Determinants of gonadal function after childhood cancer

Determinanten van gonadale functie na kinderkanker

Wendy van Dorp

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DETERMINANTS OF GONADAL FUNCTION AFTER CHILDHOOD CANCER

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Promotoren:	Prof.dr. J.S.E. Laven
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Paranimfen:	Y.V. Louwers
	S. Lie Fong

Contents

Chapter 1	General introduction	7
Chapter 2	Long-term endocrine side effects of childhood Hodgkin's lymphoma treatment: a review	25
Chapter 3	Decreased serum anti-Müllerian hormone levels in girls with newly diagnosed cancer	55
Chapter 4	Characteristics and outcome of paediatric non-Hodgkin lymphoma patients with ovarian infiltration at presentation	65
Chapter 5	Decreased ovarian function is associated with obesity in very long-term female survivors of childhood cancer	79
Chapter 6	Genetic variation may modify ovarian reserve in female childhood cancer survivors	95
Chapter 7	Recommendations for primary ovarian insufficiency surveillance for female childhood cancer survivors: a report from the international late effects of childhood cancer guideline harmonization group	111
Chapter 8	Treatment and pregnancy outcome of oocyte donation in a Dutch single-centre cohort	149
Chapter 9	Obesity independently influences gonadal function in very long-term adult male survivors of childhood cancer	165
Chapter 10	Gonadal function recovery in very long-term male survivors of childhood cancer	179
Chapter 11	Electroejaculation as a method of fertility preservation in boys diagnosed with cancer: a single-centre experience and review of the literature	193

Chapter 12	General discussion and perspectives	**207**
Chapter 13	Summary	**224**
	Samenvatting	**227**
Addendum	Authors and affiliations	**232**
	List of abbreviations	**234**
	Bibliography	**237**
	PhD portfolio	**240**
	About the author	**243**
	Dankwoord	**244**

CHAPTER 1

General introduction

1.1 | Childhood cancer and survival

Cancer is one of the most common causes of death in children aged 1–18 years in developed countries [1,2]. In the Netherlands, 600–700 children are diagnosed with cancer every year [3]. Among all age groups, one third is diagnosed with leukaemia, followed by solid tumours and tumours of the brain or central nervous system (Figure I).

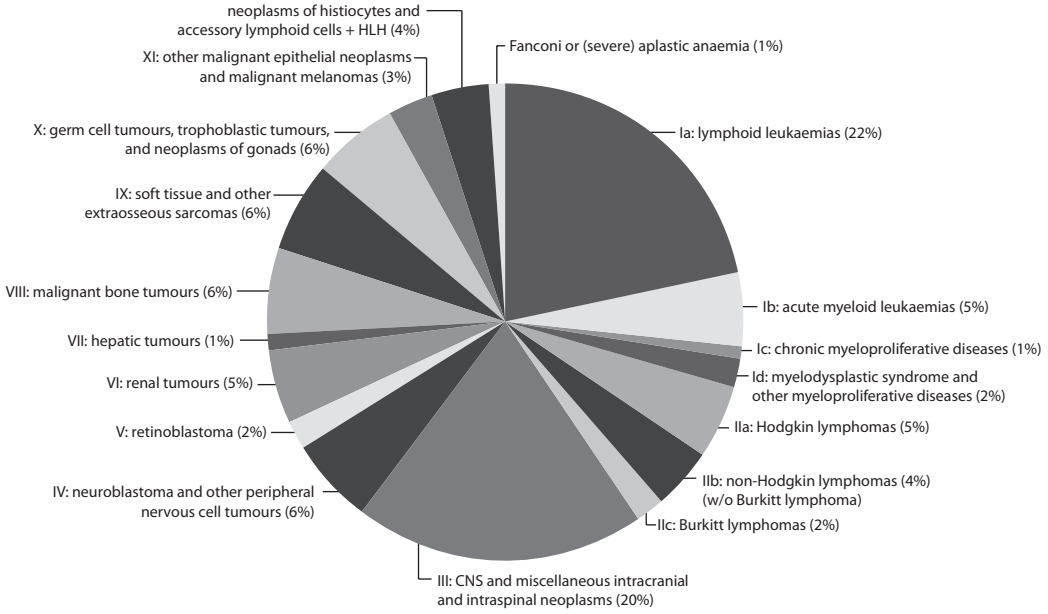

Figure I | Incidence of childhood cancer in the Netherlands in 2011.

w/o=without; CNS=central nervous system; HLH=haemophagocytic lymphohistiocytosis. Modified from SKION Basisregistratie, 2011.

Overall long-term survival of childhood cancer has steadily increased up to 75%, owing to optimised treatment regimens over the past 40 years (Figure II) [3]. As a result, approximately 7,000 long-term survivors of childhood cancer are currently living in the Netherlands. Due to the rapid expansion of the childhood cancer survivor population as well as their increased age, chronic middle- and long-term health sequelae resulting from cancer and its treatment have become more prevalent and thus require our attention [4].

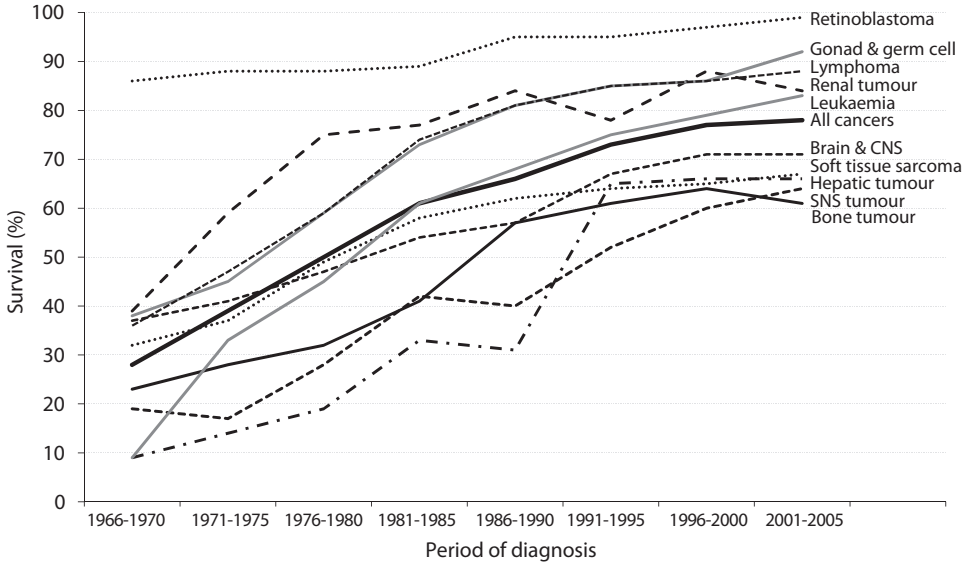

Figure II | Five-year survival by cancer type in children 0−14 years.
CNS=central nervous system; SNS=sympathetic nervous system. Modified from Stiller *et al*, 2007.

Long-term side effects of childhood cancer treatment

Mortality and morbidity rates are higher among five-year survivors of childhood cancer compared with the general population. Mortality among these survivors significantly increases over time until approximately 18% at 30 years after diagnosis compared to <5% in the general US population [5]. Treatment regimens including irradiation − especially high dose cranial and total body irradiation – as well as the use of anthracyclines and alkylating agents are associated with higher mortality rates [6,7]. In addition, treatment-related complications such as secondary malignancies and cardiac disease are associated with a significantly increased mortality risk up to 25 years after initial diagnosis [6,8].

Besides the increased mortality risk, approximately 75% of the five-year survivors have developed at least one long-term complication of their cancer treatment. One-third of these effects has even been classified as severe or life threatening [9,10]. Late effects are commonly reported in adult survivors and demonstrate an increasing prevalence with longer time elapsed from diagnosis [4]. The occurrence of late effects depends on former cancer diagnosis, and more importantly on how it was treated. Secondary malignancies are more prevalent in female than in male survivors, especially due to the relatively high incidence of breast cancer in Hodgkin lymphoma survivors treated with mantle field radiotherapy [7,11,12]. This has led to a change in the treatment regimen, in which mantle field irradiation is now replaced by involved field techniques, involved node irradiation or even chemotherapy only. Other examples of treatment-related complications are anthracycline-related cardiomyopathy and congestive heart failure, irradiation-related pulmonary toxicity and chronic kidney disease related to nephrectomy, abdominal irradiation and nephrotoxic agents.

Endocrine disorders in survivors of childhood cancer are the most common late effects, affecting 20–50% of this population [4]. Especially survivors treated with cranial, abdominal or total body irradiation or alkylating agents are at risk due to the treatment effects on the hypothalamus, pituitary gland and/or endocrine organs [13]. In particular gonadal dysfunction and its subsequent decreased fertility have frequently been reported.

1.2 | Female gonadal function

Fertility is amongst other factors determined by the ovarian reserve, defined by the actual number of primordial follicles in both ovaries [14]. A maximum number of 6–7 million oogonia is attained by 16–20 weeks gestation [15-17]. From that stage onwards, the total cortical content of germ cells falls to 1–2 million at birth as a result of a prenatal oocyte depletion (Figure III) [15,18]. Because of this fixed initial endowment of germ cells, the new-born female enters life, still far from the period at which she will gain the full reproductive potential, having lost already 80% of her primordial follicles with their oocytes [19].

At the onset of puberty, 400,000 to 500,000 primordial follicles remain [16,17,20], hence again another 1.7 million oocytes were lost form birth to puberty onwards. During a woman's reproductive life span, the number of follicles decreases gradually at a fixed rate, until the age of 35–40 years. From this point onwards, follicle loss seems to be accelerated, and finally, the primordial follicle pool is exhausted as menopause is reached, when only a few thousand remain [21].

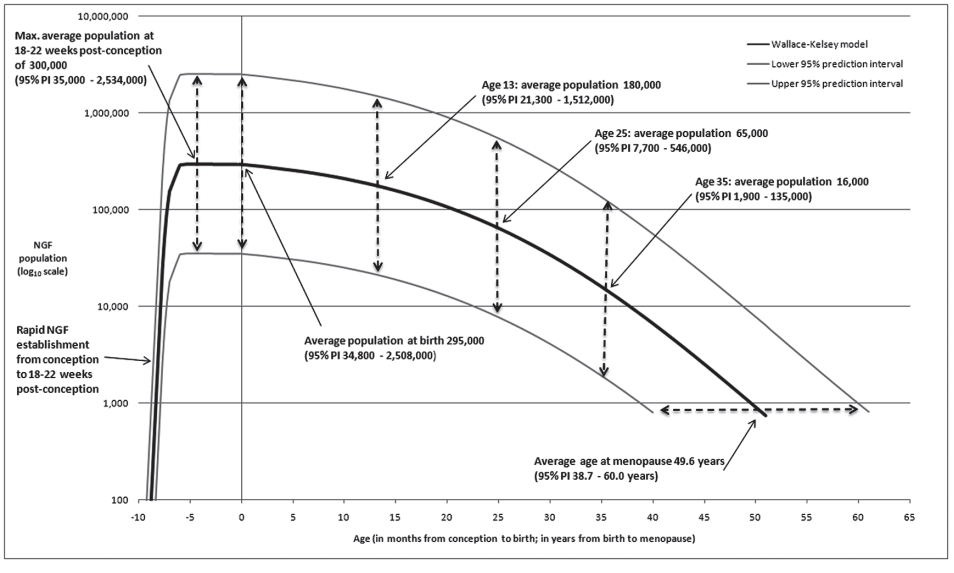

Figure III | The Wallace-Kelsey model of non-growing follicle populations from conception to menopause. This figure gives illustrative examples of NGF populations predicted by this model. At ages 20 weeks, birth, 13 years, 25 years and 35 years the average NGF population is given, together with the respective 95% prediction intervals.

NGF=non-growing follicle; PI=prediction interval (adapted, with permission, from Wallace and Kelsey, PloS One, 2010).

Normal reproductive function involves monthly follicle development, ovulation and preparation of the endometrium for implantation [22]. Both gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are produced in the anterior pituitary gland and play a principal role in the regulation of ovarian function. Their secretion is regulated by gonadotropin-releasing hormone (GnRH), a decapeptide secreted by the neurons of one of the basal nuclei in the hypothalamus. Both gonadotropins are necessary for follicular development and steroid hormone production in the ovary. In turn, oestrogens and inhibins regulate gonadotropin secretion and GnRH pulsatility by positive and negative feedback during the menstrual cycle (Figure IV).

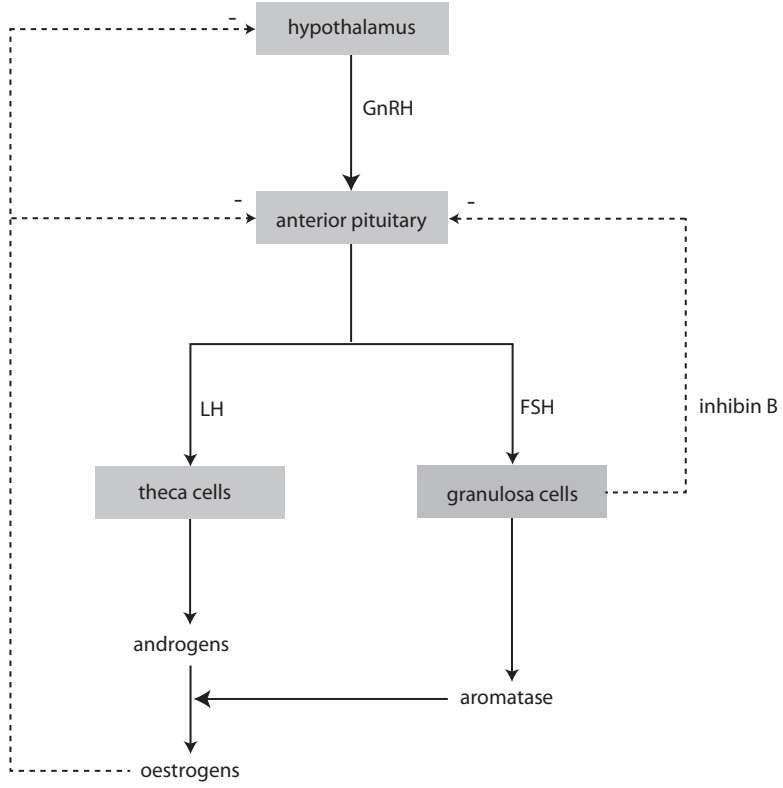

Figure IV | Female hypothalamic-pituitary-gonadal axis.
GnRH=gonadotropin-releasing hormone; LH=luteinizing hormone; FSH=follicle-stimulating hormone.

The interval extending from the late luteal decline in oestradiol as well as progesterone production to the selection of the dominant follicle is critical and decisive (Figure V) [23]. Due to the demise of the corpus luteum and the subsequent decrease in oestrogen and inhibin A production [24], FSH levels rise at the end of the luteal phase of the human menstrual cycle [25]. Initiation of growth of primordial follicles occurs continuously and in a random fashion. Follicle growth will eventually end

and follicles will enter atresia if the appropriate endocrine signal is lacking. Each growing follicle has a threshold for FSH. This so-called FSH threshold for a given follicle is not fixed but depends on its developmental stage. The threshold level should be surpassed to ensure ongoing follicle development. However, due to the profound rise in inhibin B serum levels during the early follicular phase, FSH serum concentrations tend to fall at the end of the early follicular phase. Hence, inhibin B limits the duration of the FSH rise, and thereby narrowing the FSH window i.e. the time span during which the FSH concentration is above the threshold, through negative feedback at the pituitary level. As a consequence the remaining circulating FSH levels are only enough to maintain growth of the largest follicle and this will lead to monofollicular growth which is typical in humans. In other words FSH serum levels steadily decrease during the mid- to late follicular phase of the menstrual cycle. The follicle that has gained dominance is less dependent on continued support by high early follicular phase FSH levels. Circulating FSH levels are suppressed to a concentration below the threshold for remaining follicles from the recruited cohort. These follicles will therefore cease to mature and undergo atresia. Hence, development of the most mature follicle secures selection of a single dominant follicle [26].

(Early) menopause and primary ovarian insufficiency

In the European population, the age at natural menopause varies between 40 and 60 years, with a median of 52 years, and is largely heritable. Early menopause (EM) is defined as menopause before the age of 45 years and occurs in 5−10% of the general population [27-30]. Primary ovarian insufficiency (POI), formerly known as premature ovarian failure, is a fertility disorder characterised by amenorrhoea, increased gonadotropin levels (i.e. a monotropic rise in FSH) and low oestrogen levels in women before the age of 40 years [30]. It affects approximately 1% of the general population, though its prevalence in women with primary amenorrhoea rises up to 10−28% [19]. The aetiology is unknown in most cases. Some of the identified causes of POI are genetic (Turner syndrome and Fragile X syndrome), iatrogenic (after chemotherapy or radiotherapy), and autoimmune disorders (polyglandular endocrine syndromes) [31,32].

Hormone replacement therapy is recommended to prevent long-term health problems associated with the premature loss of sex steroid activity, including osteoporosis and cardiovascular disorders. It is noteworthy that spontaneous ovulations may occur. Therefore, when pregnancy is not desired, women should be appropriately counselled to use contraception [19]. Usually, the final option for achieving pregnancy in women diagnosed with POI is oocyte donation.

As mentioned above, the occurrence of menopause shows a large variation between women and has been found to be largely heritable [33,34]. Menopausal age is a complex trait, indicating that it is the result of a complex interplay of environmental and genetic factors. The latter have received growing attention in recent years, as these factors may help to identify the various processes that underlie ovarian ageing and its variation. It may also aid in a more accurate prediction of a woman's fertility.

In large genome-wide association (GWA) studies in the general population, age at natural menopause has been associated with variants of genes involved in DNA repair, DNA maintenance and immunity [35-37]. A recently published meta-analysis of GWA studies revealed that 17 variants

previously associated with age at natural menopause were also associated with EM [38]. This indicates that EM and natural menopause have an overlapping genetic aetiology and are at least partly explained by the effects of the same polygenic variants [38].

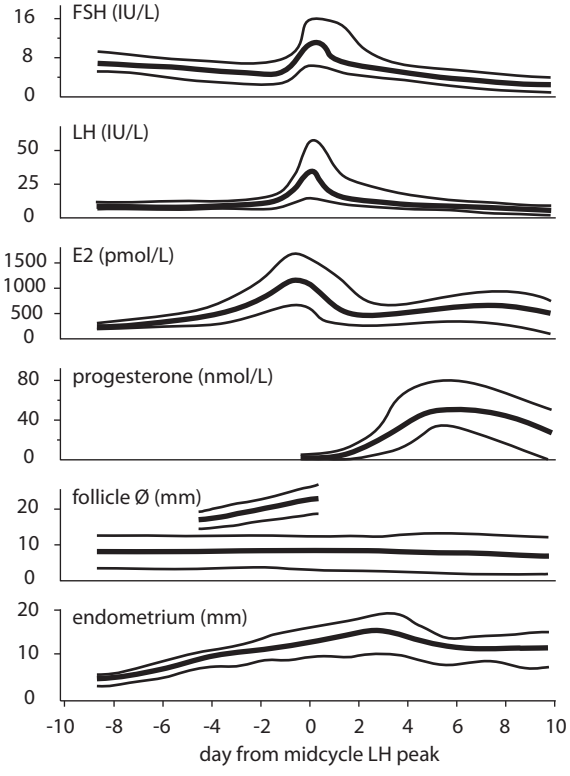

Figure V | Mean serum concentrations with 95 percentiles for LH, FSH, oestradiol and progesterone from 8 days prior to the mid-cycle LH peak until 10 days thereafter. The lower panels show the endometrium thickness and emergence and growth of the dominant follicle in relation to the time of the mid-cycle LH peak. Figures based on data from regularly cycling women.

FSH=follicle-stimulating hormone; LH=luteinizing hormone; E2=oestradiol; prog=progesterone. Modified from Macklon and Fauser, 2000 and Bakos *et al*, 1994.

Similarly, POI has also been found to be at least partly heritable. Although the majority of POI cases have idiopathic aetiologies, mutations directly causing POI have been identified in a small group of patients (*NOBOX, GDF9, LDX8*) [39]. Recently, the first two GWA studies have revealed a candidate region linked to the *ADAMTS19* gene [23] as well as an unknown region in between genes on chromosome 8 (8q22.3) [40]. Moreover, the 17 variants previously identified for natural and early menopause were similarly significantly associated with POI [38]. Indeed, POI as well as EM showed

considerable overlap. Finally, the number of risk alleles associated with the age of menopause seemed to be increased in women with POI compared to women who had their menopause between 45 and 60 years of age [38].

Assessing ovarian reserve

Despite the evident changes in quantity and quality of follicles in women aged 35–45, ovarian ageing remains largely unnoticed since menstrual cycles remain regular until perimenopausal transition. Between the onset of cycle irregularity and the occurrence of menopause, a fixed temporal relationship is believed to be present, although longitudinal data are scarce. Because the age at which women become sterile shows the same degree of variation as observed for menopause, it is generally assumed that this event carries a fixed temporal relationship with the subsequent onset of cycle irregularity and menopause, with a presumed interval of ~5 and 10 years, respectively. The same fixed relationship might also be true for the age at which women start to become subfertile (defined as failing to achieve a vital pregnancy within one year; assumed mean age 31 years) (Figure VI).

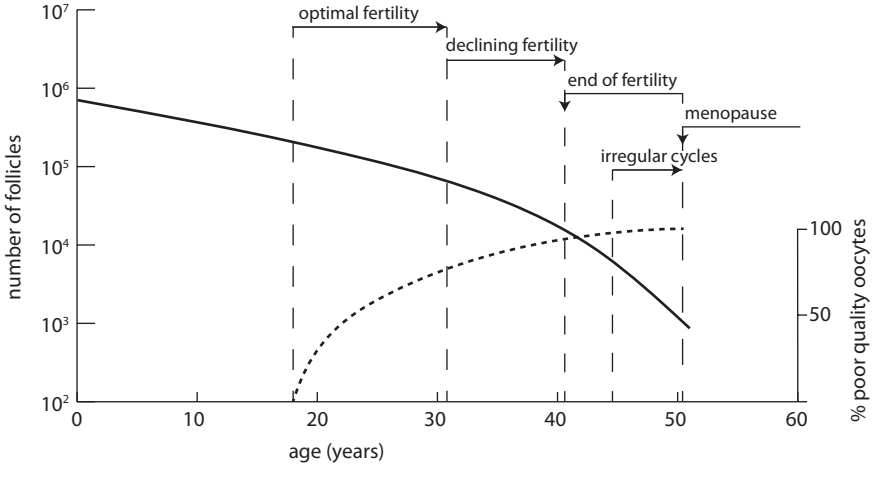

Figure VI | The decline in follicle number (unbroken line) and the increase in poor-quality oocytes (dotted line) in relation to reproductive events with increasing female age.
Modified from Klinkert, 2005, PhD thesis.

Therefore, cycle irregularity does not seem to be a reliable marker for the onset of decreasing fertility. As the number of primordial follicles is indirectly reflected by the number of growing follicles [41], the antral follicle count (AFC) is a reliable marker for ovarian ageing. AFC includes all follicles between 2 to 10 mm in diameter. Transvaginal ultrasound provides the most direct way to determine the AFC. However, intra- and inter-observer reproducibility of the AFC seems to be rather limited [42,43] and transvaginal ultrasounds are not feasible in young children en adolescents. Moreover, due to the fact that ultrasound machines become more and more sophisticated with higher resolutions, the need for new normative data is an emerging problem.

