for fertility after chemotherapy or radiation for neoplastic disease. *Fertility and sterility.* 1986;45(4):443-459.
159. Wallace WH, Thomson AB, Saran F, Kelsey TW. Predicting age of ovarian failure after radiation to a field that includes the ovaries. *International journal of radiation oncology, biology, physics.* 2005;62(3):738-744.
160. Appelman-Dijkstra NM, Kokshoorn NE, Dekkers OM, et al. Pituitary dysfunction in adult patients after cranial radiotherapy: systematic review and meta-analysis. *The Journal of clinical endocrinology and metabolism.* 2011;96(8):2330-2340.
161. Littley MD, Shalet SM, Beardwell CG, Robinson EL, Sutton ML. Radiation-induced hypopituitarism is dose-dependent. *Clinical endocrinology.* 1989;31(3):363-373.
162. Critchley HO, Wallace WH, Shalet SM, Mamtora H, Higginson J, Anderson DC. Abdominal irradiation in childhood; the potential for pregnancy. *Br J Obstet Gynaecol.* 1992;99(5):392-394.

163. Green DM, Peabody EM, Nan B, Peterson S, Kalapurakal JA, Breslow NE. Pregnancy outcome after treatment for Wilms tumor: a report from the National Wilms Tumor Study Group. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* 2002;20(10):2506-2513.
164. http://oncofertility.northwestern.edu/resources/assessing-ovarian-reserve-after-cancer-treatments.
165. Wright KD, Green DM, Daw NC. Late effects of treatment for wilms tumor. *Pediatr Hematol Oncol.* 2009;26(6):407-413.
166. http://vavnad.se/wp-content/uploads/2016/11/framjande-av-reproduktionsformaga-unga-v1-00.pdf.
167. Leporrier M, von Theobald P, Roffe JL, Muller G. A new technique to protect ovarian function before pelvic irradiation. Heterotopic ovarian autotransplantation. *Cancer.* 1987;60(9):2201-2204.
168. Fawcett SL, Gomez AC, Barter SJ, Ditchfield M, Set P. More harm than good? The anatomy of misguided shielding of the ovaries. *Br J Radiol.* 2012;85(1016):e442-447.
169. Bedaiwy MA, Abou-Setta AM, Desai N, et al. Gonadotropin-releasing hormone analog cotreatment for preservation of ovarian function during gonadotoxic chemotherapy: a systematic review and meta-analysis. *Fertility and sterility.* 2011;95(3):906-914 e901-904.
170. Del Mastro L, Boni L, Michelotti A, et al. 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. *JAMA : the journal of the American Medical Association.* 2011;306(3):269-276.
171. Moore HC, Unger JM, Phillips KA, et al. Goserelin for ovarian protection during breast-cancer adjuvant chemotherapy. *The New England journal of medicine.* 2015;372(10):923-932.
172. Gosden R. Cryopreservation: a cold look at technology for fertility preservation. *Fertility and sterility.* 2011;96(2):264-268.
173. Oktay K, Buyuk E, Veeck L, et al. Embryo development after heterotopic transplantation of cryopreserved ovarian tissue. *Lancet.* 2004;363(9412):837-840.

174. Oktay K, Karlikaya G. Ovarian function after transplantation of frozen, banked autologous ovarian tissue. *The New England journal of medicine.* 2000;342(25):1919.
175. Resetkova N, Hayashi M, Kolp LA, Christianson MS. Fertility Preservation for Prepubertal Girls: Update and Current Challenges. *Curr Obstet Gynecol Rep.* 2013;2(4):218-225.
176. Klüver Jensen A MK, Fedder J, Ernst E, Yding Andersen C. 86 successful births and 9 on-going pregnancies worldwide in women transplanted with cryopreserved frozen-thawed ovarian tissue: focus on birth and perinatal outcome in 40 of these children births. *J. Assisted Reprod. Genetic (in press).*
177. Rodriguez-Wallberg KA, Karlstrom PO, Rezapour M, et al. Full-term newborn after repeated ovarian tissue transplants in a patient treated for Ewing sarcoma by sterilizing pelvic irradiation and chemotherapy. *Acta Obstet Gynecol Scand.* 2015;94(3):324-328.
178. http://www.bbc.com/news/health-38312995.
179. Demeestere I, Simon P, Dedeken L, et al. Live birth after autograft of ovarian tissue cryopreserved during childhood. *Human reproduction.* 2015;30(9):2107-2109.
180. Dolmans MM, Iwahara Y, Donnez J, et al. Evaluation of minimal disseminated disease in cryopreserved ovarian tissue from bone and soft tissue sarcoma patients. *Human reproduction.* 2016;31(10):2292-2302.
181. Dolmans MM, Luyckx V, Donnez J, Andersen CY, Greve T. Risk of transferring malignant cells with transplanted frozen-thawed ovarian tissue. *Fertility and sterility.* 2013;99(6):1514-1522.
182. Dolmans MM, Marinescu C, Saussoy P, Van Langendonckt A, Amorim C, Donnez J. Reimplantation of cryopreserved ovarian tissue from patients with acute lymphoblastic leukemia is potentially unsafe. *Blood.* 2010;116(16):2908-2914.
183. Abir R, Feinmesser M, Yaniv I, et al. Occasional involvement of the ovary in Ewing sarcoma. *Human reproduction.* 2010;25(7):1708-1712.
184. http://www.survivorshipguidelines.org.
185. http://www.cancercentrum.se/samverkan/cancerdiagnoser/barn/vardprogram/.
186. http://www.sign.ac.uk/pdf/sign132.pdf.