In most longitudinal studies, gonadal function is evaluated by measuring serum FSH and LH levels [44]. During the perimenopausal transition FSH as well as LH levels increase due to a decrease in steroid feedback. Due to the diminishing number of follicles and a concomitant decrease in granulosa cells, inhibin levels start to decrease too. Hence, the negative feedback signal for FSH is also decreased leading to a rise in FSH secretion. Therefore, FSH levels generally are higher than LH levels. This so-called monotropic rise in FSH is the hallmark of the menopausal transition [39]. One has to keep in mind that FSH starts to increase above normal levels when the ovarian reserve has already been reduced significantly, and it therefore constitutes a relative late sign of ovarian ageing [45]. Hence, menstrual history, assessment of the AFC and the measurement of serum FSH as well as serum inhibin B concentrations constitute late markers of ovarian ageing and do have the disadvantage of late detection, underlining the need for a more easily obtained and more reliable early marker of ovarian reserve.

Anti-Müllerian hormone (AMH) has been suggested to be such an earlier and more accurate marker for ovarian reserve. AMH, also known as Müllerian inhibiting substance, is a dimeric glycoprotein exclusively produced by granulosa cells of small growing follicles. It indirectly reflects the size of the primordial follicle pool in the ovaries. In rodents, AMH expression starts after birth and increases until follicles reach the small antral stage. From the antral stage onwards, AMH expression decreases again and is absent in the pre-ovulatory stage (Figure VII) [46-48]. This specific pattern between initial and cyclic recruitment indicates that AMH may have a role in these two important steps during folliculogenesis [18,46].

Because of the specific expression pattern solely in small growing follicles, it has been suggested that AMH could be a more accurate marker for ovarian reserve since it indirectly represents the size of the primordial pool. Indeed, AMH has been shown to be a reliable serum marker for ovarian reserve in mice, women and adult survivors of childhood cancer [49-54]. AMH can be easily determined in post-pubertal women, since its serum levels are relatively stable during and between menstrual cycles, in contrast to the cyclic fluctuations observed in serum FSH levels [55]. In children, AMH levels rise during infancy, whereas AMH levels show only minor fluctuations during childhood and adolescence. In addition, the negative AMH–FSH correlation in pre-pubertal girls supports the notion that AMH is a quantitative marker of ovarian follicles even in young girls [56]. Moreover, AMH has been found to be highly predictive for the onset of menopause [45,57,58]. Recent studies have shown that several years prior to menopausal transition, serum AMH levels were low or undetectable and were more predictive for the onset of menopause compared to estimates based on chronological age (82-84). Nomograms predicting the age at menopause have been successfully reported [59,60]. Hitherto, serum AMH levels are the earliest identified marker of ovarian ageing and best reflect the reproductive decline.

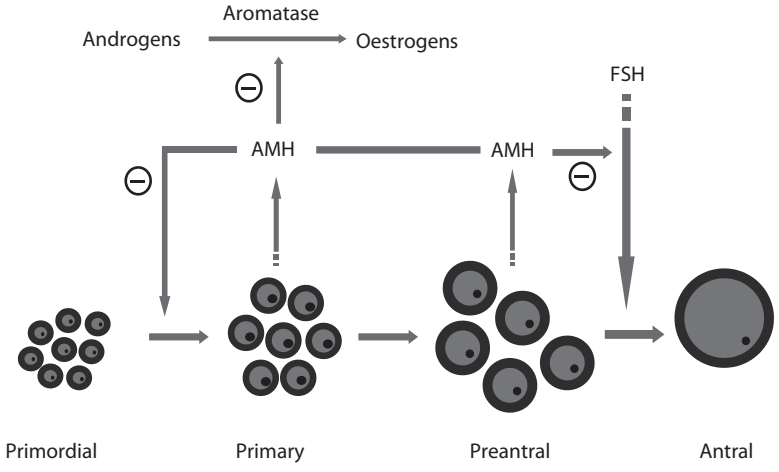

Figure VII | Initial recruitment and cycle recruitment during stages of folliculogenesis, with AMH expression and AMH function.

AMH=anti-Müllerian hormone; FSH=follicle-stimulating hormone. Modified from Visser *et al*, 2006.

Female gonadal function after childhood cancer

In female survivors of childhood cancer, loss of gonadal function is one of the major long-term side effects of treatment. Damage to the ovaries causes follicle loss and may result in POI. Both alkylating agents and abdominal irradiation can have a deleterious, dose-dependent effect on ovarian function [22,54,61]. In addition, multi-drug regimens may have a cumulative toxic effect on reproductive function. In case of the use of different alkylating agents, the alkylating agent dose (AAD) score can be used to sum the total impact of alkylating agents [61,62].

Radiotherapy may also cause damage to the uterus in addition to its gonadotoxic effects. In turn, this may lead to premature labour due to decreased distension of the uterine cavity. Moreover, low birth weight due to defective placentation might be encountered in women after abdominal radiotherapy. Finally, a higher incidence of post-partum haemorrhage was observed probably as a result of decreased contractility of the smooth muscle fibres of the uterus [61,63-66]. Therefore, close monitoring during pregnancy and intra-partum is warranted in women treated with abdominal radiotherapy [65,67].

Options for fertility preservation, such as cryopreservation of embryos after an IVF procedure are available for adult women with partners undergoing cancer treatment [68]. Ovarian tissue preservation or oocyte vitrification are newly emerging options for young women with a menstrual cycle. However, embryo and oocyte cryopreservation cannot be offered to younger pre-pubertal girls, and cryopreservation of ovarian tissue is only offered to these girls in an experimental setting. Still it is important to counsel patients on the effects their cancer treatment might have on their ovarian function and the subsequent fertility before treatment is initiated.

Although cancer treatment has been associated with loss of gonadal function, the extent of gonadal damage differs between equally treated cancer survivors. Knowledge on other potential determinants, such as obesity and genetic influence, is lacking but required to improve childhood cancer care.

1.3 | Male gonadal function

The healthy male hypothalamic-pituitary-gonadal axis controls two functions that are essential for male reproduction: the production of appropriate quantities of sex steroid hormones (i.e. androgens) and the generation of healthy mature male gametes capable of fertilising an oocyte. In boys, the onset of puberty is associated with alterations in the circulating concentrations of reproductive hormones, i.e. FSH, LH, testosterone and inhibin B.

The gonadotropins FSH and LH are produced by the pituitary gland in response to stimulation by GnRH from the hypothalamus (Figure VIII). LH in turn stimulates the Leydig cells in the testicular interstitium to synthesize and secrete testosterone. The actions of LH are indirectly supported by FSH, which induces the expression of LH receptors on testicular Leydig cells [69]. Testosterone is secreted both into the circulation and into the lumen of the seminiferous tubules, where it is highly concentrated in order to support spermatogenesis in the germinal epithelium and sperm maturation in the epididymis. Hence, concentrations within the testis are 50–100 times higher than in blood [70,71]. This concentration is aided by the fact that Sertoli cells produce androgen binding protein (ABP) upon FSH stimulation [71]. In addition, the Sertoli cells produce inhibin B after stimulation by FSH [19]. The production of inhibin B and testosterone results in a negative feedback to the pituitary and hypothalamus and thereby reducing the secretion of the pituitary gonadotropins.

Assessing male gonadal function

Semen analysis is the gold standard to establish a men's fertility potential. However, both in adolescents and young adults without a wish to father yet, obtaining semen for analysis can be a challenge due to the inability to produce semen due to stress, embarrassment or immaturity. Hence, the use of an alternative first screening method would be of value.

In the past, serum levels of LH, FSH and testosterone have been used to assess male gonadal function and fertility. An increased serum FSH is considered to be the first indicator of testicular dysfunction [72]. However, FSH is an indirect and late marker of gonadal function since it increases, similar to the female system, because of a compensatory response of the pituitary gland to loss of negative feedback as a result of low levels of testosterone and, especially falling levels of inhibin B.

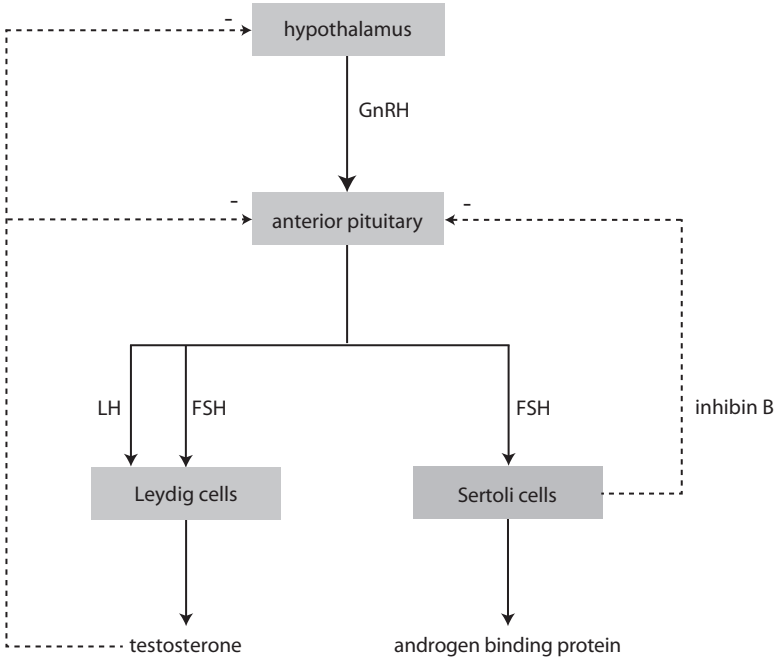

Figure VIII | Male hypothalamic-pituitary-gonadal axis.
GnRH=gonadotropin-releasing hormone; LH=luteinizing hormone; FSH=follicle-stimulating hormone.

Over the last decades, inhibin B has been identified as a reliable direct marker for assessment of the testicular function. It is produced by Sertoli cells in the testes, and inhibits the production of FSH in the pituitary. Inhibin B is strongly correlated with sperm counts in both healthy and subfertile men [72-76]. Nevertheless, in current medical practice where assisted reproductive technology (ART) is available, lower inhibin B levels do not necessarily mean infertility, since nowadays only low numbers of motile spermatozoa are required for successful ART. Although inhibin B can be used as marker for gonadal function, it cannot reliably predict successful cryopreservation of semen in boys diagnosed with cancer [77].

Male gonadal function after childhood cancer

In male childhood cancer survivors, gonadal dysfunction is largely determined by cancer treatment (Table I). Especially survivors who were treated with alkylating agents and/or testis irradiation are at risk for gonadal dysfunction, independent of the age at which the cancer treatment is administered [74,77-79].

The testicular germinal epithelium is particularly sensitive to radiation. Spermatogenesis can be impaired by direct testicular irradiation, but also by scatter radiation resulting from other treatment fields such as the pelvis and bladder [80]. Impaired spermatogenesis is observed after testicular doses as low as 0.1 Gy, and recovery is unlikely after a single testicular dose exceeding 4 to 6 Gy [81].

Table I | Gonadal dysfunction in male survivors of childhood cancer and treatment-related risk factors.

Complication	Therapy		
Hypoandrogenism:	Alkylating agents		
– delayed/arrested puberty	Radiation:	≥ 20 Gy:	– testis
– low testosterone			– pelvis
		≥ 30 Gy:	– cranial-neuroendocrine axis
			– orbital/eye
			– ear/infratemporal
			– nasopharyngeal
			– Waldeyer's ring
		Other fields combined with alkylating agents:	– flank/hemi abdomen
			– whole abdomen
			– inverted Y
			– prostate/bladder
			– TBI
	Surgery:	Orchiectomy/ Hypothalamic pituitary axis	
Reduced fertility:	Alkylating agents		
– oligospermia	Radiation:	Any testicular dose	– Flank/hemi abdomen
– azoospermia			– Whole abdomen, inverted Y, pelvic, prostate/bladder/ iliac
			– Inguinal/femoral
			– TBI
		≥ 30 Gy:	– cranial-neuroendocrine axis
			– orbital/eye
			– ear/infratemporal
			– nasopharyngeal
			– Waldeyer's ring
	Surgery:	Orchiectomy/ Hypothalamic pituitary axis	

TBI=total body irradiation. Modified from Kenney *et al*, 2012.

Studies on recovery of spermatogenesis in long-term survivors of childhood cancer are scarce. In 2/12 survivors of childhood Hodgkin lymphoma recovery has been observed even after 12–15 years following treatment. However, another study in 19 survivors did not show any recovery of gonadal function [82,83]. Larger studies are required to investigate whether and in which cases long-term recovery of spermatogenesis is possible.

1.4 | Outline of this thesis

The aim of this thesis is threefold. First of all we would like to identify determinants of gonadal dysfunction apart from cancer treatment in male and female survivors of childhood cancer. Secondly, we would like to study if and how such parameters of gonadal dysfunction recover in men after childhood cancer treatment (Figure IX). Thirdly, we would like to evaluate alternative fertility options for survivors of childhood cancer.

Chapter two reviews the literature on long-term endocrine effects of childhood Hodgkin's lymphoma treatment. From chapter three onwards, studies on determinants of gonadal function after childhood cancer are presented. In **chapter three**, we describe a study on pre-treatment AMH levels in girls with newly diagnosed cancer. A study on characteristics and outcome of ovarian infiltration in girls with non-Hodgkin lymphoma is discussed in **chapter four**. **Chapter five** focusses on the association between obesity and gonadal function in female survivors of childhood cancer. In **chapter six**, we describe a study on genetic variation of ovarian reserve in female survivors of childhood cancer. **Chapter seven** describes the international harmonised primary ovarian insufficiency surveillance recommendations for female childhood cancer survivors. Treatment and pregnancy outcomes of oocyte donation, as final fertility option for women with primary ovarian insufficiency, are described in **chapter eight**. The study on the association between obesity and gonadal function in male survivors of childhood cancer is presented in **chapter nine**. In **chapter ten**, we studied gonadal function recovery and its predictors in male survivors of childhood cancer. **Chapter eleven** focusses on procedures to preserve semen in boys with newly diagnosed cancer, in particular electroejaculation. Finally, **chapter twelve** covers the general discussion and overall conclusions of this thesis, and provides recommendations for future research.

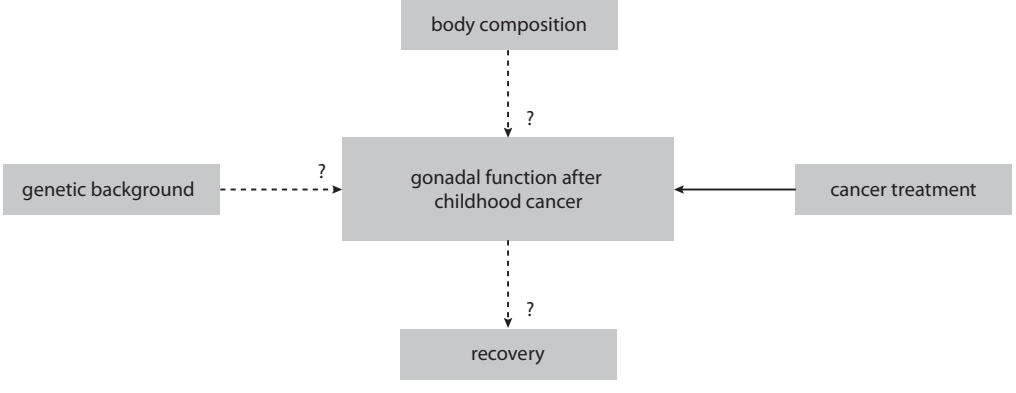

Figure IX | Gonadal reserve after childhood cancer and its potential determinants.

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CHAPTER 2

Long-term endocrine side effects of childhood Hodgkin's lymphoma treatment: a review

W van Dorp & RD van Beek, JSE Laven, R Pieters,
SMPF de Muinck Keizer-Schrama, MM van den Heuvel-Eibrink

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ABSTRACT

Background: Since childhood cancer survival has increased, long-term effects of treatment have gained interest. Childhood Hodgkin's lymphoma has been treated successfully for decades now. We provide an overview of the literature on long-term endocrine side effects, such as gonadal dysfunction and growth retardation, as a result of childhood Hodgkin's lymphoma treatment.

Methods: A comprehensive search of the Pubmed database was performed.

Results: We identified 16 studies (10 studies: 298 male survivors and 6 studies: 230 female survivors) about gonadal dysfunction. In survivors treated with alkylating agents or pelvic radiotherapy, severe gonadal damage is described. Recovery was rarely described. Seven studies (481 survivors) about bone mineral density (BMD) and growth were identified. The effects on BMD appear to be small. Data on growth are scarce, but show that radiotherapy in a dose of >30 Gy including the spine, especially in pre-pubertal children, results in reduced height. We included 10 studies (4,012 survivors) about thyroid complications. Hypothyroidism is the most common thyroid disorder after radiotherapy. There is also a significantly increased incidence in thyroid carcinoma after low-dose radiation. In survivors treated with chemotherapy only, hypothyroidism and thyroid cancer have not been reported.

Conclusion: The severity of endocrine toxicity after childhood Hodgkin's lymphoma depends on the type of treatment. Gonadal dysfunction seems to be the most severe endocrine long-term effect, especially after treatment with alkylating agents or pelvic radiotherapy. The knowledge obtained in specific follow-up programmes for paediatric cancer survivors will help to find the optimal balance between curability and long-term side effects.

INTRODUCTION

Childhood cancer has become a curable disease. Currently, two-third of all children with cancer reach long-term survival [1-6]. Hence, long-term effects of treatment in childhood cancer survivors have been acknowledged to be of major importance. It has been estimated that 70% of survivors have developed at least one of the long-term sequelae resulting from treatment. One-third of these effects have even been classified as severe or life threatening [7,8]. In Hodgkin lymphoma survivors, 70% reported at least one chronic condition [9].

Hodgkin's lymphoma is a malignancy of the lymph nodes and lymphatic system with possible involvement of other organs. It has two incidence peaks. The first peak occurs between the ages of 15 and 30 years and the second between 45 and 55 years. Hodgkin's lymphoma is very rare in children under 15 years of age [10]. Paediatric Hodgkin's lymphoma has a very good prognosis: an event free survival (EFS) up to 93% and an overall survival of even 96% have been reported [11-17]. The treatment of childhood Hodgkin's lymphoma consists of chemotherapy, radiotherapy or a combination of both. In former years, treatment of paediatric Hodgkin's lymphoma often involved extended field high-dose radiation therapy, which was associated with several severe side effects, such as secondary malignancies [18-20]. Currently, most paediatric oncology centres use a risk-adapted treatment schedule consisting of both chemotherapy and low-dose, involved field or even involved node radiotherapy with high EFSs [21-28]. In a few centres, it was decided to treat childhood Hodgkin's lymphoma with chemotherapy only in order to minimize the risk of severe late effects, such as infertility, while preserving good cure rates [13,17].

Both chemotherapy and radiotherapy have serious potential side effects, especially when used in children. In both Canadian and US studies, a high prevalence of self-reported somatic health problems is followed by a higher risk of psychosocial late effects, such as a lower educational level, unemployment, low income and fewer close friends [29-32]. In a Dutch cohort, there were only small differences in health-related quality of life and the burden of self-reported somatic disease [33]. Several follow-up studies have been performed to investigate the incidence of long-term side effects (such as abnormal cardiac function, auditory dysfunction and endocrine disruption) in all childhood cancer survivors. In general, long-term side effects of chemotherapy depend on the patient characteristics (e.g. age), type of chemotherapy (e.g. alkylating agents, anthracyclines) and cumulative dose, whereas the toxicity of radiotherapy is related to the dosage and extent of the irradiated field [8,34,35].

In this paper, we present the available literature on endocrine late sequelae after childhood Hodgkin's lymphoma treatment with a special focus on gonadal dysfunction, growth, bone mineral density (BMD), body composition and thyroid dysfunction.

METHODS

To provide an overview and summary of the known long-term endocrine effects of Hodgkin's lymphoma treatment, a comprehensive search of the Pubmed database was performed for all articles published until February 2011. Search-criteria relevant to childhood cancer survivors and long-term endocrine effects were used: childhood cancer survivors, childhood, adolescent, Hodgkin's lymphoma, endocrine effects, growth, BMD, thyroid function, gonadal function and ovarian reserve. If not included initially, cross-references picked up during the review procedure were also selected. Only articles published in the English language were included. In this way, we identified 188 papers. After carefully reading the abstracts, 166 papers were excluded because they describe the late effects of all childhood cancer treatments instead of the effects of Hodgkin's lymphoma treatment only. The 22 papers remaining and another 11 papers identified through cross-reference searches resulted in a total of 33 papers concerning specific late endocrine effects of the treatment of childhood Hodgkin's lymphoma.

RESULTS

Gonads

An important side effect of both radiotherapy and chemotherapy is gonadal dysfunction or complete loss of gonadal activity. This might result in reduced fertility or even infertility. Moreover, in women it might also cause a subsequent loss of bone mass due to the depletion of oestrogens.

Male survivors

We identified 10 studies, involving 298 male survivors, concerning male gonadal dysfunction after childhood Hodgkin's lymphoma treatment in Table I. Azoospermia and oligospermia are common long-term side effects in male childhood Hodgkin's lymphoma patients after radiotherapy and chemotherapy, especially when alkylating agents, e.g. mustine or procarbazine, were used.

Endocrine markers for male fertility. In most follow-up studies of long-term childhood cancer survivors, combinations of testicular volume, semen analysis and/or serum levels of LH, FSH and testosterone are assessed to evaluate gonadal function and fertility. An increase in serum FSH is considered to be the first indirect indicator of testicular dysfunction [36] and is reported more frequently after alkylating chemotherapy compared with treatment without alkylating chemotherapy [12,37-39]. In general, the levels of LH and FSH are positively associated with the cumulative dose of alkylating agents [12,40,41]. No LH/FSH changes were found after chemotherapy with low dosages of alkylating agents [37,38].

Recently, inhibin B has been identified as a good direct marker for assessment of testicular function. This hormone is produced by Sertoli cells of the testis, and inhibits the production of FSH in the pituitary. Inhibin B is strongly correlated with sperm counts [36,41,42]. In current medical practice where assisted reproductive technology (ART) is available, lower inhibin B levels do

not necessarily mean infertility, but lower levels are associated with lower sperm counts in both healthy and subfertile men [36,42]. Inhibin B, as a marker for gonadal impairment, was found to be lower in long-term survivors of childhood cancer [43] (Figure I). Only two studies in childhood Hodgkin's lymphoma survivors used inhibin B as a marker for male gonadal function. One small study reported decreased inhibin B levels in childhood Hodgkin's lymphoma survivors treated with mechlorethamine, vincristine, procarbazine and prednisone (MOPP) and adriamycin, bleomycin, vinblastine, dacarbazine (ABVD) or cyclophosphamide, vincristine, procarbazine and prednisolone (COPP) and ABVD compared with controls [44]. We showed in male survivors of childhood Hodgkin's lymphoma that inhibin B was decreased after treatment with MOPP but not after epirubicin, bleomycin, vinblastine and dacarbazine (EBVD). Moreover, inhibin B was a better indicator for spermatogenesis in long-term childhood Hodgkin's lymphoma survivors than FSH [41].

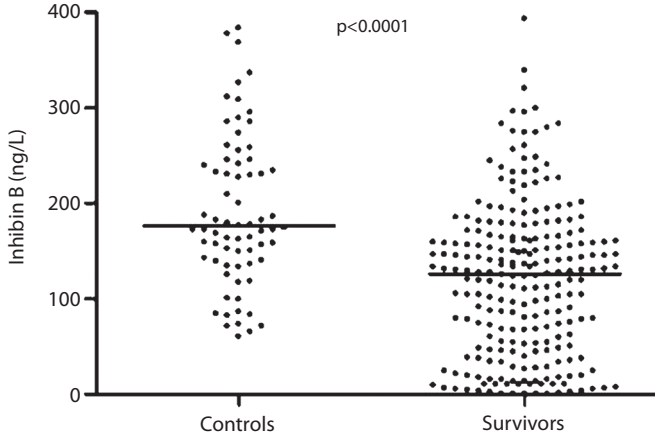

Figure I | Inhibin B values of controls and childhood cancer survivors. Each dot represents one person. The horizontal lines represent the median values of inhibin B in the normospermic controls (176.5 ng/L) and in the survivors (123.0 ng/L, p<0.0001) (adapted, with permission, from: van Casteren *et al*, Pediatr Blood Cancer 2009).

Sperm quality. Nearly all male Hodgkin's lymphoma survivors treated with alkylating chemotherapy only, suffer from oligospermia or azoospermia. This is the case in both adult Hodgkin's lymphoma survivors [45-48] and childhood Hodgkin's lymphoma survivors [41,49]. We showed that 12 out of 17 (70%) long-term male survivors of childhood Hodgkin's lymphoma treated without radiotherapy but with MOPP had azoospermia or severe oligospermia, whereas all survivors treated without alkylating chemotherapy had normospermia [41]. After 10 years of follow-up in a small group of survivors, no recovery of spermatogenesis was observed [49]. In adult Hodgkin's lymphoma survivors, recovery of spermatogenesis after alkylating chemotherapy (such as procarbazine) has only been reported in a small proportion within 5–15 years follow-up, but survivors treated with

ABVD appear to be at a significant advantage over survivors treated with MOPP in terms of testicular function, as demonstrated by the return to normal fertility in the vast majority of survivors treated with ABVD [47,50-53]. Although animal studies suggest a protection against the cytotoxic effects of chemotherapy before puberty in both males and females [54], recent clinical studies in male patients treated for Hodgkin's lymphoma show no difference in the severity of the gonadal damage [41] or the chance of recovery of spermatogenesis between boys treated before puberty and those treated during or after puberty [55,56]. Reduction of alkylating agents reduces the risk of gonadal damage as detected by increased serum FSH levels and decreased inhibin B levels [37]. We recently reported, using sperm analyses combined with fertility markers like inhibin B, that gonadal damage was significantly related to the cumulative dosages of alkylating agents in childhood Hodgkin's lymphoma survivors [41].

Since the testis is one of the most radiosensitive tissues, very low dosages of radiation can cause severe gonadal damage. When treated with testicular irradiation dosages below 0.2 Gy, no significant effect on FSH levels or sperm counts was observed in adult patients treated for Hodgkin's lymphoma. When treated with dosages between 0.2 and 0.7 Gy, a transient dose-dependent increase in FSH and reduction in sperm concentration were observed, with a return to normal values within 12–24 months (n=17) [57]. Ten adult Hodgkin's lymphoma patients treated with testicular doses of radiation of 1.2–3 Gy were all azoospermic following treatment, with no recovery up to 15 months of follow-up. After 15 months, four patients showed recovery and up to 40 months later recovery was observed in the another [58]. After 17–43 months, no recovery was observed in patients treated with 1.4–2.6 Gy, but a return of fertility was seen in two patients treated with testicular radiation dosages of 1.2 Gy. This may represent a threshold for permanent testicular damage [59]. In survivors of childhood acute lymphoblastic leukaemia treated with total body irradiation (TBI) or testicular irradiation (n=7), severe gonadal damage was observed [60]. In childhood Hodgkin's lymphoma survivors, pelvic radiotherapy in a small group of adult males caused azoospermia that was reversible in most cases (66%–96%) within 2 years after cessation of therapy [61,62]. Ortin *et al* reported a small group of boys treated with pelvic radiotherapy (30–45 Gy). Recovery of spermatogenesis occurred after long-term follow-up, and recovery to normal levels was less frequent (2 out of 12: 17%) than reported in patients treated during adulthood [56].

In childhood Hodgkin's lymphoma survivors in whom radiotherapy and chemotherapy were combined, spermatogenesis was disturbed in up to 75–100% of the male patients, similar to studies with chemotherapy including alkylating agents [55,56,63,64] (Table I).

In conclusion, in male childhood cancer survivors treated with alkylating agents or pelvic radiotherapy, severe gonadal damage is described. In survivors treated without alkylating agents, normal gonadal function was observed. Gonadal damage is significantly related to the cumulative dosages of alkylating agents. Recent studies with a long (>10 years) follow-up have shown recovery of spermatogenesis in a small proportion of childhood Hodgkin's lymphoma survivors. Therefore, prolonged follow-up studies are recommended to investigate recovery and to determine possible fertility treatment in male survivors.

Table I | Studies on testicular function in male childhood Hodgkin's lymphoma survivors.

Author	n[a]	Age (range)[b]	Median follow-up	Therapy		Gonadal evaluation						
						Semen analysis			Serum markers			
				CT	RT	n	Azo	Oligo	n	LH	FSH	Inh B
Heikens *et al* (1996)	19	11 years (5–15 years)	10 years	MOPP	0	18	67%	33%	19	↑	↑	–
Ortin *et al* (1990)	20	13 years (2–15 years)	9 years	MOPP	12	15	60%	15%	11	↑	↑	–
Mackie *et al*(1996)	46	12.2 years (8.2–15.3 years	6 years	ChlVPP	0	–	–	–	46	↑	↑	–
Shafford *et al* (1993)	30	10 years (4–15 years)	11.8 years	ChlVPP	10	13	91%	9%	30	↑	↑	–
Papadakis *et al* (1999)	36	13 years (2–22 years)	6.8 years	MDP	6	2	100%	–	36	↑	↑	–
Ben Arush *et al* (2000)	20	2–16 years		MOPP/ABVD COMP MOPP	4	20	40%	40%	20	↑	↑	–
Cicognani *et al* (2000)	11	10.3 years (3–15 years)	5.5 years	ABVD MOPP/ ABVD COPP/ ABVD	0	–	–	–	11	↑	↑	↓[d]
van den Berg *et al* (2004)	14	14 years (5–18 years)	5.1 years	MOPP/ ABVD	0	–	–	–	14	=[c]	=[c]	–
Gerres *et al* (1998)	46	14.9 years	1.9 years	OEPA	0	–	–	–	46	↑[d]	↑[d]	–
van Beek *et al* (2007)	56	11.4 years (3.7–15.9 years)	15.5 years	OEPA/ COPP A(or E)BVD MOPP/A(or E)BVD	0	21	53%[d]	6%[d]	56	↑[d]	↑[d]	↓[d]

-=information not available; CT=chemotherapy; MOPP=mechlorethamine*, vincristine, procarbazine*, prednisolone; COPP=MOPP cyclophosphamide* replaces mechlorethamine; COMP=COPP, methotrexate replaces procarbazine; ABVD=adriamycin, bleomycin, vinblastine, dacarbazine*; MDP=doxorubicin, procarbazine*, prednisolone, vincristine, cyclophosphamide*; ChlVPP=chlorambucil, vinblastine, procarbazine*, prednisolone; OEPA=vincristine, etoposide, prednisolone, doxyrubicin; OPPA=OEPA procarbazine* replaces etoposide (*=alkylating agent), RT=number of patients with gonadal radiotherapy; n=number of patients tested; azo=azoospermia; oligo=oligospermia; LH=luteinizing hormone, FSH=follicle-stimulating hormone; inh B=inhibin B. Arrows indicate increased (↑), decreased (↓) or normal values (=); a) number of male survivors; b) age at diagnosis (median and range); c) Only 2 male survivors with increased levels of FSH and 1 with an increased level of LH; d) Only in patients treated with MOPP or COPP.

Female survivors

We identified six studies involving 230 female survivors concerning female gonadal dysfunction after childhood Hodgkin's lymphoma treatment (Table II). In female childhood cancer survivors both alkylating agents and abdominal radiotherapy can cause severe ovarian damage, eventually leading to premature ovarian failure (POF) [65-68].

Endocrine markers for female fertility. Usually, gonadal function is measured in follow-up studies by analysing serum LH and FSH levels [69]. Both FSH and LH are predictive for the ovarian reserve although FSH presumably only increases above the normal range with severe loss of ovarian function [70,71]. In recent years, two new markers to assess ovarian function became available. The first one, inhibin B, which in females is solely produced by granulosa cells of small antral follicles, is decreased in women with known fertility problems (e.g. imminent ovarian failure) and undetectable in postmenopausal women [72-74]. Inhibin B is one of the first endocrine markers to change in perimenopausal women and is the cause for the monotropic rise in FSH which is a characteristic of the perimenopausal period [75]. The second marker is anti-Müllerian hormone (AMH), which is similarly produced by granulosa cells of early developing (pre-) antral follicles, and levels decrease coinciding with a decrease in the number of developing follicles with age [76-78]. A strong correlation between irregular cycles (as a surrogate for menopausal transition) and AMH levels randomly measured during the reproductive lifespan in a group of healthy women was observed [79]. In addition, AMH was shown to be a good predictor for the success of ART [80-84]. However, results of different studies should be carefully interpreted, because international standards for AMH assays are lacking. Recently, it was reported that long-term ovarian function after chemotherapy can be predicted by pre-treatment serum AMH levels in women with early breast cancer [85]. We showed that AMH is a good early marker for a decreased ovarian reserve in female Hodgkin's lymphoma survivors, even when LH and FSH are still within normal ranges and menstrual cycles are still regular [65]. These results were recently confirmed in a larger cohort of survivors of other types of childhood cancer in our institute [66] (Figure II).

Ovarian function. A small study showed that 8 out of 10 girls with Hodgkin's lymphoma, treated with pelvic irradiation only, had a normal ovarian function. It is important to note that in that study nine girls did have an oophoropexy prior to irradiation to transfer the ovaries out of the radiation field. None of the girls treated with radiotherapy to other sites than the pelvis suffered from POF [56]. AMH levels were not measured in this study. Abdominal radiotherapy can cause decreased levels of AMH, thereby reflecting critically impaired ovarian reserve even in the presence of, as yet, normal menstrual cycles [66]. Another study showed that exposure to increasing dosages of radiation to the ovaries and a diagnosis of Hodgkin's lymphoma are risk factors for nonsurgical POF [67]. The studies that describe the reproductive status of women after chemotherapy and/or radiotherapy for childhood Hodgkin's lymphoma are reported in Table II. The incidence of POF depends on several factors: chemotherapy types and dosages, pelvic radiotherapy and age at diagnosis. Several hypotheses have been postulated about the exact mechanisms of chemotherapy-induced ovarian damage, but the exact mechanisms still remain unknown. However it is clear that the damage

Table II | Studies on ovarian function in female childhood Hodgkin's lymphoma survivors.

Author	n[a]	Age (range)[b]	Median follow-up	Therapy		Gonadal evaluation						
				CT	RT	Amenorrhoea irregular cycle[c]	Pregnancies	Serum markers				
								n	LH	FSH	Inh B	AMH
Hudson *et al* (1993)	37	14.6 years (4.3–20.1 years)	4.1 years	COP/ABVD	18	6/37	17 in 10 women	–	–	–	–	–
Ortin *et al* (1990)	86	13 years (2–15 years)	9 years	MOPP MOPP/ABVD ABVD PAVe VMB	28	11/86	40 in 86 women	–	–	–	–	–
van den Berg *et al* (2004)	14	14 years (5–18 years)	5.1 years	MOPP ABVD MOPP/ ABVD	0	2/14	1 in 14 women	14	=[d]	=[d]	–	–
Mackie *et al* (1996)	32	13.0 years (9.0–15.2 years)	4.3 years	ChlVPP	0	10/32	11 in 9 women	32	↑[e]	↑[e]	–	–
Papadakis *et al* (1999)	29	14.1 years (6.1–20.0 years)	5.2 years	MDP	6	–	8 in 6 women	29	↑	↑	–	–
van Beek *et al* (2007)	32	11.6 years (5.7–24.5 years)	15.5 years	A(or E)BVD MOPP/A(orE)BVD	0	1/32	17 in 11 women	32	=	↑[f]	↓[f]	↓[f]

-=information not available; CT=chemotherapy: MOPP=mechlorethamine*, vincristine, procarbazine*, prednisolone; COP=cyclophosphamide*, vincristine, procarbazine*; ChlVPP=chlorambucil, vinblastine, procarbazine*, prednisolone; ABVD=adriamycin, bleomycin, vinblastine, dacarbazine*; PAVe=procarbazine*, alkeran*, vinblastine; VMB=vinblastine, bleomycin, methotrexate (*=alkylating agent); RT=number of patients with gonadal radiotherapy; a) number of female survivors; b) age at diagnosis (median and range); c) amenorrhoea or irregular cycle only in female survivors treated with alkylating therapy or pelvic irradiation; d) only two female survivors with increased levels of FSH and one with an increased level of LH; e) increased FSH in 17/32 and LH in 15/32 female survivors; f) only in women with MOPP.

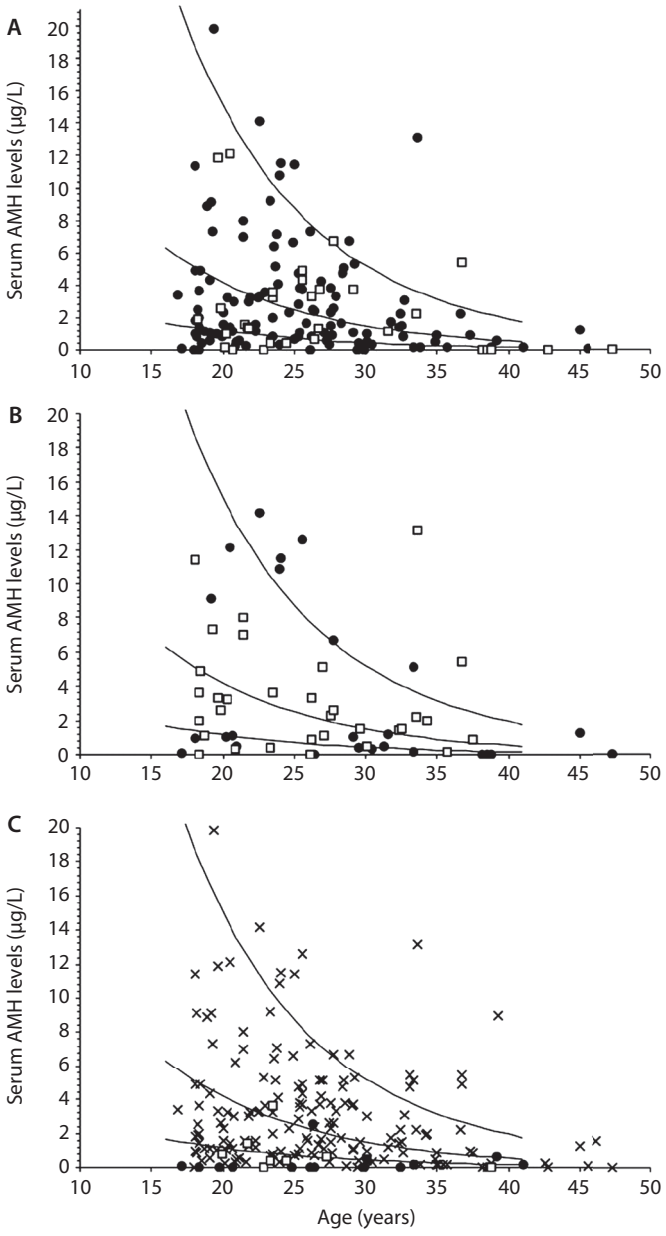

Figure II | Serum AMH levels in subgroups of survivors versus 10th, 50th and 90th percentile of AMH levels in controls (——). (**A**) Survivors treated prior (•) to or after (☐) menarche. (**B**) Survivors with regular menstrual cycles (•) or oligo- or amenorrhoea (☐). (**C**) Survivors treated with total body irradiation or abdominal radiotherapy (•), more than three MOPP cycles (☐) or another treatment regimen (×). AMH=anti-Müllerian hormone; MOPP=mustargen oncovin procarbazine prednisone (adapted from: Lie Fong *et al*, Hum Reprod 2009).

caused by chemotherapy leads to a relatively poor pregnancy outcome in survivors [86]. When treated with pelvic irradiation, the dose of radiation required to destroy 50% of the primordial follicles was determined to be <2 Gy [87]. One third of the Hodgkin's lymphoma survivors treated during childhood with chemotherapy containing procarbazine suffered from POF or had a high risk of entering menopause before the age of 40 years [39,88]. Recovery of ovarian function was reported rarely [39] and was not seen in patients treated with pelvic irradiation [64]. However Ortin *et al* reported less than 10% POF after chemotherapy only, and the other survivors had normal menstruation function [56]. We showed that ovarian damage occurs in the majority of women treated with MOPP as reflected by severely decreased AMH serum levels [65]. These childhood Hodgkin's lymphoma survivors had significantly reduced AMH levels when compared with survivors of other types of childhood cancer [66]. Recently, AMH serum levels were measured prior to, during and after treatment in adult women treated with chemotherapy for lymphoma. Women treated with ABVD showed recovery of AMH levels at 12 months after cessation of treatment, while women treated with non-ABVD but with cyclophosphamide showed no recovery of AMH levels at 12 months after cessation of treatment [89].

Childhood Hodgkin's lymphoma survivors treated with chemotherapy with a limitation of MOPP therapy to three courses seem to suffer less from gonadal damage compared with six MOPP courses [37,65]. Cumulative risk of menopause at the age of 40 did not differ between childhood cancer survivors treated at a younger age compared with those treated at an older age [88], nor between treatment before and after menarche [66].

Several studies reported up to 40% POF in female Hodgkin's lymphoma survivors treated during childhood with both pelvic irradiation and alkylating chemotherapy (mustine and procarbazine) [24,56], while none of the women treated without pelvic irradiation (n=18) had POF [24]. Some studies in adult female Hodgkin's lymphoma survivors suggest less POF when patients were treated before the age of 25 years when compared with those patients treated after that age [90], and had a better chance of recovery of ovarian function [91]. Two large childhood cancer survivor cohorts reported increased risks of premature menopause or ovarian failure within 5 years after cessation of therapy after exposure to procarbazine and pelvic irradiation (≥20 Gy), based on information obtained from questionnaires [92,93].

In female childhood cancer survivors, both alkylating agents and abdominal radiotherapy can cause severe ovarian damage. Recovery of the gonadal function was reported rarely.

Pregnancy outcome. Fertility is decreased among female childhood cancer survivors compared with female siblings (RR: 0.81), and survivors more frequently deliver preterm compared with their siblings when exposed to radiation [67]. Chemotherapy alone does not appear to have an independent effect on the uterus [94], but radiotherapy may cause damage to the uterus, which may lead to premature labour, low birth weight and a higher incidence of post-partum haemorrhage [67,94-98]. Also, a small increased risk of miscarriage was reported in survivors [97]. Recently, an increased risk of stillbirth and neonatal death has been reported after uterine and ovarian irradiation [99]. Therefore, a close monitoring during pregnancy and inpatient labour is warranted in women previously treated with abdominal radiotherapy [98,99].

Data about pregnancies in childhood Hodgkin's lymphoma survivors are scarce. The percentage of women becoming pregnant after treatment for Hodgkin's lymphoma in previous studies varies from <10% to almost 50% [24,37,39,56,64,65]. It is important to report that a proportion of these survivors are proven to be fertile. Confounders such as age and fertility of the partner, family planning and the time to pregnancy were not available.

Differences in toxicity between male and female survivors

In the past, it has been suggested that gonadal damage was less frequent in female childhood Hodgkin's lymphoma survivors than in male survivors [56]. A reason for the less frequently found gonadal damage in female survivors may be the lack of reliable markers for measuring ovarian function in the past. Recent studies using AMH as a marker of ovarian reserve showed impaired gonadal function in a higher proportion of childhood Hodgkin's lymphoma survivors treated with more than three MOPP cycles, abdominal irradiation or TBI [65,66]. The extent of gonadal damage seems to depend mostly on the type of treatment, rather than age at treatment [66]. Previously, female patients younger than 25 years of age were thought to be protected against the toxic effects of alkylating agents [90,91,100], but none of these studies used AMH. Therefore, the damage to the ovaries might have been underestimated. Early menopause may have been a disguised problem in female childhood Hodgkin's lymphoma survivors, especially after MOPP and abdominal irradiation, and therefore we proclaim that more attention should be paid to this issue, as it could have a major impact on fertility counselling and family planning of these young female childhood Hodgkin's lymphoma survivors treated with alkylating agents or abdominal irradiation.

Prevention

In some clinical trials, gonadotrophin-releasing hormone-analogues (GnRH-a) were used to prevent gonadal damage in patients treated with alkylating agents, and a possible protected function was observed [101-103]. However, none of these studies were prospective, randomized clinical trials, and hence must be considered inconclusive [104]. Recently, a prospective randomized study of hormonal co-treatment with oral contraception or GnRH-a during treatment for advanced-stage Hodgkin's lymphoma in young women reported no protection of ovarian reserve [105]. It is important to note that these studies have not evaluated long-term outcomes and the group of women was small. Therefore, this treatment should not replace alternative options for the protection of fertility, such as gonadal preservation, which should be offered to young female Hodgkin's lymphoma patients before treatment. Trials with GnRH-a in male patients showed no protection of GnRH-a treatment against cytotoxic damage [106-108].

Fertility preservation

Studies to prevent gonadal dysfunction in women still are the subject of research. Hence other options for fertility preservation remain very important. Ovarian cryopreservation in pre-pubertal girls, oocyte vitrification in adults, and embryo cryopreservation in adults with a partner are possible options. However, the success rate of ovarian cryopreservation is unclear since the number of women in whom frozen-thawed ovarian tissue has been reimplanted is unknown [109]. The

American Society of Clinical Oncology guideline recommends that ovarian cryopreservation and transplantation procedures should only be performed in centres with the necessary expertise [104]. However, in Europe and North America, a small number of research-based ovarian tissue storage facilities can provide the best treatment. To provide success rates of cryopreservation, all procedures should be documented and analysed [109].

Growth, osteoporosis and body composition

We identified seven studies involving 481 survivors concerning growth, osteoporosis and body composition after Hodgkin's lymphoma treatment during childhood (Table III).

Growth

In general, reduced growth in children during treatment for cancer is either caused by the malignancy itself, or by treatment-related morbidity, such as recurring infections, malnutrition during treatment and the treatment per se (surgery, chemotherapy and radiotherapy) [110-112]. Chemotherapy induced growth impairment might be, at least in part, the result of growth hormone deficiency [112] or direct interference with bone turnover [113]. Irradiation of parts of the spine in children contributes to poor growth by decreasing the growth of individual bones of the spine. This may result in reduced final height, but also in disproportional growth. Most of the loss in height after radiotherapy or chemotherapy affects the upper part of the body, reflected by loss in sitting height [114]. This is not surprising since the spine contains large numbers of epiphyses and if chemotherapy has a direct effect on the epiphyseal growth plate, it seems likely that this would result in greater loss in sitting height than leg length [115].