187. http://www.uhb.nhs.uk/Downloads/pdf/CancerPbTherapyBasedLongTermFollowUp.pdf.
188. https://www.siope.eu/wp-content/uploads/2013/09/6.-The-Survivorship-Passport.pdf.
189. Hjorth L, Haupt R, Skinner R, et al. Survivorship after childhood cancer: PanCare: a European Network to promote optimal long-term care. *European journal of cancer.* 2015;51(10):1203-1211.
190. van Dorp W, Mulder RL, Kremer LC, et al. Recommendations for Premature Ovarian Insufficiency Surveillance for Female Survivors of Childhood, Adolescent, and Young Adult Cancer: A Report From the International Late Effects of Childhood Cancer Guideline Harmonization Group in Collaboration With the PanCareSurFup Consortium. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* 2016;34(28):3440-3450.
191. van Dorp W, van den Heuvel-Eibrink MM, de Vries AC, et al. Decreased serum anti-Mullerian hormone levels in girls with newly diagnosed cancer. *Human reproduction.* 2014;29(2):337-342.
192. Schleiermacher G, Peter M, Oberlin O, et al. Increased risk of systemic relapses associated with bone marrow micrometastasis and circulating tumor cells in localized ewing tumor. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* 2003;21(1):85-91.
193. Su HC, Haunschild C, Chung K, et al. Prechemotherapy antimullerian hormone, age, and body size predict timing of return of ovarian function in young breast cancer patients. *Cancer.* 2014;120(23):3691-3698.
194. Anderson RA, Rosendahl M, Kelsey TW, Cameron DA. Pretreatment anti-Mullerian hormone predicts for loss of ovarian function after chemotherapy for early breast cancer. *European journal of cancer.* 2013;49(16):3404-3411.
195. Rosendahl M, Andersen CY, la Cour Freiesleben N, Juul A, Lossl K, Andersen AN. Dynamics and mechanisms of chemotherapy-induced ovarian follicular depletion in women of fertile age. *Fertility and sterility.* 2010;94(1):156-166.
196. Gupta AA, Lee Chong A, Deveault C, et al. Anti-Mullerian Hormone in Female Adolescent Cancer Patients Before, During,

and After Completion of Therapy: A Pilot Feasibility Study. *J Pediatr Adolesc Gynecol.* 2016;29(6):599-603.
197. Mulrooney DA, Dover DC, Li S, et al. Twenty years of follow-up among survivors of childhood and young adult acute myeloid leukemia: a report from the Childhood Cancer Survivor Study. *Cancer.* 2008;112(9):2071-2079.
198. Liesner RJ, Leiper AD, Hann IM, Chessells JM. Late effects of intensive treatment for acute myeloid leukemia and myelodysplasia in childhood. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* 1994;12(5):916-924.
199. Anders C, Marcom PK, Peterson B, et al. A pilot study of predictive markers of chemotherapy-related amenorrhea among premenopausal women with early stage breast cancer. *Cancer investigation.* 2008;26(3):286-295.
200. Anderson RA, Themmen AP, Al-Qahtani A, Groome NP, Cameron DA. The effects of chemotherapy and long-term gonadotrophin suppression on the ovarian reserve in premenopausal women with breast cancer. *Human reproduction.* 2006;21(10):2583-2592.
201. Henry NL, Xia R, Schott AF, McConnell D, Banerjee M, Hayes DF. Prediction of postchemotherapy ovarian function using markers of ovarian reserve. *The oncologist.* 2014;19(1):68-74.
202. Chai J, Howie AF, Cameron DA, Anderson RA. A highly-sensitive anti-Mullerian hormone assay improves analysis of ovarian function following chemotherapy for early breast cancer. *European journal of cancer.* 2014;50(14):2367-2374.
203. Carlsson IB, Scott JE, Visser JA, Ritvos O, Themmen AP, Hovatta O. Anti-Mullerian hormone inhibits initiation of growth of human primordial ovarian follicles in vitro. *Human reproduction.* 2006;21(9):2223-2227.