Most of the data regarding the negative effects of childhood Hodgkin's lymphoma treatment on growth are collected from survivors treated with combined modality treatment (Table III). In a study of 124 childhood Hodgkin's lymphoma survivors, treated at the age of 9–16 years with MOPP with or without ABVD, a height loss of 13 cm (-2 SDs) was described more than 2 years after cessation of therapy [116]. This loss was most severe in pre-pubertal-treated children who had received high dose radiotherapy (≥33 Gy) to the entire spine [116]. In survivors treated before the age of 14, a small, but significant, loss of final height of 0.4 SD after doxorubicin, procarbazine, prednisone, vincristine, cyclophosphamide (MDP) and radiotherapy (n=69) was observed [117], which is a quite modest loss of height when compared with losses reported in patients treated for other common paediatric malignancies, such as acute leukaemia and brain tumours. Nevertheless, patients treated with chemotherapy and radiation at younger age and with higher radiation dosages are likely to sustain clinically significant decrements in height potential and therefore should be counselled accordingly. Nysom *et al* showed a reduced height after radiotherapy to the spine 11 years after diagnosis of childhood Hodgkin's lymphoma and non-Hodgkin lymphoma [118].

Scarce data are available on growth in childhood Hodgkin's lymphoma survivors after chemotherapy only. The 11 children reported by Papadakis *et al* treated with chemotherapy only had normal final heights [117]. Our own series of 88 Dutch childhood Hodgkin's lymphoma survivors treated with chemotherapy only, showed that only male survivors treated with MOPP had reduced height [119]. This might be explained by the fact that the men were younger at diagnosis and more

often treated at or before the time of peak height velocity compared with the women within this study.

From these studies, we can conclude that radiotherapy in a dose of >30 Gy including the spine (with or without chemotherapy) results in loss of height, most severe in pre-pubertal children. When lower dosages of radiotherapy are used, the impairment of skeletal growth appears to be minimal. Data on growth after chemotherapy only are scarce. Treatment with MOPP seems to cause a reduction in height as well [119]. Therefore, extended follow-up studies are necessary to determine the long-term effects on growth. Overall, when comparing these findings with other common paediatric malignancies (such as leukaemia and brain tumours), the loss of height after treatment for Hodgkin's lymphoma seems to be quite modest.

Osteoporosis and osteopenia

As bone mass is acquired during childhood and adolescence, disturbance of this process can result in a lower peak bone mass which subsequently results in osteoporosis and higher fracture rate in later life. In general, BMD is determined by several factors, such as gender, race, physical activity, calcium intake, smoking and alcohol consumption [120,121]. In young girls, the pubertal stage is the most important determinant of BMD, whereas in boys weight is the most important determinant [122].

Corticosteroids, which are frequently used in the treatment for Hodgkin's lymphoma, can cause osteopenia and osteoporosis by interference with both osteoblast and osteoclast function [123-125]. Apart from these direct effects, also indirect effects of chemotherapy, such as gonadal damage, may affect bone turnover [126-128]. Gonadal damage may cause impaired oestrogen production necessary to stimulate osteoblast activity and bone mass acquisition during puberty in females, but also in males [129-131]. Some chemotherapeutic agents such as cyclophosphamide and cisplatin can cause renal damage. This may cause deregulation of the calcium and vitamin D metabolism resulting in lower BMD [132]. In addition, it has been described that patients during therapy, but also cancer survivors, are generally physically less active in comparison with healthy controls [133]. In children with cancer, the lack of physical activity can potentially cause decreased BMD [134,135].

A decreased BMD is reported in female survivors of adult Hodgkin's lymphoma with POF as well as in male survivors [1,126-128,136]. Three studies reported a slightly reduced BMD, as measured by dual-energy X-ray absorptiometry (DXA), in survivors of childhood Hodgkin's lymphoma 9.4–15.5 years after diagnosis [118,119,137]. Compared with local reference values, the size-adjusted bone mass in one study was normal [118]. We have shown that female childhood Hodgkin's lymphoma survivors treated with alkylating chemotherapy (MOPP) without radiotherapy had a slightly, but significantly reduced BMD of the total body and, after correction for bone size, also of the lumbar spine [119]. Similarly, Sala *et al* described a reduced BMD of the lumbar spine in childhood Hodgkin's lymphoma survivors, which was correlated with the cumulative dose of corticosteroids [137]. Another study in childhood Hodgkin's lymphoma survivors reported negligible BMD deficits overall. However, males diagnosed at 14 years or older, were at 6.5 times higher risk than females for BMD deficits [138]. In a small series of children treated with prednisolone, chemotherapy and radiotherapy, anthropometric

traits or bone mass did not differ from those in the reference group and mediastinal or abdominal irradiation was not associated with BMD [139].

Overall, the effects of childhood Hodgkin's lymphoma treatment on BMD appear to be small but longer follow-up studies are needed to assess the consequences, especially the incidence of osteoporosis, and subsequent fractures in later life. The real impact of childhood Hodgkin's lymphoma treatment on BMD during the post-menopausal period is unknown, since it has only been possible to treat childhood cancer successfully over the last few decades. In addition, genetic variation, as reflected by allelic variation in specific single nucleotide polymorphisms, is an important denominator of bone mass in healthy post-menopausal women and also during therapy in certain types of childhood cancer [140-143]. As yet, it is unknown whether this allelic variation contributes to a decrease in BMD after the age of 50 in childhood cancer survivors.

Body composition

We and others reported that treatment with prednisolone is a risk factor for an increased percentage of body fat in different groups of childhood cancer survivors, especially in acute lymphoblastic leukaemia (ALL) survivors [144,145]. Only scarce data are available on body composition in survivors of childhood Hodgkin's lymphoma (Table III). Knowledge on this is important as higher fat mass and BMI increase the risk of metabolic syndrome and cardiovascular incidents in later life [146,147].

Nysom *et al* showed an increased percentage body fat in a small series of long-term survivors of childhood Hodgkin's lymphoma who were treated with combined modality treatment [148]. Our study in childhood Hodgkin's lymphoma survivors treated with chemotherapy only also revealed an increased percentage body fat [119]. The percentage body fat of patients treated with MOPP (a prednisolone-containing regimen) was comparable with that of patients treated without MOPP, indicating that prednisolone is not an important determinant of increased percentage body fat in these patients. The different influences of steroids in Hodgkin's lymphoma survivors when compared with ALL survivors may be due to the fact that the cumulative dose of prednisolone is substantially lower in Hodgkin's lymphoma than in ALL [124]. In childhood Hodgkin's lymphoma survivors, Nysom *et al* reported normal BMI, despite an increased percentage fat and explained this by a decreased lean body mass, although the lean body mass was not directly measured [148]. In our childhood Hodgkin's lymphoma survivor study, the median BMI was increased, while lean body mass was normal, indicating that increased fat mass indeed plays an important role [119]. In conclusion, although data is scarcely available, survivors of childhood Hodgkin's lymphoma tend to have an increased fat mass, which might increase the risk of developing the metabolic syndrome.

Thyroid

We identified 10 studies involving 4,012 survivors about thyroid complications after Hodgkin's lymphoma treatment during childhood (Table IV). After cervical region irradiation, a large proportion, up to 40%, of the childhood Hodgkin's lymphoma survivors reveal thyroid disorders, such as hypothyroidism, thyroid nodules and thyroid cancer [24,91,149-155] (Table IV). In most of the protocols, the mean radiation dose on the thyroid region was ≥35 Gy. Although hyperthyroidism (mainly Graves' disease) may occur after radiotherapy, it is less frequent than hypothyroidism

Table III | Studies on bone mineral density and growth in childhood Hodgkin's lymphoma survivors.

Author	n[a]	Age (range)[b]	Median follow-up	Therapy		Outcome		
				CT	RT	Growth	BMD	Body composition
Willman *et al* (1994)	124	9–16 years	>2 years	MOPP MOPP/ ABVD	124[2]	Loss of height with ≥33 Gy radiotherapy in pre-pubertal children	–	–
Papadakis *et al* (1996)	80	9.7 years (2.4–14.0 years)	>3 years	MDP	11[1], 58[4]	Reduced final height after radiotherapy. Lowest in children treated youngest	–	–
Nysom *et al* (2001)	44 (23 HL)	11.1 years (3.9–15.0 years)	10.5 years	Prednisone Methotrexate	1[1], 10[2] 6[3], 27[4]	Reduced height	Normal	Increased percentage fat
Sala *et al* (2007)	22	14.7 years (5.6–17.4 years)	>1 years	–	17[4]	–	Reduced BMD related to cumulative dose corticosteroids	–
Kaste *et al* (2009)	109	15.1 years (3.1–20.7 years)	7.5 years	Procarbazine, cyclophosphamide, methotrexate, predisone	39	–	Normal, males increased risk for SDS <–1.5	–
van Beek *et al* (2009)	88	11.6 years (3.7–17.2 years)	15.5 years	A(or E)BVD MOPP/ A(or E)BVD	18[4]	Reduced height in males treated with MOPP	Reduced BMD in females treated with MOPP	Increased percentage fat (female without MOPP) Normal lean body mass
Muszynska-Roslan *et al* (2009)	35	11.6 years (7.8-15.4 years)	6.3 years	Steroids CT without MTX	35	–	Normal	–

-=information not available; HL=Hodgkin's lymphoma; CT=chemotherapy; MOPP=mustine, vincristine, procarbazine, prednisone; ABVD=adriamycin, bleomycin, vinblastine, dacarbazine; MDP=doxorubicin, procarbazine, prednisone, vincristine, cyclophosphamide; EBVD=ABVD, epiadriamycin replaces adriamycin; RT=number of patients with [1]=gonads in radiation field, [2]=lumbar spine in radiation field, [3]=cranial irradiation, [4]=other fields of irradiation; a) number of survivors; b) age at diagnosis (median and range).

Table IV | Studies on thyroid complications in childhood Hodgkin's lymphoma survivors.

Author	n[a]	Age (range)[b]	Median follow-up	Therapy		Outcome			
				CT	RT	Hypo thyroidism	Hyper thyroidism	Carcinoma	Ultrasound abnormalities
Van den Berg *et al* (1997)	21	14 years (5–18 years)	5.0 years	MOPP/ ABVD	1	1/21	0/21	0/21	–
Soberman *et al* (1991)	18	14 years	6.4 years	–	18	7/18	–	1/18	16/18
Hancock *et al* (1991)	1787[c]	28 years (2–82 years)	9.9 years	MOPP MVP ABVD	1,677	513/1,677	32/1,677	6/1,677	44/1,671
Healy *et al* (1996)	46	12.5 years (4–16 years)	10.3 years	None	46	28/46	–	2/46	46/46
Sklar *et al* (2000)	1791	14 years (2–20 years)	>5 years	–	1,210	456/1,791	82/1,791	20/1,791	146/1,791
Atahan *et al* (1998)	46	8.5 years (2–18 years)	10.5 years	COPP COPP/ ABVD	46	22/46	1/46	–	–
Hudson *et al* (1993)	79	14.6 years (4.3–20.1 years)	4.1 years	COP/ABVD	79	19/79	0/79	2/79	0/79
Solt *et al* (2000)	26	10.8 years (2.1–16.4 years)	11.3 years	MOPP MOPP/ABVD	26	14/26	0/26	0/26	14/26
Van Beek *et al* (2009)	88	11.6 years 3.7–17.2 years	15.5 years	A(or E)BVD MOPP/ A(or E)BVD	18	5/88	0/88	0/88	–
O'Brien *et al* (2010)	110	11.3 years	20.6 years	ABVD/MOPP or MOPP	110	–	–	5/110	–

-=information not available; CT=chemotherapy; MOPP=mustine, vincristine, procarbazine, prednisone; COPP=cyclophosphamide, vincristine, procarbazine, prednisone; COP=cyclophosphamide, vincristine, procarbazine; ABVD=adriamycin, bleomycin, vinblastine, dacarbazine; EBVD=ABVD, epiadriamycin replaces adriamycin; MVP=melphalan, procarbazine, vinblastine; RT=number of patients with radiotherapy to the neck; a) number of survivors; b)age at diagnosis (median and range); c) paediatric patients (<17 yr): n=272 (analysis not specified for paediatric patients only).

[149,152,153]. Altogether, up to 40% of the childhood Hodgkin's lymphoma survivors treated with chemotherapy have impaired thyroid function [149,151,153]. In patients under the age of 17, the radiation dose has been shown to be the most important risk factor for developing hypothyroidism [149]. Female sex and older age at diagnosis have been reported as independent, predisposing risk factors increasing the risk for hypothyroidism [153].

In contrast, in studies of survivors of childhood Hodgkin's lymphoma treated with chemotherapy only, no hypothyroidism was reported [13,17,156] except for one single case [119]. However, when patients received additional radiation to the thyroid or mediastinum, six of seven either had abnormal levels of TSH or free thyroid hormone, or used thyroid hormones [13,119,156]. In a cohort of 205 childhood cancer survivors (of which 28.5% had either Hodgkin's lymphoma or non-Hodgkin's lymphoma), van Santen *et al* showed that the addition of chemotherapy did not increase the damage to the thyroid axis already caused by radiotherapy [157].

The risk of thyroid cancer after radiotherapy is also markedly increased compared with the otherwise healthy population. A large study in more than 1,700 childhood Hodgkin's lymphoma survivors reported 20 cases of thyroid carcinoma (RR 18.3) after a median follow-up of 15 years [153]. All these 20 patients received radiotherapy to the cervical region. Hancock *et al* reported that 6 out of 1,677 Hodgkin's lymphoma survivors (both children and adults) after combined modality treatment had thyroid cancer (RR 15.6) after a mean follow-up of 10 years [149,158]. However, this follow-up is relatively short for developing a secondary malignancy [13,17,119]. Recently, 5 out of 112 cases of thyroid carcinoma were reported in children treated with MOPP/ ABVD and lower dose radiotherapy after a median follow-up of 20 years [159]. The significant incidence of thyroid carcinoma in this low-dose radiation group is not surprising given the nonlinear radiation dose-response in which the thyroid second malignant neoplasm (SMN) risk increases from 0 to 20 Gy and then decreases, with few cases occurring at dosages above 40 Gy due to extensive cell killing [160]. In childhood Hodgkin's lymphoma survivors treated with chemotherapy only, to our knowledge, no cases of thyroid cancer have been reported so far. We studied 88 childhood Hodgkin's lymphoma survivors not treated with radiotherapy and a median follow-up of 15 years, and no cases of thyroid cancer were found [119]. Since the risk of thyroid cancer increases even after follow-up of more than 20 years [161], prolonged surveillance is necessary.

Altogether, hypothyroidism is the most common thyroid disorder after radiotherapy. There is a significant incidence of thyroid carcinoma in the low-dose radiation group, which is consistent with the hypothesis in which thyroid SMN risk increases from 0 to 20 Gy and then decreases due to cell killing. In survivors treated with chemotherapy only, hypothyroidism does not seem to occur and no cases of thyroid cancer have been reported. However, prolonged follow-up is necessary to evaluate the incidence after 20 years.

RECOMMENDATIONS AND PERSPECTIVES

Screening

No consensus exists yet on how to monitor long-term endocrine side effects after childhood Hodgkin's lymphoma treatment. The identification of novel markers opens new options for screening. However, there are several ways of detecting long-term endocrine effects (Table V).

Table V | Recommendations: screening for long-term endocrine effects in childhood Hodgkin's lymphoma survivors.

Recommendations	Patients	Physical examination	Advanced screening
Growth, osteoporosis	Irradiation to the spine	Height, sitting height, weight	Dual energy X-ray absorptiometry
Thyroid	Cervical region irradiation TBI	Palpation, TSH and free thyroid hormone	Thyroid ultrasound
Gonads: female	All, especially alkylating agents, pelvic/ TBI	Tanner stadium Day 2-5: LH, FSH, inhibin B, AMH	Gynaecologist: antral follicle count
Gonads: male	All, especially alkylating agents, pelvic/ TBI	Tanner stadium Sperm analysis, testis volume, LH, FSH, inhibin B	–

TBI=total body irradiation; AMH=anti-Müllerian hormone; LH=luteinizing hormone; FSH=follicle-stimulating hormone.

Gonads

In male survivors, evaluation of testicular function should consist of assessment of Tanner stage, testicular volume and consistency and sperm analysis as well as assessment of LH, FSH and inhibin B serum measurement. Inhibin B, produced by the Sertoli cells, is a marker that correlates strongly with spermatogenesis. So far, semen analysis has been the gold standard. However, inhibin B is less invasive and seems to be a reliable marker for gonadal (dys)function. Inhibin B serum levels <150 ng/L seems to be indicative for gonadal damage. Consequently, a sperm analysis should be performed and the patient should be referred to an andrologist.

In female patients, the evaluation should consist of the Tanner stage and the assessment of LH, FSH, inhibin B and AMH. A transvaginal ultrasound might be performed to determine the antral follicle count. LH, FSH and inhibin B should be determined on day 2–5 of the menstrual cycle. If the patient uses oral contraceptives, hormone measurements should be performed a minimum of 1 week after the last pill was taken to minimize the effects of the pills on the gonadal markers [162]. In contrast to FSH, AMH levels seem to be constant during the menstrual cycle [163-167]. Serum AMH is currently the most reliable serum marker for ovarian reserve [65,76,168]. Recently, the correlations between ovarian primordial follicle count and antral follicle count and AMH were described, where the antral follicle count (r=0.78) showed a greater correlation with the ovarian primordial follicle count than AMH [169]. However, the difference was relatively small. First, the

predictive value of AMH in identifying those women with a diminished ovarian reserve and hence absolute infertility remains to be established. Therefore, ovarian ultrasound to assess the number of small antral follicles remains important.

Growth and osteoporosis

Proper history taking and complete physical examination (including height, sitting height and weight) should be performed in patients treated with radiotherapy that included the spinal areas. Survivors of childhood Hodgkin's lymphoma treated with lower dosages of radiotherapy do not seem to be prone to develop growth disorders and osteoporosis. If a patient is treated with high dosages of radiotherapy and is at risk for osteoporosis after taking a proper history and complete physical examination, DXA scans should be performed, which is an easy and reliable way to assess BMD and body composition. If the BMD is decreased with clinical impact, treatment with bisphosphonates could be considered with caution since they remain in the skeleton for decades. Whether the long-term inhibition of bone turnover can be harmful over time remains unresolved [170]. In human, no studies that investigate teratogenicity have been performed, but in animal studies, teratogenicity was suggested [171]. Hence, bisphosphonates should not be used during pregnancy. First, it should be investigated whether the decreased BMD is caused by POF. If this is the case, hormone replacement therapy should be considered first.

Thyroid

Since patients treated with irradiation to the cervical region or total body are at risk for developing thyroid disorders such as hypothyroidism, thyroid nodules and thyroid cancer, these survivors should be included in a specific follow-up programme. Thyroid function can easily be followed using TSH and free thyroid hormone in both male and female survivors. To detect morphological changes of thyroid tissue, an ultrasound of the thyroid can be useful, especially when radiotherapy to the cervical region was administered [151,154]. However, it is important to note that benign changes will be observed that may lead to unnecessary invasive procedures. There is an ongoing debate about implementing this screening because of the high rate of false-positive findings. We advise performing the physical examination (palpation of the thyroid) and measuring TSH and free thyroid hormone levels. If these levels are abnormal, supplementation should be started in order to normalize thyroid function.

Prevention

There is no worldwide consensus on the prevention of long-term endocrine side effects. National and international experts are in the process of formulating guidelines. Most important is to adjust treatment to decrease toxicity as much as possible without increasing risk of relapse. Because this is difficult, preservation of gonadal material is an attractive option. Commonly used techniques to preserve gonadal function include sperm, embryo and oocyte cryopreservation and are advised for all applicable patients who are at risk for infertility [104]. Semen cryopreservation in boys is a feasible method to preserve spermatozoa before gonadotoxic therapy is started and should be offered to all pubertal boys from 12–13 years of age despite their young age [172]. Semen is

obtained after masturbation in the majority of the boys. When this is impossible, penile vibration or electroejaculation should be considered [173].

No protection was observed when using GnRH-a in young women treated for advanced-stage Hodgkin's lymphoma [105]. Preventive therapeutic methods such as oocyte vitrification, preservation of entire ovaries and oophoropexy are promising, but remain subject of further research [174,175].

CONCLUSIONS

Childhood cancer survival is increasing. Childhood Hodgkin's lymphoma survival has reached 90% and therefore long-term effects of treatment have gained increasing interest. As shown in this review, the severity of the endocrine toxicity after childhood Hodgkin's lymphoma depends mainly on the type of treatment. Gonadal dysfunction seems to be the most severe endocrine long-term effect. In survivors treated with alkylating agents or pelvic radiotherapy, severe gonadal damage is described and seems to be irreversible in women and partly in men. However, prolonged follow-up is necessary to determine potential recovery over the years. Nowadays, many centres have specific follow-up programmes to detect and, if necessary, treat the long-term side effects of cancer treatment during childhood. The knowledge obtained in these outpatient clinics can help in the search for the optimal balance between cure and late effects in treatments for childhood and adolescent Hodgkin's lymphoma.

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CHAPTER 3

Decreased serum anti-Müllerian hormone levels in girls with newly diagnosed cancer

W van Dorp, MM van den Heuvel-Eibrink, ACH de Vries,
SMF Pluijm, JA Visser, R Pieters, JSE Laven

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ABSTRACT

Study question: Are anti-Müllerian hormone (AMH) levels reduced in girls with newly diagnosed cancer before the start of treatment?

Summary answer: AMH levels are already compromised in girls at the time of cancer diagnosis compared with healthy girls.

What is known already: In women diagnosed with cancer, evidence of reduced ovarian function has been described even before treatment has started. In girls with newly diagnosed cancer, no data are available.

Study design, size, duration: We performed an age-matched case-control study in girls with newly diagnosed cancer.

Participants/materials, setting, methods: We determined serum AMH levels in a cohort of 208 girls with newly diagnosed cancer, up to 18 years of age at diagnosis, and compared them with AMH levels of 250 age-matched healthy girls. The diagnoses included were acute lymphoblastic leukaemia, acute myeloid leukaemia, Hodgkin lymphoma, non-Hodgkin lymphoma, nephroblastoma, sarcoma, and neuroblastoma.

Main results and the role of chance: The median age was 6.6 years (range 0.0-17.4), comparable with that in the control group (median 6.3 years, range 0.3-18.0). Girls with childhood cancer presented with significantly lower serum AMH levels compared with healthy age-matched controls (standard deviation scores (SDS) -0.8, $p<0.001$). Median AMH level in patients was 1.4 µg/L (0.1–10.2) versus 3.0 µg/L (0.1–18.3) in controls. Specifically, 84% of all patients had AMH levels below the 50th percentile of normal AMH levels, and 19% below the 10th percentile. Surrogate markers of general health status (temperature, C-reactive protein and haemoglobin levels at diagnosis) were significantly correlated with AMH SDS.

Limitations, reasons for caution: Some caution is warranted because AMH levels increase with age in healthy children but the cases and controls were age-matched in our study. Although our sample size was large, additional studies are still required in an independent cohort.

Wider implications of the findings: Our study shows that AMH levels are reduced in girls with newly diagnosed cancer even before the cancer treatment has started. AMH levels correlate with impairment of general health status in girls. Therefore, besides (pre) antral follicle number, other factors may influence serum AMH levels. Longitudinal studies during and after childhood cancer are currently being performed in order to evaluate possible ovarian recovery after discontinuation of treatment.

INTRODUCTION

Overall survival of childhood cancer has increased dramatically over the past decades, which has urged clinicians to pay attention to short- and long-term adverse effects of cancer treatment [1,2]. A well-known long-term side effect of cancer treatment in both adult and childhood cancer survivors is gonadal dysfunction, often resulting in impaired fertility [3,4]. The magnitude of this impairment depends on the treatment modality, the total cumulative dosages, as well as on the genetic susceptibility of a cancer survivor [3,5-8]. However, data addressing the potential influence of the disease itself on ovarian function are sparse [9].

The assessment of ovarian reserve in adults – including a clinical evaluation of secondary sexual characteristics, menstrual history, transvaginal ultrasound during the early follicular phase including an antral follicle count of the ovaries, and FSH, LH, oestradiol and progesterone levels – is not informative in pre-pubertal girls since the hypothalamic-pituitary-gonadal axis has not been activated yet.

Recently, it has been suggested that anti-Müllerian hormone (AMH) constitutes as a reliable indirect serum marker of ovarian function in healthy pre-pubertal and post-pubertal girls, as well as in girls with childhood cancer [9,10]. AMH is produced by granulosa cells of small growing follicles and indirectly reflects the size of the primordial follicle pool in the ovaries [11]. In peri-pubertal girls, minor fluctuations in AMH with slightly increasing levels prior to pubertal onset and decreasing levels after onset of puberty have been described [12]. After puberty, serum AMH levels are stable within and between menstrual cycles, in contrast to FSH [13-16]. Up to an age of 25 years, AMH levels gradually increase and thereafter a linear decrease occurs with low or undetectable levels at the time of menopause [17].

In adult women with Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL) (n=64), it has been shown that AMH levels are already reduced before the start of cytotoxic chemotherapy [18]. This indicates that not only therapy but also the disease or subsequent health status may influence ovarian function later in life. Moreover, also in adolescent and young adult women diagnosed with HL significant damage to gametes has been observed before the start of treatment [19].

Likewise in boys a considerable percentage had oligospermic or even azoospermic counts at the time of cancer diagnosis [20]. The reason for this impaired testicular function is unclear, but a direct effect of the disease and compromised general health status seem to play an important role.

In girls with newly diagnosed cancer, only scarce data on AMH levels before cancer treatment are available. Knowledge regarding this issue is however important because it might relate to potential recovery after treatment. Brougham and colleagues observed slight but not significant lower AMH levels before treatment in a limited number of youngsters with various types of cancer [9]. To address this issue in more detail, we studied AMH levels in a large single-centre cohort of girls at the time of diagnosis of childhood cancer, before the start of treatment.

MATERIALS AND METHODS

Subjects

We included girls up to the age of 18 years with newly diagnosed childhood cancer at our Paediatric Oncology Centre. Patients with brain tumours were excluded due to possible hypothalamic-pituitary-axis dysfunction, and patients with germ cell tumours were excluded because of the localisation of the tumour in the ovary and/or direct influence on hormone production.

Details on age, diagnosis and pubertal stage were retrieved from our local database. Pubertal status was assessed clinically at diagnosis and classified as pre-pubertal (Tanner stage 1), mid-pubertal (Tanner stage 2–3), or late pubertal (Tanner stage 4–5) [21]. AMH levels were measured in left-over serum after diagnostic work-up. Informed consent was obtained from all included patients and parents who agreed to use left-over material for this study according to the standards of the Institutional Review Board.

Controls

As a control group, we included healthy girls and adolescents aged up to 18 years (n=250) from our previously published nomogram study [17]. Detailed information on recruitment strategy and study populations is available in the original paper.

Laboratory measurements

Serum AMH levels in both subjects and controls were measured at the diagnostic endocrine laboratory at the Erasmus Medical Centre (Rotterdam, the Netherlands). AMH levels were measured using an in-house double-antibody enzyme-linked immunosorbent assay (commercially available as the GenII Beckman Coulter, Beckman Coulter, Inc., Webster, TX, USA) as previously described [22]. All samples were stored using the same protocol at -20°C until assayed. AMH immunoreactivity in serum samples was stable after repeated freeze-thaw cycles. Intra- and inter-assay coefficients of variation were <5 and <10%, respectively [22,23].

Statistics

AMH levels were normally distributed after log-transformation. AMH levels were analysed as continuous variables, as well as standard deviation scores (SDS) and percentiles. AMH percentiles were based on the percentiles of 250 healthy girls: <10^{th} percentile, 10^{th} percentile–50^{th} percentile, 50^{th} percentile–90^{th} percentile, and >90^{th} percentile [17]. As AMH is age-dependent, SDS were calculated to correct for age, based on previously reported AMH levels of (age-matched) 250 healthy girls from our institute [17]. Nonparametric and chi-square tests were used to compare included and excluded patients, and to compare AMH levels of patients and controls. SDS of girls with childhood cancer were compared with healthy references using the one sample t-test. Linear regression was used to compare AMH SDS between subsets of diagnoses, and to study the association between the surrogate markers of general health status (temperature at diagnosis, haemoglobin (Hb) levels and C-reactive protein (CRP) levels), and AMH SDS. AMH SDS according to Tanner stages were evaluated using the Mann-Whitney *U* test. P-values of <0.05 were considered significant. All statistical analyses

were performed using the IBM Statistical Package for Social Sciences version 20 (IBM Corp., Armonk, NY, USA).

RESULTS

In 208 girls with cancer AMH levels were measured at the time of diagnosis at the Erasmus Medical Centre-Sophia's Children's Hospital Rotterdam. Seventy-seven girls (36.5%) were diagnosed with acute lymphoblastic leukaemia, 19 (9.1%) with acute myeloid leukaemia, 32 (15.4%) with HL, 18 (8.7%) with NHL, 22 (10.6%) with nephroblastoma, 23 (11.5%) with sarcoma and 17 (8.2%) with neuroblastoma.

The median age was 6.6 years (range 0.0–17.4) and comparable with the median age of girls in the control group (median 6.3 years, range 0.3–18.0, p=0.44). Girls diagnosed with cancer had significantly lower pre-treatment AMH levels compared with the age-matched controls (SDS -0.8, p<0.001) (Figure I). Median AMH level in patients was 1.4 µg/L (0.1–10.2) versus 3.0 µg/L (0.1–18.3) in the controls. In all tumour types median AMH SDS was below zero, but no significant differences were observed between the malignancies (Figure II).

Of all girls with newly diagnosed cancer, 83.7% had AMH levels below the 50th percentile, and 19.2% had AMH levels below the 10th percentile (Table I).

Table I | Anti-Müllerian hormone levels in girls with newly diagnosed cancer compared with the 10th, 50th and 90th percentiles in age-matched controls.

	n	<p10	%	p10-<p50	%	<p50	%	p50-p90	%
ALL	77	14	18.2	49	63.6	63	81.8	14	18.2
AML	19	4	21.1	14	73.7	18	94.7	1	5.3
HL	32	9	28.1	18	56.3	27	84.4	5	15.6
NHL	18	4	22.2	10	55.6	14	77.8	4	22.2
Nephroblastoma	22	1	4.5	17	77.3	18	81.8	4	18.2
Sarcoma	23	3	13.0	15	65.2	18	78.3	5	21.7
Neuroblastoma	17	5	29.4	11	64.7	16	94.1	1	5.9
Total	208	40	19.2	134	64.4	174	83.7	34	16.3

ALL=acute lymphoblastic leukaemia; AML=acute myeloid leukaemia; HL=Hodgkin lymphoma; NHL=non-Hodgkin lymphoma.

Tanner stages were reliably documented in 161 patients. Fourteen girls (8.7%) were classified as being late pubertal, 15 as being mid-pubertal (9.3%) and 132 girls (82.0%) were pre-pubertal. AMH SDS were not significantly different between the Tanner categorical subgroups (p=0.15).

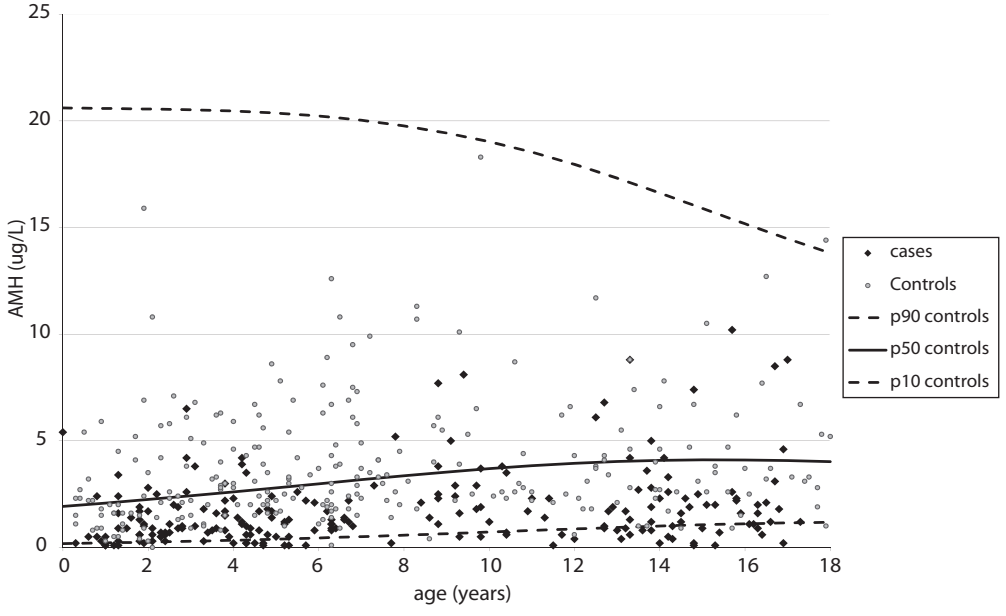

Figure I | Pre-treatment anti-Müllerian hormone (AMH) levels in girls with newly diagnosed childhood cancer (n=208) when compared with AMH levels in a cohort of 250 age-matched healthy girls (p<0.001, one sample t-test). p90, p50 and p10 refer to 90th, 50th and 10th percentiles, respectively.

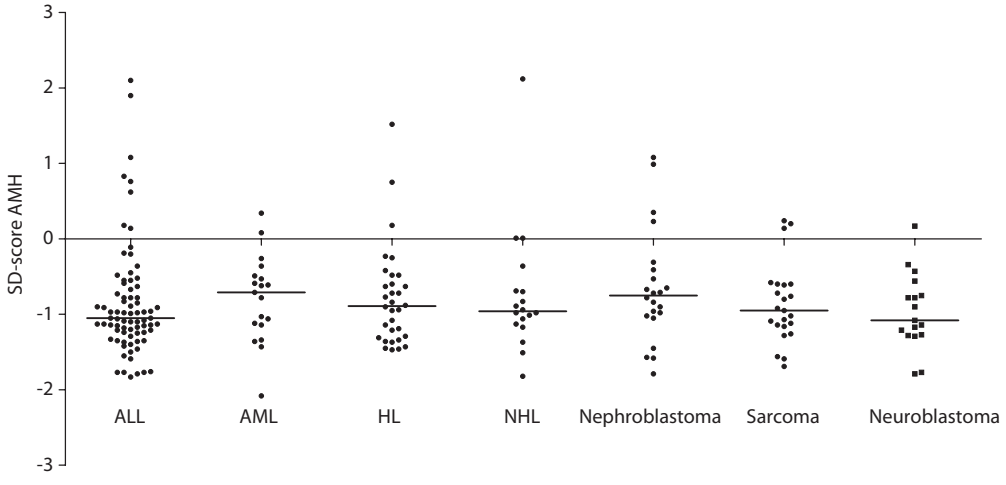

Figure II | Pre-treatment AMH standard deviation scores (SDS) by diagnosis for girls diagnosed with childhood cancer (n=208).

ALL=acute lymphoblastic leukaemia; AML=acute myeloid leukaemia; HL=Hodgkin lymphoma; NHL=non-Hodgkin lymphoma.

Temperature at diagnosis, a surrogate marker of general health status, was inversely correlated with AMH SDS (β -0.119, 95% confidence interval (CI) -0.235; -0.003, p=0.044) as was CRP with AMH SDS (β -0.002; 95% CI: -0.004; -0.000, p=0.016), while Hb levels and AMH SDS were positively correlated (β 0.064, 95% CI: 0.005; 0.123, p=0.033).

DISCUSSION

In the present study, we have shown that AMH levels in girls with newly diagnosed cancer are reduced compared with age-matched controls, even before chemotherapy or radiotherapy has started.

The rationale for these reduced levels is unclear. Previous studies in adult women with HL or NHL, but also in adults with systemic diseases, such as diabetes mellitus, systemic lupus erythematosus and cardiovascular diseases, suggest that the compromised general health status due to the consumptive character of the disease decreases AMH levels. In these patients, a decreased AMH production in the small growing follicles inflicted by the chronic disease, an increased metabolism of AMH in cancer patients, or a more rapid decline of the primordial follicle pool might be present [24-27]. In order to gain at least some insight into the influence of a compromised health status, we studied some indirect markers of general illness, i.e. temperature, CRP and Hb levels. Indeed, we found a negative association between temperature, CRP and AMH SDS, while a positive association was observed between Hb levels and AMH SDS. These findings indicate that the pre-treatment general health status might contribute to the decreased AMH levels, suggesting that factors other than (pre) antral follicle number may influence serum AMH levels.

Another potential cause of the decreased AMH SDS independently of childhood cancer subtype might be impaired granulosa cell function due to an impaired DNA repair mechanism. Genetic variation mapping to DNA repair genes has been associated with both cancer and ovarian ageing [28,29]. Other factors, such as stress, cannot be excluded. It would be interesting to compare AMH levels in different groups of young girls including those with other severe illnesses in order to study the effect of illness and stress. Whether the reduced AMH levels are reversible and what the mechanism of this accelerated reduction of AMH levels is, is currently unknown. In the normal situation AMH levels reflect the number of growing follicles, and hence AMH has been suggested to be a marker of the quantitative aspect of ovarian reserve. However, when health status is compromised, this may affect granulosa cell function, leading to decreased serum AMH levels without affecting the follicle count. Hence, AMH might be a marker of ovarian function, a reduced primordial follicle pool, or a combination thereof in girls with newly diagnosed cancer.

We analysed AMH levels in a substantial sample of girls with newly diagnosed cancer. Other ovarian function markers, such as FSH and inhibin B, were not measured as it has been reported that FSH and inhibin B are no reliable markers in pre-pubertal girls with cancer [9]. Inhibin B levels were undetectable in the majority of pre-pubertal female patients with cancer, and FSH levels did not increase during cancer treatment illustrating the quiescence of the hypothalamic-pituitary axis during pre-puberty. In adults, AMH is strongly correlated with the antral follicle count and is

relatively constant during and between menstrual cycles [13-16]. Nevertheless, some caution is warranted since AMH levels increase with age in healthy children and thereafter remain stable until early adulthood [17,30,31]. Moreover, it is important to recall that AMH is an indirect marker for the number of primordial follicles in the ovary. Nelson *et al* showed variation in AMH levels in the first 2–3 years of life before an accelerated increase from 4 years onwards [31]. However, further data show that AMH rises until the age of 25 years, after which AMH decreases until the age of menopause [17]. Hagen *et al* [10] showed the same increase, but all three models do show considerable variability during infancy [17,30,31]. It has been suggested that the increase and the variability during infancy might be explained by an increase of the number of granulosa cells producing AMH or that the number of small growing follicles, in which AMH expression is strongest, increases [32]. Hagen *et al* observed a post-natal peak in AMH levels, which was also reported by others [10,33]. A suggested explanation for this increase was shown by the temporal relationship between increased follicular growth and post-natal AMH levels, probably in response to the post-natal FSH surge stimulating ovarian folliculogenesis and AMH transcription [33]. The variation in the timing of this process might at least partly explain the AMH variability. In order to minimize the influence of age and variation during the early years, we compared AMH levels in girls with childhood cancer to age-matched controls. However, the effect of variability cannot be completely ruled out, and therefore caution is warranted when extrapolating these results to other populations. Lastly, although the number of patients analysed in this study is larger than in previous studies, the number is still relatively small. Therefore, we recommend to replicate our study in a larger independent cohort of survivors of childhood cancer.

In adults, pre-treatment AMH levels seem to be important for the interpretation of treatment-related ovarian damage during follow-up. It has been shown that baseline AMH levels affect the magnitude of acute changes in ovarian reserve from chemotherapy, and that the rate of recovery of AMH is determined by pre-treatment levels [34]. The present study is the first large study on AMH levels in girls with newly diagnosed cancer. However, data on recovery are not yet available. Longitudinal studies during and after childhood cancer are currently being performed to determine whether AMH levels recover (i.e. increase) with improved health status.

In conclusion, we show that AMH levels in girls with newly diagnosed cancer are already compromised before treatment starts, suggesting that the disease itself and/or the subsequent health status affects AMH levels. Therefore, besides (pre) antral follicle number, other factors may influence serum AMH levels.

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CHAPTER 4

Characteristics and outcome of paediatric non-Hodgkin lymphoma patients with ovarian infiltration at presentation

W van Dorp & C Owusuaa, JSE Laven,
MM van den Heuvel-Eibrink & A Beishuizen

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ABSTRACT

Background: Ovarian infiltration in paediatric non-Hodgkin lymphoma (NHL) at presentation is rare and information on outcome is scarce and mainly based on case reports and small series.

Procedure: Evaluation of clinical characteristics and outcome of ovarian infiltrated paediatric NHL cases of a single centre, and an extensive review of all cases reported so far in literature.

Results: At presentation, 6/60 female NHL cases of our centre had ovarian infiltration, and combining these cases with previous case reports, a total of 42 cases were identified. Median age at presentation was 10.9 years (range 0−18), and all but one had a B-cell immunophenotype, with 32/42 cases being classified as Burkitt. Bilateral involvement was reported in 26/41 cases, of which 22 were bilaterally ovariectomized as first treatment. All cases were treated with chemotherapy. Relapses were reported in 9/36 and death in 16/36. After follow-up in our centre (median 13.4 years), in 2 cases with ovarian infiltration anti-Müllerian hormone (AMH) values were available (2.1 and 0.9 µg/L). In non-infiltrated cases, median AMH level was 2.2 µg/L.

Conclusions: We conclude that in case of ovarian tumours with negative tumour markers (AFP and β-HCG), NHL should be considered in order to avoid unnecessary surgery.

INTRODUCTION

Lymphomas account for 10–15% of all paediatric cancers of which the non-Hodgkin lymphoma (NHL) group represents 60% [1]. The most common sites of presentation of NHL are the mediastinum, neck, and abdomen [2]. At initial presentation, lymphomas rarely involve the ovaries, in children as well as in adults. The scarce information on clinical presentation of ovarian infiltrated paediatric NHL is mainly based on case reports and small series combining ovarian infiltration in adults and children, and the frequency based on cohort studies is unknown [3,4]. In addition, it is unknown what the influence is on gonadal function later in life. In the current report, we present (1) a retrospective single-centre analysis of the frequency, clinical features and outcomes of ovarian infiltration at presentation of girls diagnosed with NHL in our institution, and (2) an extensive review of the clinical characteristics and survival of all well-documented ovarian infiltrating NHL cases in childhood reported in the literature.

METHODS

Single-centre data analysis

From 1966 to 2012, 160 consecutive paediatric NHL cases were diagnosed at the Paediatric Oncology/ Haematology department of the Erasmus MC-Sophia Children's Hospital, of which all 60 girls were included in this retrospective survey. Medical records were reviewed for clinical characteristics, i.e. sex, age at diagnosis, presenting symptoms, tumour characteristics, subtype (radiological, histopathological and immunophenotypical reports), disease stage (computed tomography (CT) or ultrasound) at baseline, upfront chemotherapy, and other types of treatment (ovariectomy, other surgery, abdominal radiotherapy, and stem cell transplantation), as well as outcome parameters. The diagnosis of ovarian infiltration of NHL was derived by findings on abdominal ultrasound, which is together with CT, MRI and PET scans a common diagnostic tool in the staging procedure for NHL in children, and confirmed in 4/6 by CT/ MRI scans. In 2 cases (diagnosed in 1982 and 1990), only ultrasound was performed.

Serum AMH level assessment

Serum anti-Müllerian hormone (AMH) was used as a proxy for gonadal function in our female childhood cancer survivors (CCS) [5-8]. Serum AMH levels were measured using an in-house double-antibody enzyme-linked immunosorbent assay; intra- and interassay coefficients of variance (CVs) were <10% and <5%, respectively [9,10], and compared to AMH levels of women from the general population. These women were proven fertile or had regular menstrual cycles [11].

Literature review

We conducted searches in the electronic databases PubMed, Embase, Medline, Cochrane, and Web of Science in January 2012, using the following key words and their synonyms: ovary, non-Hodgkin lymphoma, child. Studies were eligible for selection if NHL cases were individually well-documented;

aged less than 19 years at diagnosis; NHL at baseline; ovarian infiltration at presentation; the article was published in a peer-reviewed scientific journal written in the English or Dutch language. After removing the duplicates, the author screened titles and abstracts to select eligible studies. Full text papers were obtained of the selected papers, and were excluded by the author if studies did not met the inclusion criteria. The complete search strategy is available on request.

RESULTS

Descriptives of single centre study

Six of the 60 girls (10%) diagnosed with NHL in our centre had ovarian infiltration at diagnosis (Table I). The median age at diagnosis of these cases was 10.5 years (range 3.1–15.7). All six (four unilateral, two bilateral) were stage III, according to the Murphy's staging system [12], and were immunophenotyped as B-cell (Burkitt type) NHL. The most common presenting symptoms were abdominal pain and/or mass (n=6/6), and one case was complicated by hydronephrosis (n=1/6). Alpha-fetoprotein (AFP) and beta-human chorionic gonadotropin (β-HCG) were within the normal range in all six cases. All patients were treated with multi-agent chemotherapy. One patient had upfront bilateral ovariectomy, which was performed in 1982 because of 'malignant appearance' during laparotomy at presentation according to the surgical reports. Two patients were treated with unilateral ovariectomy; the first for debulking of the tumour and the second because of incomplete response to chemotherapy. Abdominal radiotherapy was administered in one patient, seven months post diagnosis due to progression of the disease. None received stem cell transplantation (SCT). Two out of six patients had disease progression within six months from diagnosis and eventually died due to progressive and incurable disease. The characteristics and outcome of the ovarian infiltrated as well as the other NHL cases are depicted in Table I.

Table I | Patient characteristic at baseline in the single-centre cohort.

	Ovarian infiltration	No ovarian infiltration	Total
n total	6	54	60
Median age at diagnosis (in years)	10.5 (3.1–15.7)	9.1 (0.4–16.4)	9.1 (0.4–16.4)
Presenting symptoms/physical exam			
– General (fever, fatigue, weight loss)	2 (33.3)	25 (46.3)	27 (45.0)
– Abdominal symptoms	5 (83.3)	19 (35.2)	24 (40)
– B-symptoms	1 (16.7)	4 (7.4)	5 (8.3)
– Palpable nodes	1 (16.7)	16 (29.6)	17 (28.3)
– Abdominal mass	4 (66.7)	24 (44.4)	28 (46.7)
– Ascites	1 (16.7)	1 (1.9)	2 (3.3)
– Hepatosplenomegaly	1 (16.7)	7 (13.0)	8 (13.3)
– Pleural fluid	0	4 (7.4)	4 (6.7)

Table I | *Continued*

	Ovarian infiltration	No ovarian infiltration	Total
Subtype			
– Burkitt lymphoma	6 (100.0)	11 (20.4)	17 (28.3)
– DLBCL	0	7 (13.0)	7 (11.7)
– T-LBL	0	17 (31.5)	17 (28.3)
– Precursor B-NHL	0	4 (7.4)	4 (6.7)
– ALCL	0	13 (24.1)	13 (21.7)
– Unclassified	0	2 (3.7)	2 (3.3)
Organ infiltration			
– Ovary	6	0	6 (10.0)
– Kidney	2 (33.3)	5 (9.3)	7 (11.7)
– Liver	1 (16.7)	2 (3.7)	3 (5.0)
– Spleen	0	2 (3.7)	2 (3.3)
– Pancreas	0	1 (1.9)	1 (1.7)
– Mesentery	0	4 (7.4)	4 (6.7)
– Intra-abdominal	0	5 (9.3)	5 (8.3)
– Mediastinum	1 (16.7)	19 (35.2)	20 (33.3)
– Lymph noduli	1 (16.7)	24 (44.4)	25 (41.7)
– Bone marrow	0	8 (14.8)	8 (13.3)
– CNS	0	4 (7.4)	4 (6.7)
– Skin	0	4 (7.4)	4 (6.7)
– Other	0	3 (5.6)	3 (5.0)
Stage			
– Stage I	0	12 (22.2)	12 (20.0)
– Stage II	0	9 (16.7)	9 (15.0)
– Stage III	6 (100.0)	16 (29.6)	22 (36.7)
– Stage IV	0	17 (31.5)	17 (28.3)
Other therapy			
– Abdominal radiotherapy	1 (16.7)	0 (0.0)	1 (1.7)
– Stem cell transplantation	0	3 (5.6)	3 (5.0)
– Ovariectomy	3 (50.0)*	2 (3.7)	5 (8.3)
– Ileum resection	0	5 (9.3)	5 (8.3)
Event			
– Relapse	0	11 (20.4)	11 (18.3)
– Death	2 (33.3)	14 (25.9)	16 (26.7)
Median time diagnosis – relapse (in months)	-	6.5 (2.6–12.1)	6.5 (2.6–12.1)
Median time diagnosis – death (in months)	6.9 (6.4–9.0)	11.0 (6.4–140.1)	9.6 (4.8–140.1
Median time relapse – death (in months)	2.1 (1.0–3.1)	4.3 (0.3–137.1)	3.9 (0.3–137.1)

Data is presented in median (range) or n (%). N=number; DLBCL=diffuse large B-cell lymphoma; T-LBL=T-cell lymphoblastic lymphoma; Precursor B-NHL=pre-B-cell lymphoblastic lymphoma; ALCL=anaplastic large cell lymphoma; CNS=central nervous system; AMH=anti-Müllerian hormone; *=of which two were unilateral ovariectomy and one was bilateral at diagnosis in 1982.

In the long-term survivors (n=44), serum AMH levels were available in 25 cases, including 3/6 cases with ovarian infiltration. Two of these three cases, aged 14.8 and 16.3 years at time of screening, had been treated with chemotherapy only (AMH levels 2.1 and 0.9 µg/L), and one case aged 28.4 years at screening had been treated with a bilateral ovariectomy (AMH level <0.1 µg/L). Follow-up times were respectively 1.3, 7.3, and 24.3 years. None of these three women underwent abdominal radiotherapy. The 22 female CCS without ovarian infiltration had a median serum AMH level of 2.2 µg/L (range <0.1−8.1) at a median follow-up time of 13.4 years (range 0.3−27.7) and a median age of 22.9 years (range 4.5−40.3) at screening. Figure I shows the serum AMH levels of our single-centre female NHL CCS, in comparison with serum AMH levels of Dutch controls [11].

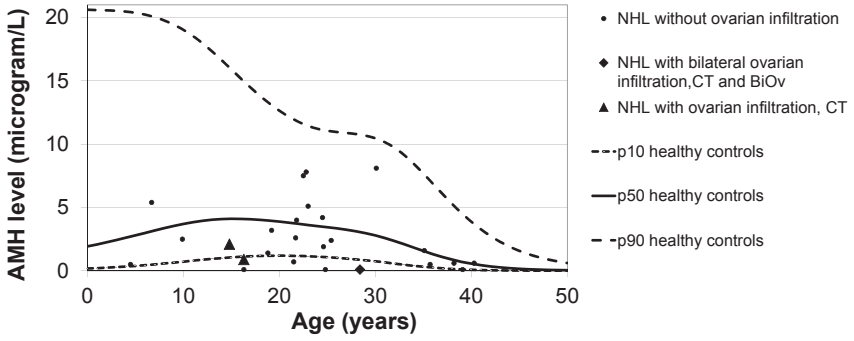

Figure I | Serum AMH levels in NHL survivors in single-centre cohort.

Anti-Müllerian hormone levels in NHL patients as compared to the p10, p50 and p90 in 250 healthy controls [11]. NHL=non-Hodgkin lymphoma; CT=chemotherapy; BiOv=bilateral ovariectomy.

Literature review of ovarian infiltrated NHL

The combined search resulted in 537 articles. After screening the titles and abstracts, 64 articles met the inclusion criteria and were retrieved for further assessment. Twenty manuscripts were selected based on inclusion and exclusion criteria. Of the selected articles, a cross-reference of related articles, references and citing articles was performed. This yielded no further manuscripts for inclusion. This search resulted in 36 well documented cases of childhood ovarian infiltrated NHL, enabling a review of clinical characteristics and outcome of 42 cases in total (Table II). The median age was 10.9 years (range 0.9−18.0) at presentation. Except for one incidental finding, all cases presented with abdominal pain and/or abdominal mass. Ascites at presentation was found in 9/42 cases, fever in 10/42 cases, and 5/42 cases had respiratory symptoms. The immunophenotype was described in 42 cases. All but one had a B-cell immunophenotype, with 32/42 cases being classified as Burkitt and 9/42 as B-NHL. One patient was diagnosed with ALCL with a t(2;5) fusion gene, with unavailable specific information on the immunophenotype.

Bilateral ovarian involvement at diagnosis was described in 26/41 cases (63.4%). In one case, the side of ovarian infiltration was not specified. Concomitant infiltration of other organs than the ovary was described in 22/42 cases, and included other intra-abdominal sites (uterine tubes, uterus, small

intestine, appendix, mesentery, retroperitoneum, liver and the stomach; n=22/42), orbita (n=1/42) and chest (mediastinal enlargement and pleural effusion; n=6/42). Data on disease stage, CNS and BM involvement at presentation were available for respectively 34, 32 and 36 cases. Based on the Murphy's staging system 4/34 stage II and 29/34 cases were stage III. Only one case was diagnosed with stage IV due to CNS involvement. None had BM infiltration (Table II).

Chemotherapy was administered in 38/42 cases, ovariectomy in 34/41 and abdominal radiotherapy in 6/41 cases. Upfront ovariectomy was performed in 31/41 cases (9 were performed unilateral and the other 22 bilateral). Upfront chemotherapy was administered in 10/41 and one case was initially treated with abdominal radiotherapy. Two other cases were treated with unilateral ovariectomy after failure or incomplete response to chemotherapy. Other types of surgery because of complications or infiltration of the disease, such as hemicolectomy, cystectomy, hysterectomy, appendectomy, and omentectomy, were described in 14/41 cases (Figure II). 36/42 cases had available data on clinical outcome. Relapses occurred in 9/36 of the well-described cases; all but one resulted in death. Eight other patients were reported to die of extreme toxicity, that is, extreme progressive cachexia, complete intestinal obstruction, and sepsis.

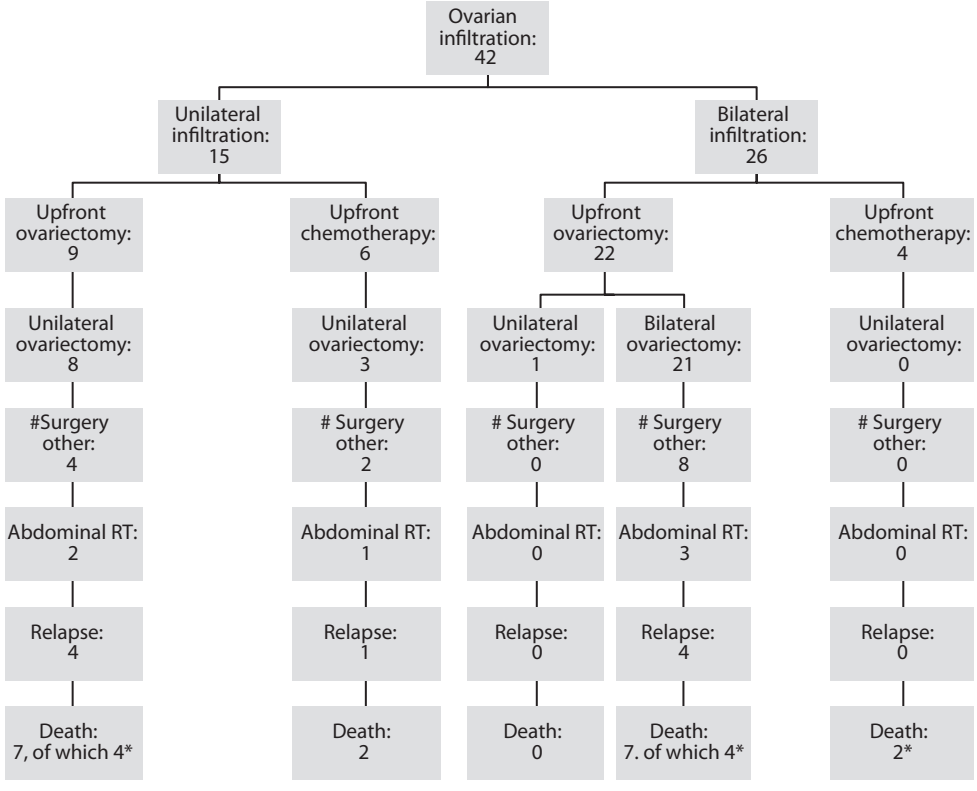

Figure II | Treatment algorithm of paediatric ovarian infiltrated cases.

In one case, ovarian infiltration is not specified in unilateral or bilateral.*=death due to treatment toxicity such as extreme progressive cachexia, complete intestinal obstruction or sepsis; #=other types of surgery performed, such as hemicolectomy, cystectomy, hysterectomy, appendectomy and omentectomy.

Table II | Clinical features of cases of paediatric ovarian non-Hodgkin lymphoma.

Author	n	Age at Dx (yr)	Symptoms & examination at presentation					Sub-type	Stage	Ovarian infiltration	
			Fever	Abd.	Re.	Abd. mass	Ascites			Uni	Bi
Seed, 1966 [21]	1	15	0	1	0	1	0	BL	III	1	0
Jamra, 1970 [22]	1	12	1	0	0	1	0	BL	NA	0	1
Bluming, 1971 [23]	1	4	0	1	0	1	0	BL	NA	0	1
Pickleman, 1972 [24]	1	7	1	1	1	1	0	BL	III	0	1
Nkrumah, 1973 [25]	5	10	0	0	0	5/5	0	5x BL	4x III, 1x IV	2/5	3/5
Aggio, 1974 [26]	1	4	0	1	0	1	0	BL	NA	0	1
Kuramoto, 1979 [27]	1	15	0	1	0	1	1	BL	III	1	0
Piura, 1986 [28]	1	16	0	1	0	1	0	B-NHL	NA	0	1
Weekes, 1986 [29]	1	15	1	1	0	1	1	BL	III	0	1
Monterroso, 1993 [3]	12	12	1/12	3/12	1/12	10/12	2/12	9 BL, 3 B-NHL	3x II, 9x III,	4/11*	7/11*
Creatsas, 1997 [30]	2	16	1/2	2/2	0	2/2	0	BL, N-NHL	II, III	1/2	1/2
Mitsumori, 1999 [31]	1	12	0	1	0	1	0	B-NHL	NA	0	1
Turken, 2000 [32]	1	0.9	0	0	0	1	0	NHL	III	0	1
Azizoglu, 2001 [17]	1	16	0	1	0	1	0	BL	III	0	1
Eren, 2004 [33]	1	4.5	0	1	0	1	1	BL	NA	0	1
Koksal, 2005 [34]	1	9	0	1	1	1	1	B-NHL	III	1	0
Ray, 2008 [35]	1	8	0	1	0	1	0	B-NHL	NA	0	1
Chong, 2009 [18]	1	14	1	1	1	1	1	ALCL	III	1	0
Cyriac, 2010 [36]	1	13	1	1	0	1	1	BL	NA	0	1
Chakrabarti, 2011 [37]	1	1.7	1	0	0	1	0	BL	III	0	1
Present report	6	10.5	2/6	6/6	1/6	4/6	1/6	6x BL	6x III	4/6	2/6
Total	42	10.9	10/42	25/42	5/42	38/42	9/42	-	-	15/41	26/ 41

Data is presented in median or n. N=number; Dx=diagnosis; Abd=abdominal symptoms; re=respiratory symptoms; BL=Burkitt lymphoma; B-NHL=B-non-Hodgkin lymphoma; ALCL=anaplastic large cell lymphoma; Uni=unilateral; bi=bilateral; intra-abd=intra-abdominal; CNS=central nervous system; BM=bone marrow; thor=thoracal; CT=chemotherapy; ov=ovariectomy; RT=radiotherapy; Rel=relapse; D=death; mo=months; yr=year; CCR=complete clinical remission; NA=not available;*=in one case site not specified.

Other sites of involvement at diagnosis					Treatment				Outcome			CCR postDx
Intr-abd.	CNS	BM	Orbita	Thor	Uni-ov.	Bi-ov.	CT	Abd RT	Rel.	D	D (mo)	(mo)
1	0	0	0	0	1	0	1	1	0	1	4	
0	0	0	0	0	0	0	1	0	0	0		NA
0	NA	NA	0	0	1	0	1	0	0	0		
0	0	0	0	0	0	1	1	0	0	1	7	
1/5	1/5	0	0	0	2/5	3/5	5x1	0	3/5	4/5	3,8,18.5,28	>26
1	NA	0	0	0	0	1	1	0	0	1	0.5	
1	NA	NA	1	0	1	0	1	0	0	1	1.5	
0	NA	0	0	0	0	1	1	1	0	0		>120
1	0	0	0	1	0	1	1	0	NA	NA		
11/12	0/12	1/12	0	3/12	2/11	8/11	9/11	3/11	6/9	5/9	1 6,7,10, 15	53,72, 105, 197
0	0	0	0	0	1/2	1/2	2/2	0	0	0		36,60
0	NA	NA	0	0	0	0	1	0	0	0		
0	NA	0	0	0	0	1	1	0	0	0		8
0	NA	NA	0	0	0	1	0	0	NA	NA		
1	NA	NA	0	0	0	1	1	0	0	1	0.5	
0	0	0	0	1	1	0	1	0	0	0		6
0	NA	NA	0	0	0	1	1	0	NA	NA		
1	0	0	0	1	1	0	1	0	0	0		24
0	NA	0	0	0	0	0	1	0	0	0		6
1	0	0	0	0	0	1	1	0	0	0		28
3/6	0/6	0	0	1	2/6	1/6	6x1	1/6	0	2/6	5, 9	12,24, 84,>25yr
22/42	1/32	1/36	1/42	6/42	12/41	22/41	38/42	6/41	9/36	16/36		

DISCUSSION

NHL in children usually presents with lymph node involvement with consequent symptoms and to a lesser extend with organ infiltration. The current report indicates that infiltration of the ovaries at presentation of paediatric NHL is rare, which is similar to what has been reported in adults [13]. Therefore, specific clinical characteristics are difficult to describe. From the observation of the 42 reviewed cases including our own particular cohort, all but one paediatric cases presented with abdominal pain and/or abdominal mass, which again is comparable with the presentation as described in adult cases [3,14].

Most paediatric cases showed a B-cell NHL immunophenotype, of which the majority had a Burkitt type, which may be a reflection of the most common lymphoma found in children [15]. This is in line with reported adult ovarian infiltrated NHL cases, where mainly diffuse large B-cell lymphoma (DLBCL) is found, followed by Burkitt lymphoma [3,14,16]. Nevertheless, it may also stress the specific tendency of homing of malignant B-cell NHL cells rather than T-NHL cells to infiltrate ovarian tissue, which suggests that Burkitt lymphomas preferentially involve the ovaries. The explanation of this is unknown. Only three adult primary ovarian anaplastic large-cell lymphoma (ALCL) cases have been reported of which two immunophenotypically showed T-cell lineage monoclonality [3,16-18]. The paediatric case that was cytogenetically confirmed as ALCL was not immunophenotyped.

BM involvement was not, and CNS infiltration was reported only once in the 42 paediatric ovarian infiltrated NHL cases. This could be an illustration of the fact that B-NHL, that infiltrates ovarian tissue, represents a distinct biological entity with a different homing biology. However, in general, BM and CNS involvement in B-cell NHL in children occurs only in approximately 5% and 10%, respectively [19,20], and considering the limited number of patients, this finding should be interpreted with caution.

Bilateral ovarian infiltration was reported in over half of the ovarian infiltrated paediatric NHL cases. Upfront ovariectomy was performed in 65% of the paediatric cases, of which strikingly 71% bilateral, even in recent years after improving diagnostic processes of NHL, and even after confirming negative gonadal tumour markers (α-fetoprotein (AFP) and human chorionic gonadotropin (β-HCG)). It is important to take into account the year of diagnosis, but even after 2000, the majority of the patients (7/8) have been treated with ovariectomy. Moreover, even in the early seventies authors discussed the upfront ovariectomy as first treatment and advised surgeons to be aware of ovarian infiltration in NHL patients since treatment was advised to be predominantly non-operative [24]. As chemotherapy alone is often sufficient to cure NHL in children, these findings urge the need to consider the diagnosis of NHL in such cases, and to perform needle biopsies in a selected subset of children with ovarian tumours that do not reveal positive tumour markers (AFP and b-HCG), especially in children with bilateral ovarian infiltration. This however points to a diagnostic dilemma, as it should be kept in mind that there is a potential risk of tumour spread when performing needle biopsies in such cases. This risk should be taken into account, but especially given the strong curing potential of intensive chemotherapy, it has to be balanced against the risk of performing an ovariectomy upfront, which can substantially impair gonadal function and fertility as illustrated by the decreased serum AMH level in the bilaterally ovariectomized patients. Subsequent premature

loss of ovarian function does have an incredible detrimental impact on a woman's general health later on in life.

In conclusion, ovarian infiltration in paediatric NHL is rare, and is predominated by the B-NHL phenotype. Considering the current excellent outcome using intensive chemotherapy only, awareness of NHL in case of ovarian tumours with negative tumour markers (AFP and b-HCG) is important in order to avoid unnecessary upfront surgery, especially in case of bilateral ovarian infiltration. Nevertheless, gonadal function should be carefully monitored in all long-term survivors of paediatric NHL, due to the potential risk of post-surgery-, chemo- and radiotherapy-related impairment.

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CHAPTER 5

Decreased ovarian function is associated with obesity in very long-term female survivors of childhood cancer

W van Dorp & K Blijdorp, JSE Laven, R Pieters, JA Visser,
AJ van der Lely, SJCMM Neggers, MM van den Heuvel-Eibrink

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ABSTRACT

Objective: Obesity and gonadal dysfunction are known major side effects of treatment in adult childhood cancer survivors (CCS). In the general population, obesity has a negative influence on female fertility. We aimed to evaluate whether obesity and serum insulin are associated with decreased ovarian reserve markers in CCS.

Design: Retrospective single-centre cohort study.

Methods: Data of 191 female survivors of childhood cancer were analysed. Median follow-up time was 18.8 (2.3–48.8) years. Outcome measures were serum anti-Müllerian hormone (AMH) and total follicle count (FC). Potential risk factors were body mass index (BMI); body composition measures, determined by dual energy X-ray absorptiometry (total fat percentage, lean body mass and visceral fat percentage); and fasting insulin.

Results: Lower serum AMH was found in obese subjects (β (%) -49, p=0.007), and in subjects with fasting insulin in the highest tertile (β (%) -43, p=0.039). Total fat percentage tends to be associated with serum AMH (β (%) -2.1, p=0.06). Survivors in the highest tertile of insulin had significantly lower FC than survivors in the lowest tertile (β -6.3, p=0.013). BMI and other measures of body composition were not associated with FC. Correlation between serum AMH and antral follicle count (AFC) was ρ=0.32 (p=0.08).

Conclusions: Obesity and insulin resistance are associated with gonadal damage, as reflected by decreased AMH and reduced FC, in adult survivors of childhood cancer. In contrast to its highly predictive value for AFC in the healthy female population, serum AMH does not seem to correlate as well with AFC in childhood cancer survivors.

INTRODUCTION

The prevalence of obesity has increased dramatically since the 1970s [1]. It is a well-described risk factor for diabetes, hypertension, heart disease, stroke, and cancer [2]. In healthy women, high body mass index (BMI) also affects reproductive health [3,4] as reflected by impaired ovulatory function and a lower pregnancy rate [5]. In childhood cancer survivors (CCS), obesity is a major side effect, occurring in 9–30%, and mainly depending on previous treatment [6,7]. In adult female CCS, the risk of obesity is increased by 50% when compared with the general population [8].

Gonadal dysfunction is an important side effect of cancer treatment in CCS. Alkylating agents and abdominal radiotherapy in particular, can have a deleterious effect on ovarian function [9]. To determine ovarian reserve, anti-Müllerian hormone (AMH) was identified as a reliable serum marker [10]. It is produced by the granulosa cells of small growing follicles, reflects the size of the primordial follicle pool in the ovaries, and is an indicator of a woman's reproductive capacity [11]. It is stable during and between menstrual cycles, in contrast to FSH [12-14], and is considered to be a valued marker for ovarian reserve, as it corresponds well with antral follicle count (AFC), which reflects reproductive status [10].

In their late reproductive years, the gonadal function of women seems to be affected by obesity [15,16]. Insulin resistance, which occurs at a higher frequency in adult CCS, may affect granulosa cell function [17,18]. The association between obesity, insulin resistance, and gonadal dysfunction, which are more prevalent in CCS than in the general population, has never been explored. Therefore, the aim of this study was to determine the association between BMI, body composition, insulin levels, and ovarian reserve as reflected by AMH and follicle counts in a substantial single-centre cohort of female adult survivors of childhood cancer.

MATERIALS AND METHODS

Subjects

A retrospective single-centre cohort study was performed among female survivors that visited our late-effects outpatient clinic for long-term CCS. Inclusion criteria were: age ≥18 and <50 years and female CCS diagnosed and treated between 1964 and 2005, who had both serum AMH levels and BMI determined at the same moment at least 5 years after cessation of cancer treatment. Exclusion criteria were ovariectomy, polycystic ovary syndrome (PCOS) and AMH >5 µg/L. One hundred and five survivors visited the gynaecological outpatient clinic for screening. In the remaining 180 CCS, we were not able to distinguish between PCOS and non-PCOS subjects, because data on hyperandrogenism (clinical and biochemical) and total follicle counts were not available. As we were not able to use the Rotterdam criteria in these remaining cases, and Dewailly *et al* suggested a cut-off limit of AMH >5 µg/L to define PCOM [19], we used this marker and cut-off limit for the presence of PCOM as one of the criteria of PCOS and excluded these survivors.

Outcome measures (AMH/FC)

Serum samples were taken randomly during the menstrual cycle as AMH has been shown not to fluctuate during the menstrual cycle and during OCP use [12]. AMH was measured using an in-house double-antibody enzyme-linked immunosorbent assay (ELISA) (commercially available through Beckman-Coulter) [10,20]. The intra- and inter-assay coefficients of variation were <10 and <5% respectively [10,20]. A subset of patients (n=91) underwent a standardised gynaecological examination. Clinical examination was performed after an overnight fasting period on a random day and included menstrual history, current cycle length, cycle regularity, height, and weight. Transvaginal ultrasonography was performed to assess ovarian volume and total follicle count (FC) for both ovaries and to exclude other genital abnormalities. FC was called antral follicle count (AFC) if the FC was performed during the follicular phase (days 2–5) or during amenorrhoea. PCOS was diagnosed according to the revised Rotterdam 2003 criteria [21]. The presence of polycystic ovaries was defined as ≥12 follicles in one or both ovaries and/or increased ovarian volume (>10 ml), without the presence of a cyst (>10mm) [22].

Obesity variables

Follow-up data of the most recent visit included the following variables: weight, height, BMI calculated from height and weight [23], and waist:hip ratio (WHR), as measured by waist circumference divided by hip circumference [24]. Serum insulin (pmol/L) was measured using a chemiluminescence-based immunoassay (Immulite 2000, Siemens DPC, Los Angeles, CA, USA) after an overnight fasting period. Glucose levels were measured using a Hitachi 917 analyzer (Roche Diagnostics). Insulin resistance was determined by calculation of the homeostatis model assessment (HOMA) score as plasma glucose (mmol/L) x plasma insulin (mU/L) /22.5 [25]. Lean body mass (kg) and percentage of body fat were measured by dual energy X-ray absorptiometry (DXA, Lunar Prodigy, GE Healthcare, Madison, WI, USA). Visceral fat percentage was calculated from intra-abdominal fat (kg) and total fat (kg) measured by DXA [26]. Waist, WHR, and visceral fat percentage were not analysed in the subset of survivors treated with abdominal radiotherapy because of impairment of local body fat and the frequent occurrence of scoliosis.

Potential confounders

Data concerning treatment protocols, disease, and patient characteristics were retrieved from our local database and were completed using the medical records where necessary. Follow-up time was defined as time since cessation of treatment. Among patients exposed to alkylating agents, the alkylating agent dose (AAD) score was calculated by determining the drug dose tertile distribution in our entire cohort of survivors and adding the tertile scores (1, 2 and 3) for each of the alkylating agents given to a particular patient as previously described by Tucker *et al* and Green *et al* [27,28]. An AAD score of zero was assigned to patients not exposed to any alkylating agent.

Statistics

To examine the associations between obesity variables and AMH or FC we used univariate and multiple linear regression analyses. In all multiple linear regression models, age, age at diagnosis, total

body irradiation, abdominal radiotherapy, and AAD score were included as potential confounders. The analyses were performed in several steps. First, BMI, body composition, and insulin were entered as continuous variables. Secondly, to evaluate whether there was an exponential association with AMH, squared variables of BMI, body composition measures, and insulin were added to the relevant model. Subsequently, all variables were divided into quintiles or tertiles (depending on sample size) and added to the relevant model as dummy variables, with the lowest quintile/tertile as reference category. Additionally, BMI was divided into four categories: BMI >30 kg/m^2 (obese), 25–30 kg/m^2 (overweight), 18–25 kg/m^2 (normal weight), and <18 kg/m^2 (underweight) and added to the model as dummy variables with normal weight as reference category. Associations are expressed as standardised regression coefficients because this measure allows direct comparison of the strengths of associations between different determinants. The distribution of AMH was normalised by ^{10}log transformation to improve the plots of the residual analyses and expressed as percentage. P-values <0.05 (two-tailed) are considered statistically significant. SPSS 17.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis.

RESULTS

Gonadal function

The cohort consisted of 425 female survivors of childhood cancer, of whom 292 visited the late-effects outpatient clinic. Seven survivors were excluded because of previous one-sided or two-sided ovariectomy (n=6 and n=1 respectively) and two because they were >50 years old. Sixteen survivors were clinically diagnosed with PCOS by a gynaecologist and were therefore excluded. Another 76 survivors were excluded because their AMH levels were >5 µg/L. Finally, 191 female survivors were included in the analysis.

Clinical characteristics and treatment details of the total cohort of female CCS of our centre and the survivors included in the study are shown in Table I. The included sample is representative for the total cohort of female CCS of our centre.

At the time of inclusion, 42 of 191 included survivors (22.0%) had regular menstrual cycles, whereas eight survivors (4.2%) had oligo- or amenorrhoea. One survivor had shortening of mean menstrual cycle length (1.3%). No data on menstrual cycles were available in one survivor (1.3%) who delivered recently and one survivor (1.3%) who was pregnant. In 23 of 191 survivors (30.3%), data on menstrual cycle at time of screening were not available. All other survivors used oral contraceptive pills (99/191, 51.8%) or were under hormonal replacement therapy (16/191: 8.4%) at the time of follow-up.

Median AMH level of the total group was 1.7 µg/L (range 0.1–4.9). Median FC was 10 (0–25). The Spearman correlation coefficient (ρ) between AMH and AFC was 0.32 (p=0.08) in survivors who were screened during the follicular phase or during amenorrhoea. FSH was significantly inversely correlated with AMH (ρ=-0.30, p<0.001) and was significantly higher in survivors with AMH <0.1 µg/L compared with survivors with AMH >0.1 µg/L (20.9 versus 4.9 U/L).

Table I | Representativeness of included survivors compared with the total group of female adult CCS and survivors in whom AMH was measured.

	Total group of adult female CCS		**Female CCS with AMH measurement**		**Included survivors in this study***	
	n=425		**n=292**		**n=191**	
Age at diagnosis (years)	7.3 (0–17.9)		6.2 (0–16.8)		**6.3 (0–16.2)**	
Age at follow up (years)	NA		26.5 (17.7–57.4)		**27.1 (17.7–50.0)**	
Follow up time (years)	NA		17.8 (2.3–48.8)		**18.8 (2.3–48.8)**	
Diagnosis n (%)						
ALL & T-NHL	128 (30)		91(31)		**52 (27)**	
Acute myeloid leukaemia	9 (2)		9 (3)		**7 (4)**	
B-cell non-Hodgkin lymphoma	25 (6)		18 (6)		**12 (6)**	
Hodgkin lymphoma	29 (7)		21 (7)		18 (9)	
Bone tumour	22 (5)		14 (5)		**14 (7)**	
Wilms tumour	47 (11)		41 (14)		**31 (16)**	
Neuroblastoma	32 (8)		29 (10)		18 (9)	
Germ cell tumour	12 (3)		8 (3)		**2 (1)**	
Malignant mesenchymal tumour	35 (8)		27 (9)		**17 (9)**	
Brain tumour	52 (12)		18 (6)		**13 (7)**	
Other	34 (8)		16 (5)		**7 (4)**	
Therapy n (%)	**n (%)**	**TCD (Gy)**	**n (%)**	**TCD (Gy)**	**n (%)**	**TCD (Gy)**
Abdominal radiotherapy	27 (7)	25 (10–71)	24 (8)	25 (10–62)	20 (11)	25 (10–60)
Total body irradiation	13 (3)	8 (7–15)	16 (5)	8 (6–12)	11 (6)	12 (7–12)
AAD score						
0	237 (56)		150 (51)		**93 (49)**	
1	47 (11)		35 (12)		**19 (10)**	
2	47 (11)		39 (13)		**26 (14)**	
3	70 (17)		55 (19)		**42 (22)**	
4	9 (2)		5 (2)		**5 (3)**	
≥5	15 (4)		8 (2)		**6 (3)**	

* After exclusion of ovariectomized subjects (n=7), PCOS subjects as classified according to the revised PCOS Rotterdam criteria (n=16), or AMH >5 µg/L (n=76) if subjects were not classified according to the revised PCOS Rotterdam criteria since information about follicle count and hyperandrogenism was not available, and women >50 years (n=2). Data are expressed as median (range) or frequencies (%).

NA=not applicable; CCS=childhood cancer survivors; AMH=anti-Müllerian hormone; ALL=acute lymphoblastic leukaemia; T-NHL=T-cell non-Hodgkin lymphoma; TCD=total cumulative dose; AAD=alkylating agent dose.

Influence of obesity, body composition and insulin on AMH

Twenty subjects (10%) were defined as obese (BMI ≥30 kg/m^2), 44 (23%) as overweight (BMI 25–30 kg/m^2), seven (4%) as underweight (BMI <18 kg/m^2), and 120 (63%) had normal weight (Supplementary Table I). AMH levels were significantly inversely associated with obesity (BMI ≥30 kg/m^2), high fasting insulin (>54 pmol/L), total fat percentage, waist circumference, WHR, and visceral fat percentage but not with HOMA and lean body mass (Table II). After adjustment for confounders (age, age at diagnosis, treatment with abdominal or total body irradiation, and AAD score) obesity (BMI >30 kg/m^2) and high fasting insulin (>54 pmol/L) remained significantly associated with AMH (β (%) -49, p=0.007, and β (%) -43, p=0.039, respectively) (Table II, Figure I, Figure IIa, IIb).

Table II | Univariate and multivariate linear regression analyses illustrating the influence of BMI, measures of body composition, and insulin on AMH levels.

	AMH (%) n=191 (122) [a]					
	Univariate model			Multivariate model		
	β (%)	95% CI	p	β (%)	95% CI	p
Obesity (BMI ≥30 kg/m^2)	-55*	-75 to -18	0.009	-49*	-68 to -17	0.007
Overweight (BMI 25–30 kg/m^2)	1	-35 to 56	0.974	15	-20 to 64	0.450
Underweight (BMI <18 kg/m^2)	-17	-68 to 116	0.698	-21	-64 to 76	0.568
BMI	-3.3	-6.8 to 0.4	0.081	-2.0	-5.0 to 1.0	0.191
Total fat percentage	-3.7*	-6.4 to -1.0	0.009	-2.1	-4.2 to 0.1	0.06
Lean body mass (kg)	2.0	-11.3 to 5.2	0.22	0.4	-2.2 to 3.0	0.76
Waist circumference (cm)[b]	-2.0*	-3.7 to -0.2	0.025	-0.5	-1.8 to 0.9	0.511
Waist:hip ratio[b]	-2.5*	-4.3 to -0.2	0.037	0.1	-1.8 to 2.6	0.894
Visceral fat percentage[b]	-13*	-22 to -2	0.02	-1	-10 to 8	0.79
Insulin	-0.4	-0.9 to 0.1	0.132	-0.2	-0.6 to 0.3	0.48
Insulin 2nd tertile (24–54 pmol/L)	2	-47 to 96	0.952	-8	-45 to 55	0.761
Insulin 3rd tertile (>54 pmol/L)	-54*	-76 to -12	0.019	-43*	-67 to -3	0.039
HOMA	-13	-28 to 5	0.135	-6	-18 to 9	0.387
HOMA 2nd tertile (0.58–1.40)	17	-40 to 127	0.636	-8	-46 to 9	0.753
HOMA 3rd tertile (>1.40)	-45	-71 to 8	0.080	-41	-65 to 0.3	0.051

AMH=anti-Müllerian hormone; CI=confidence interval; HOMA=homeostatis model assessment; *P<0.05.

[a]Number of available DXA scans; [b]For the dependent variables waist, waist hip ratio and visceral fat percentage, survivors treated with abdominal radiotherapy are excluded from the analysis (n=19). Corrected for age, age at diagnosis, total body irradiation, abdominal radiotherapy and alkylating agent dose (AAD) score.

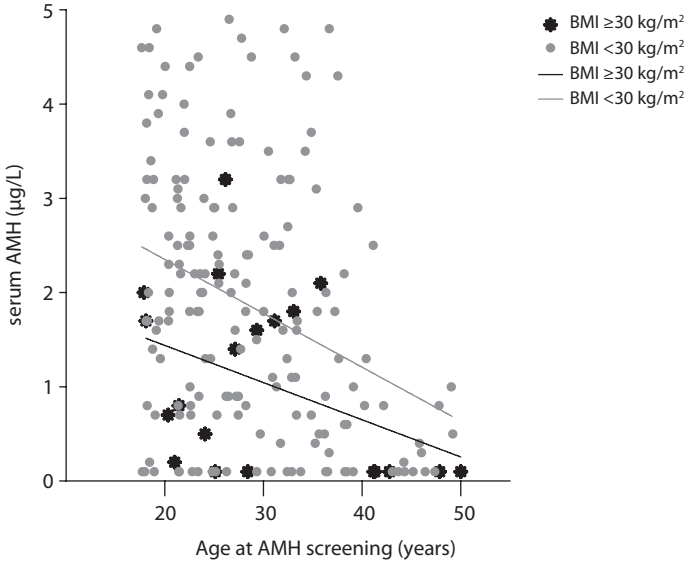

Figure I | Anti-Müllerian hormone (AMH) in obese (BMI ≥30 kg/m²) survivors compared with non-obese survivors.

Table III | Multivariate linear regression analyses illustrating the lacking influence of BMI, measures of body composition, and insulin on total follicle count.

	Total follicle count n=54 (34)[a]					
	Univariate model			Multivariate model		
	β (%)	95% CI	p	β (%)	95% CI	p
Obesity (BMI ≥30 kg/m²)	-0.6	-7.8 to 6.6	0.867	-0.9	-8.0 to 6.2	0.804
Overweight (BMI 25–30 kg/m²)	-2.1	-7.7 to 3.5	0.452	-2.1	-8.1 to 3.8	0.471
Underweight (BMI <18 kg/m²)	-3.0	-13.9 to 7.9	0.584	-5.4	-16.4 to 5.6	0.330
Body mass index	-0.03	-0.43 to 0.38	0.902	-0.01	-0.42 to 0.40	0.978
Total fat percentage	-0.09	-0.44 to 0.26	0.614	-0.06	-0.44 to 0.32	0.747
Lean body mass (kg)	0.18	-0.15 to 0.51	0.266	0.20	-0.17 to 0.57	0.278
Waist circumference (cm)[b]	-0.02	-0.24 to 0.21	0.890	0.04	-0.18 to 0.26	0.732
Waist – hip ratio[b]	-0.06	-0.37 to 0.25	0.696	0.05	-0.26 to 0.35	0.759
Visceral fat percentage[b]	-0.58	-2.44 to 1.28	0.529	-0.18	-2.10 to 1.75	0.851
Insulin	-0.03	-0.07 to 0.00	0.083	-0.02	-0.06 to 0.01	0.192
Insulin 2nd tertile (25–48 pmol/L)	-3.1	-7.8 to 1.5	0.186	-3.9	-8.6 to 0.8	0.104
Insulin 3rd tertile (>48 pmol/L)	-6.6*	-11.3 to -2.0	0.006	-6.3*	-11.2 to -1.4	0.013
HOMA	-0.98	-2.24 to 0.27	0.121	-0.70	-2.00 to 6.00	0.283
HOMA 2nd tertile (0.66–1.24)	-2.47	-7.22 to 2.28	0.302	-3.42	-8.40 to 1.57	0.175
HOMA 3rd tertile (>1.24)	-5.06*	-9.95 to -0.18	0.043	-5.01	-10.26 to 0.23	0.061

CI=confidence interval; HOMA=homeostatis model assessment; *P<0.05.
[a]Number of available DXA scans; [b]For the dependent variables waist, waist hip ratio and visceral fat percentage, survivors treated with abdominal radiotherapy are excluded from the analysis (n=19). Corrected for age, age at diagnosis, total body irradiation, abdominal radiotherapy and AAD score.

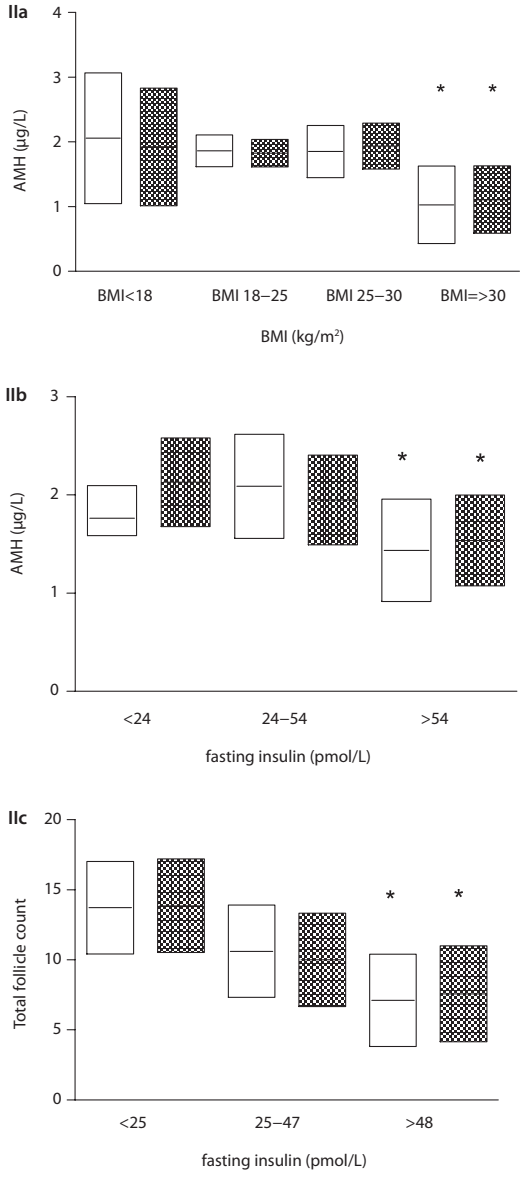

Figure II | Anti-Müllerian hormone (AMH) and total follicle count in BMI and fasting insulin subgroups. White bars: univariate analysis. Dotted bars: after adjustment for age, age at diagnosis, total body irradiation, alkylating agent dose score, and abdominal radiotherapy.

IIa Anti-Müllerian hormone (AMH) in four BMI categories, univariate and after adjustment for possible confounders expressed as mean (95% CI). *=p<0.05 compared with subjects with BMI 25–30.

IIb AMH in serum fasting insulin subgroups expressed as mean (95% CI). *=p<0.05 compared with subjects with fasting insulin <25 pmol/L.

IIc Total follicle count in serum fasting insulin subgroups expressed as mean (95% CI). *=p<0.05 compared with subjects with fasting insulin <25 pmol/L.

Influence of obesity, body composition and insulin on FC

There were no significant associations between FC and BMI or body composition measures (Table III). Survivors with insulin and HOMA in the highest tertile had significantly lower FC than others. After adjustment for confounders, no linear or exponential association between FC and BMI or measures of body composition was found. FC did not differ significantly between quintiles of BMI or body composition (data not shown). Subsequently, FC did not differ between BMI categories. However, there were only five obese subjects with available FCs (data not shown). Insulin was significantly associated with FC, i.e. survivors with an insulin level in the highest tertile (>48 pmol/L) had significantly lower FC counts when compared with survivors with insulin levels <25 pmol/L (β -6.3, p=0.013) (Table III, Figure IIc).

DISCUSSION

The current study shows that diminished ovarian reserve, as reflected by low AMH and low FC, was independently associated with obesity and high insulin levels in female CCS.

Our results show that obesity is indeed independently associated with decreased AMH in female CCS [6,29,30]. In the current study, total and visceral fat percentages were not associated with gonadal function, although we observed a trend to a negative association between total fat percentage and AMH levels. This may be due to a size effect or to the fact that we measured total fat percentage and not the fat distribution in different compartments. In fact, for that purpose, abdominal computer tomography, which is the gold standard for measuring intra-abdominal fat mass, is preferred over DXA [31].

Although no oral glucose tolerance tests were performed and therefore some cases in the pre-diabetic state might have been missed, none of the subjects were diagnosed and treated for diabetes mellitus, based on fasting glucose levels and/or medical history. Therefore, we evaluated fasting insulin levels as a measure for insulin resistance and related these to ovarian reserve markers. The negative association between fasting insulin levels and AMH was previously described in the reproductive-age women without PCOS [16] and was confirmed by our study among CCS.

We identified obesity to be negatively associated with ovarian reserve as assessed by AMH levels. There is no linear association between AMH and BMI, but above a certain threshold (in this case BMI 30 kg/m^2), AMH is significantly lower compared with normal weight subjects. The explanation for this nonlinear association could be that only above a certain threshold of BMI do metabolic changes occur, including insulin resistance, leptin resistance, and elevated levels of adipokines. These factors could play a role in affecting the pituitary-gonadal axis and damaging granulosa cells, although this hypothesis has never been proved. In this study, all subjects with obesity were evaluated, including one survivor treated for craniopharyngioma and one survivor treated with high-dose brain tumour irradiation (>35 Gy). In these subjects, hypothalamic obesity could not be excluded. To our knowledge, no other studies that assess a possible link between obesity and AMH have been performed in female CCS. In the general population, only one study among three has shown an association in women of reproductive age [15,16,32].

Based on these results, we hypothesize that obesity influences the degree of gonadal damage in female CCS. However, it is also conceivable that impaired gonadal function may lead to the development of adiposity and insulin resistance. In animal models it is known that oestradiol has an inhibitory effect on food intake via anorexigenic peptides that decrease meal size. In ovariectomized rats, the removal of oestrogens leads to changes in meal size, obesity [33,34], increased leptin sensitivity, and decreased insulin sensitivity [35]. However, insulin resistance could also decrease granulose cell function, which could lead to reduced ovarian function and therefore lower AMH levels [17,18]. Our hypothesis fits with the result of a study in type 2 diabetes mellitus (T2DM) patients, in which AFCs were significantly lower than in healthy controls [36], which was possibly a result of insulin resistance in the T2DM patients. Furthermore, the fact that stringent glycaemic control in diabetic patients improves menstrual cycles and fertility rates underlines the hypothesis that prolonged hyperglycaemia and chronic complications of diabetes negatively affect ovarian reserve [37]. Animal models have shown that ovulation was suppressed in hyperglycaemic-hyperinsulinemic conditions, due to hypovascularisation, follicular atresia and eventually involution of ovaries, caused by glucotoxicity and the cytolipotoxic effect of obesity [38]. It should, however, be stressed that due to the cross-sectional design of the current study, cause and effect could not be distinguished.

In 105/285 cases, PCOS versus non-PCOS was diagnosed according to the Rotterdam criteria, as these survivors also visited the gynaecological outpatient clinic. However, in the remaining 180 CCS, we were not able to distinguish between PCOS and non-PCOS subjects because data on hyperandrogenism (clinical and biochemical) and FCs were not available. As we were not able to use the Rotterdam criteria in these remaining cases, and Dewailly *et al* suggested a cut-off limit of AMH >5 µg/L to define PCOM [19], we used this marker and cut-off limit for the presence of PCOM. We recognise the limitation of this cut-off limit as we probably exclude more subjects than we would have done if we were able to exclude them based on the Rotterdam criteria. However, we believe that this is the best way to make our population as homogeneous as possible. Moreover, the remaining subjects were representatives of the whole cohort of female CCS according to age, age at follow-up, diagnosis, and treatment. As we agreed that the use of the cut-off value is of limited accuracy, we also performed sub-analyses in the 105 cases that were classified according to the Rotterdam criteria. Sixteen survivors were diagnosed with PCOS and were therefore excluded from this analysis. We found a trend to an association between total fat percentage and AMH (p=0.053). This is the same trend as found in our previous analyses. However, we did not find an association between BMI and AMH, which might be due to the underrepresentation of obese survivors in this subset (n=5). If we perform multivariate analyses in the whole group of survivors with AMH levels (n=285), we observe no significant correlations with obesity, which fits with the hypothesis that obese women with PCOS have raised AMH levels, while obese women without PCOS have lower AMH levels.

Despite the negative association between FC and serum insulin, we did not find an association between FC and obesity, in contrast to AMH. However, it should be stressed that FCs were available in only five obese subjects. So, power issues may be important. Larger cohorts are necessary to study this association in the future.

In healthy females AFC and AMH correlate very well [39], but the present study shows that this correlation is weak among CCS. This may be due to the fact that follicular AMH expression is lower in CCS treated with gonadotoxic therapies. To our knowledge, no large studies have been performed in female CCS in which the correlation has been studied. Therefore, we cannot draw any firm conclusions based on our small subset analyses regarding the correlation between AFC and AMH. We believe that it is important to study this association prospectively in a large nationwide cohort, such as the DCOG LATER-VEVO study [40].

We did not correct our analyses for smoking and OCP use. Smoking is linked to ovarian ageing in the general population [41]. However, we did not find a significant difference in AMH levels between smokers and non-smokers. The fact that smoking significantly influences ovarian reserve in the general population but not in female CCS may be caused by the large effect of the gonadotoxic treatment that may exceed the impact of smoking on the ovarian reserve. Whether OCP use affects AMH levels is still a matter of debate. In our study, we did not find an association between OCP and AMH. Therefore, we did not include smoking and OCP use as confounders.

In conclusion, low serum AMH is associated with obesity and high insulin levels, and low FC with high insulin levels in a large cohort of adult female CCS. Furthermore, despite its highly predictive value for AFC in the general female population, serum AMH seems to correlate only weakly with AFC in childhood cancer survivors.

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Supplementary Table I | Baseline characteristics of normal weight, overweight and obese subjects.

	Normal weight subjects		**Overweight subjects**		**Obese subjects**	
	BMI <25 kg/m²		**BMI 25–30 kg/m²**		**BMI ≥30 kg/m²**	
	n=127		**n=44**		**n=20**	
Age at diagnosis (years)	6.2 (0–16.2)		7.5 (0.4–16.1)		6.3 (1.5–15.1)	
Age at follow up (years)	26.3 (17.7–49.2)		28.8 (17.7–49.1)		27.8 (17.9–50.0)	
Follow up time (years)	18.5 (3.2–43.6)		21.2 (2.3–43.5)		18.4 (5.1–48.8)	
Diagnosis n (%)						
ALL & T-NHL	33 (26)		12 (27)		7 (35)	
Acute myeloid leukaemia	5 (4)		2 (5)		0	
B-cell non-Hodgkin lymphoma	9 (7)		1 (2)		2 (10)	
Hodgkin lymphoma	11 (9)		5 (11)		2 (10)	
Bone tumour	9 (7)		4 (9)		1 (5)	
Wilms tumour	21 (17)		9 (21)		1 (5)	
Neuroblastoma	13 (10)		4 (9)		1 (5)	
Germ cell tumour	1 (1)		1 (2)		0	
Malignant mesenchymal tumour	12 (9)		2 (5)		3 (15)	
Brain tumour	6 (5)		4 (9)		3 (15)	
Other	7 (6)		0		0	
Therapy n (%)	**n (%)**	**TCD (Gy)**	**n (%)**	**TCD (Gy)**	**n (%)**	**TCD (Gy)**
Abdominal radiotherapy	13 (10)	25 (10–50)	3 (7)	12	1	12
Total body irradiation	7 (6)	8 (7–12)	6 (14)	23 (20–60)	1	40
AAD score						
0	56 (44)		27 (61)		10 (50)	
1	15 (12)		3 (7)		1 (5)	
2	18 (14)		7 (16)		1 (5)	
3	30 (24)		6 (14)		6 (30)	
4	4 (3)		0		1 (5)	
≥5	4 (3)		1 (2)		1 (5)	

Data are expressed as median (range) or frequencies (%).
ALL=acute lymphoblastic leukaemia; T-NHL=T-cell non-Hodgkin lymphoma; TCD=total cumulative dose; AAD=alkylating agent dose.

CHAPTER 6

Genetic variation may modify ovarian reserve in female childhood cancer survivors

W van Dorp, MM van den Heuvel-Eibrink,
L Stolk, R Pieters, AG Uitterlinden, JA Visser, JSE Laven

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ABSTRACT

Study question: Are genetic polymorphisms, previously identified as being associated with age at menopause in the healthy population, associated with ovarian reserve and predicted age at menopause in adult long-term survivors of childhood cancer?

Summary answer: The CT genotype of rs1172822 in the *BRSK1 gene* is associated with lower serum anti-Müllerian hormone (AMH) levels and a younger predicted age at menopause in adult survivors of childhood cancer.

What is known already: Gonadotoxicity is a well-known late side effect of chemotherapy and radiotherapy in adult survivors of childhood cancer. In the healthy population, several genetic polymorphisms are associated with age at natural menopause. Currently, data on the impact of previously identified variants in gene loci associated with ovarian reserve in adult long-term survivors of childhood cancer are lacking.

Study design, size, duration: We performed a pilot study in a single-centre cohort of adult female Caucasian childhood cancer survivors (n=176).

Participants/materials, setting, methods: We determined serum AMH levels (a marker of ovarian reserve) in adult survivors of childhood cancer (n=176) and studied single nucleotide polymorphisms (SNPs) previously reported to be associated with age at natural menopause: *BRSK1* (rs1172822), *ARHGEF7* (rs7333181), *MCM8* (rs236114), *PCSK1* (rs271924), *IGF2R* (rs9457827) and *TNF* (rs909253). Association analysis was performed using the additive genetic model. Linear regression was conducted to assess the effect of significant polymorphisms in two previously published menopause prediction models.

Main results and the role of chance: The CT genotype of rs1172822 in the *BRSK1* (BR serine/ threonine kinase 1) gene was negatively associated with serum AMH levels in our cohort (odds ratio=3.15, 95% confidence interval 1.35–7.32, p=0.008) and significantly associated with the predicted age at menopause (p=0.04). The other five SNPs were not associated with serum AMH levels.

Limitations, reasons for caution: This is a pilot study showing preliminary data which must be confirmed. To confirm our findings and enlarge the project, a nationwide genome-wide association (GWA) project on the ovarian reserve in female survivors of childhood cancer should be performed, including a replication cohort.

Wider implications of the findings: Our findings support the hypothesis that previously identified genetic polymorphisms associated with age at menopause in healthy women may have an effect on the onset of menopause in female survivors of childhood cancer. Our study highlights a new aspect of the influences on the ovarian reserve after childhood cancer, which should be investigated further in a nationwide GWA study. Eventually, this information can help us to improve counselling on fertility preservation prior to cancer treatment based on genetic factors in individual patients.

INTRODUCTION

A well-known long-term side effect of cancer treatment in survivors of both adult and childhood cancer is gonadotoxicity [1,2]. The extent of gonadal damage depends on the treatment modality and the total cumulative dose [2-5]. In addition, the multi-drug approach may have a cumulative toxic effect on reproductive function [6,7].

Along with these iatrogenic variables, the genetic variation of individuals together with environmental influences (e.g. smoking) determines an individual's reproductive health. Hence, fertility can be regarded as a complex trait that is a result of the interplay between a person's genetic make-up and the environmental disruptors to which one was exposed.

In the general population, the age at natural menopause varies between 40 and 60 years, with a mean of 50–51 years, and is largely heritable [8,9]. Associations between age at menopause and variants of genes involved in DNA repair, DNA maintenance and immunity have been previously identified in large genome-wide association (GWA) studies [10-12].

Serum anti-Müllerian hormone (AMH) has been found to be a marker of the ovarian reserve. It is produced by granulosa cells of small, growing follicles in the ovary and is measurable in blood [13]. It has been described as a useful serum marker of ovarian reserve in mice [14], healthy women [13] and adult survivors of childhood cancer [2]. Recently, serum AMH levels were found to be highly predictive for the onset of menopause in healthy women [15-17]. Two nomograms to predict the age at menopause have been reported [18,19]: in these studies, AMH and age were significantly correlated with the time to menopause, and therefore included as predictors in the prediction models.

Variation of long-term toxicity in childhood cancer survivors (CCS) who received the same treatment suggests that, besides environmental factors, genetic variation may influence the impact of late effects. In male adult CCS, genetic polymorphisms in the oestrogen receptor were associated with an increased risk of azoospermia [20]. Data on the impact of previously identified variants in gene loci associated with the ovarian reserve in adult long-term CCS are lacking. Since these genes may have an additive influence on the ovarian reserve (together with the effects of gonadotoxic treatment regimens), the aim of this pilot study was to evaluate whether the genetic polymorphisms known to be associated with menopause were associated with serum AMH levels, and together could predict age at menopause in adult long-term CCS.

MATERIALS AND METHODS

Subjects

This retrospective study was performed in a single-centre cohort of female adult CCS treated at the Erasmus MC–Sophia Children's Hospital from October 1962 to May 2001. Participants were 18 years and younger at the time of diagnosis, 18–50 years old at the time of follow-up and in continuous complete remission. They had completed treatment at least 5 years ago. Participants were recruited during their regular visit at the late effects outpatient clinic. Only Caucasian females

were included in order to minimize ethnic influences on the study outcome. Full details of cancer treatment were collected from the late effects clinic database and medical records, i.e. type and total cumulative doses of chemotherapy; site, field and cumulative dose of radiotherapy; extent of surgery; conditioning regimen prior to stem-cell transplantation; complications and relapses. The alkylating agent dose (AAD) score can be used to sum all alkylating agents that are known to be gonadotoxic. We calculated this score by determining the drug dose tertile distribution in our entire cohort of survivors independently and adding the tertile scores (1, 2 and 3) for each of the alkylating agents given to a particular patient, as previously used in CCS [21,22] (Supplementary Table I). An AAD score of zero was assigned to patients not exposed to alkylating agents. A general health screening, including extensive history taking and physical examination, was performed. An official written informed consent was obtained from every patient that agreed to participate according to the standards of the Institutional Review Board.

Measurement of ovarian reserve

As most of our patients were of premenopausal age, a marker that is consistent during the menstrual cycle and representative of the ovarian reserve is needed. AMH is strongly correlated with the antral follicle count and is relatively constant during and between menstrual cycles, in contrast to FSH [23-26]. Therefore, AMH is a sensitive serum marker for the ovarian reserve and a proxy for menopause.

Serum samples were taken randomly during the menstrual cycle, in pregnant survivors as well as in survivors taking oral contraceptive pills or hormone replacement therapy. Serum AMH levels were measured with an in-house double-antibody enzyme-linked immunosorbent assay [13,14]. Intra- and inter-assay coefficients of variation were <10 and <5%, respectively [13,14].

Genotyping

Single nucleotide polymorphisms (SNPs) were selected based on a literature search of previous candidate gene studies and more recently published GWA studies (for minor allele frequencies (MAFs): see Table I). We genotyped seven previously identified polymorphisms associated with age at menopause (*BRSK1* (rs1172822) [11], *ARHGEF7* (rs7333181) [11], *MCM8* (rs236114) [11], *PCSK1* (rs271924) [12], *SRD5A1* (rs494958) [12], *IGF2R* (rs9457827) [12] and *TNF* (rs909253) [12]) in the samples from long-term CCS.

Genomic DNA was extracted from peripheral blood using standard DNA extraction methods. 1–2 ng of genomic DNA was dispensed into 384-well plates using a Caliper Sciclone ALH3000 pipetting robot (Caliper LS, Mountain View, CA, USA). Genotypes were determined using Sequenom iPLEX genotyping and Taqman Allelic Discrimination assays. All primers and probes are available on request.

Multiplex PCR assays were designed for the Sequenom iPLEX genotyping using Assay Designer on the website (https://mysequenom.com/tools/genotyping/default.aspx). For this, sequences containing the SNP site and at least 100bp of flanking sequence on either side of the SNP were used. As previously described [11], 2 ng of genomic DNA was amplified in a 5 µl reaction containing 1x Taq PCR Buffer (Sequenom), 2mM $MgCl_2$, 500 µM each dNTP, 100 nM each PCR primer and 0.5 U

Taq (Sequenom). The reaction was incubated at 94°C for 20 seconds, 56°C for 30 seconds, 72°C for 1 minute, followed by 3 minutes at 72°C. Excess dNTPs were then removed from the reaction by incubation with 0.3 U shrimp alkaline phosphatase (Sequenom) at 37°C for 40 minutes followed by 5 minutes at 85°C to deactivate the enzyme. Single primer extension over the SNP was carried out using a final concentration of 0.731 µM to 1.462 µM for each extension primer (depending on the mass of the probe), iPLEX termination mix (Sequenom), 10x iPLEX Buffer Plus and iPLEX enzyme (Sequenom) and subsequently cycled using the following programme: 94°C for 30 seconds followed by 94°C for 5 seconds, 5 cycles of 52° for 5 seconds and 80°C for 5 seconds, the previous three steps being repeated 40 times, then 72°C for 3 minutes. This reaction was then desalted by the addition of 6 mg clear resin (Sequenom) followed by mixing (15 minutes) and centrifugation (5 minutes, 3000 rpm) to settle the contents of the tube. The extension product was then spotted onto a 384-well spectroCHIP using the SEQUENOM MassARRAY Nanodispenser RS1000 before analysis on the MassARRAY Compact System (Sequenom). Data were collected using SpectroACQUIRE 3.3.1.13 and clustering was called using TYPER Analyser 4.0.3.18 (Sequenom). Additionally, to ensure data quality, the genotypes for each subject were also checked manually.

Genotypes for rs236114 and rs909253 were generated using Taqman Allelic Discrimination (Applied Biosystems Inc., Foster City, CA, USA). Primer and probe sequences were optimized using the SNP assay-by-design service of Applied Biosystems (for details see www.appliedbiosystems.com). Reactions were performed on the Taqman Prism 7900HT 384-well format.

Table I | Minor allele frequencies (MAFs) in previous studies and the present study.

	Locus	Chr	MAF CCS AMH≥1 µg/L	MAF CCS AMH<1 µg/L	MAF Previous studies (Stolk *et al*, 2009, He *et al*, 2010)
rs7333181 (A)	*ARHGEF7*	13	0.11	0.13	0.12
rs1172822 (T)	*BRSK1*	19	0.35	0.48	0.39
rs236114 (T)	*MCM8*	20	0.19	0.18	0.21
rs494958 (T)	*SRD5A1*	*5*	*0.15*	*0.15*	*0.16*
rs271924 (T)	*PCSK1*	5	0.32	0.34	0.38
rs9457827 (T)	*IGF2R*	6	0.07	0.07	0.06
rs909253 (G)	*TNF*	6	0.33	0.32	0.34

MAF=minor allele frequency; CCS=childhood cancer survivors Chr=chromosome; AMH=anti-Müllerian hormone.
All SNPs except rs494958 are in Hardy-Weinberg equilibrium (HWE).

Statistical analysis

The SNPs were tested for deviation from the Hardy-Weinberg equilibrium (HWE) by comparing the observed and expected genotype frequencies using the chi-square test. AMH levels were studied as continuous measures and were divided in two groups based on levels considered to be clinically relevant and acting as a proxy for the ovarian reserve: serum AMH levels below 1 µg/L versus AMH levels equal to or above 1 µg/L. A chi-squared test was used to analyse the associations between the SNPs and the two AMH groups.

Association analyses were carried out and the additive genetic model was tested. First, we pooled heterozygous and homozygous carriers of the risk alleles under a dominant inheritance model. Linear regression was performed to calculate the effect of carrying the risk allele on continuous log-transformed AMH levels. Secondly, logistic regression was performed to calculate the odds ratio (OR) and 95% confidence intervals (CI) of the three genotypes (carriers of zero, one or two risk alleles) to assess risk, adjusted for age at AMH assessment, the AAD score and the total dose of radiotherapy to the pelvis. Analyses were not performed when the number of patients per genotype subgroup was below n=5. In view of the multiple comparisons, p-values of ≤0.0083 (0.05/6 SNPs) were considered to be significant.

We estimated the age at menopause based on serum AMH levels using two prediction models of the age at menopause based on a Dutch cohort and an Iranian cohort [18,19]. These prediction models are based on age and serum AMH levels. Subsequently, the effect of significant SNPs on this predicted age of menopause was evaluated using a linear regression model, adjusted for AAD score and abdominal radiotherapy dose. P-values ≤ 0.05 were considered statistically significant. Statistical analysis was performed using the Statistical Package for the Social Sciences 17.0 software (SPSS, Chicago IL, USA).

RESULTS

The cohort of CCS consisted of 238 women, of whom 5 died during follow-up, 6 were non-responders, 6 refused participation, 3 emigrated, 3 were disabled after treatment and 2 patients were >50 years. From the remaining 213 eligible subjects, serum AMH levels and DNA for genotyping were available in 192 survivors. Sixteen non-Caucasian survivors were excluded. Finally, 176 Caucasian women were included. The median age at diagnosis was 5.4 years (range 0.1–16.8 years) in participants, and was comparable with the median age of survivors who discontinued follow-up and those in whom serum AMH levels or DNA samples were not available (median 6.7 years, range 0.8–14.0 years, p=0.98). Neither the type of malignancy (p=0.08) nor the frequency of relapse (15% versus 10%; p=0.48), or the treatment with alkylating agents (54% versus 47%, p=0.48) or radiotherapy (34% versus 29%; p=0.44) differed between participants and non-participants, respectively.

The median age at time of cessation of treatment was 6.9 years (range 0.1–17.8 years). The median age at follow-up was 24.8 years (range 18.0–46.2 years). Fifty-six survivors (32%) received chemotherapy and radiotherapy, 104 (59%) chemotherapy only, 4 (2%) radiotherapy only, and 12 (7%) surgery only. Twenty-three survivors (13%) were in second remission after relapse and six (3%) were in remission after a second malignancy. Eighty-nine survivors (51%) were treated with alkylating agents. Twelve survivors (7%) were treated with abdominal irradiation or total body irradiation.

At the time of inclusion, 48 of 176 survivors (27.3%) had regular menstrual cycles, whereas 15 survivors (8.5%) had oligo- or amenorrhoea. Three (1.7%) survivors were pregnant. In 6 survivors (3.4%), data on their menstrual cycle at time of screening were not available. All other survivors used oral contraceptive pills (55.1%) or hormonal replacement therapy (n=7: 4.0%) at the time of follow-up.

Table II presents the characteristics of the 176 female CCS. Survivors were divided into two groups on the basis of AMH levels: a level below 1 µg/L (61/176: 35%) was considered to be low; a level equal to or above 1 µg/L (115/176: 65%) was considered to be normal. Age at diagnosis, age at end of treatment, diagnosis and age at follow-up were not significantly different between the two AMH groups. As expected, significantly more survivors in the low AMH group were treated with abdominal irradiation or total body irradiation, and significantly more survivors in the normal AMH group were not treated with the known gonadotoxic alkylating agents. The median serum AMH level in all survivors was 1.8 µg/L (range < 0.1–14.3 µg/L).

Table II | Characteristics of female Caucasian patients who survived childhood cancer.

	CCS all n=176 Median (range)	**CCS AMH<1 µg/L n=61 Median (range)**	**CCS AMH≥1 µg/L n=115 Median (range)**	**p***
Age at diagnosis (yrs)	5.4 (0.1–16.8)	6.1 (0.2–15.4)	5.2 (0.1–16.8)	0.221
Age at discontinuation of treatment (yrs)	6.9 (0.1–17.8)	7.8 (1.2–17.0)	6.4 (0.1–17.8)	0.130
Age at follow-up (yrs)	24.8 (18.0–46.2)	26.4 (18.0–42.6)	24.4 (18.0–46.2)	0.061
Follow-up time (yrs)	17.4 (4.0–41.3)	17.3 (4.0–38.8)	17.5 (5.0–41.3)	0.528
Diagnosis n (%)				
Acute lymphoblastic leukaemia	66 (37.5)	25 (41.0)	41 (35.7)	0.488
Acute myeloid leukaemia	4 (2.3)	3 (3.9)	1 (0.9)	0.087
B-cell non-Hodgkin lymphoma	8 (4.5)	4 (6.6)	4 (3.5)	0.352
T-cell non-Hodgkin lymphoma	7 (4.0)	2 (3.3)	5 (4.3)	0.731
Hodgkin lymphoma	13 (7.4)	7 (11.5)	6 (5.2)	0.132
Osteosarcoma	4 (2.3)	2 (3.3)	2 (1.7)	0.515
Renal tumour	24 (13.6)	9 (14.8)	15 (13.0)	0.754
Ewing sarcoma	4 (2.3)	1 (1.6)	3 (2.6)	0.682
Neuroblastoma	17 (9.7)	4 (6.6)	13 (11.3)	0.312
Rhabdomyosarcoma	7 (4.0)	3 (4.9)	4 (3.5)	0.643
Langerhans cell histiocytosis	6 (3.4)	0 (0.0)	6 (5.2)	0.070
Brain tumour	2 (1.1)	0 (0.0)	2 (1.7)	0.302
Other	14 (8.0)	1 (1.6)	13 (11.3)	**0.025**
Therapy n (%)				
Abdominal radiotherapy (30 Gy (15–45))	7 (4.0)	6 (9.8)	1 (0.9)	**0.004**
Total body irradiation (8 Gy 7–12))	5 (2.8)	5 (8.2)	0 (0.0)	**0.002**
AAD score (%)				
0	87 (49.4)	23 (37.7)	64 (55.7)	**0.027**
1	25 (14.2)	11 (18.0)	14 (12.2)	0.301
2	23 (13.1)	7 (11.5)	16 (13.9)	0.634
3	32 (18.2)	15 (24.6)	17 (14.8)	0.116
4	4 (2.3)	3 (4.9)	1 (0.9)	0.089
5–7	5 (2.8)	2 (3.2)	3 (2.6)	0.807

*P-values indicate the difference in serum AMH levels in survivors with AMH<1 µg/L and AMH≥1 µg/L. Values are absolute numbers (%) or medians (range). AAD score=alkylating agent dose score; CCS=childhood cancer survivors; AMH=anti-Müllerian hormone.

All polymorphisms were in HWE except for rs494958 (*SRD5A1*) (Table I). Consequently, this polymorphism was not used for comparison of allele frequencies and haplotype distributions in this study.

Table III | Distribution of genotypes associated with age at menopause and minor allele frequencies (MAFs) in 176 Caucasian CCS.

Genotype	CCS AMH<1 µg/L n (%)	CCS AMH≥1 µg/L n (%)	OR	(95% CI)	p
rs7333181					
GG	48 (78.7)	91 (79.8)	(ref)	–	–
GA	10 (16.4)	20 (17.5)	1.14	0.46–2.83	0.777
AA[a]	3 (4.9)	3 (2.6)			
rs1172822					
CC	11 (18.0)	45 (40.5)	(ref)	–	–
CT	41 (67.2)	54 (48.6)	3.15	1.35–7.32	**0.008**
TT	9 (14.8)	12 (10.8)	3.45	1.06–11.27	0.040
rs236114					
CC	38 (69.1)	71 (65.7)	(ref)	–	–
CT	14 (25.5)	33 (30.6)	0.96	0.44–2.11	0.919
TT[a]	3 (5.5)	4 (3.7)	–	–	–
rs271924					
AA	27 (44.3)	49 (43.4)	(ref)	–	–
AT	27 (44.3)	55 (48.7)	0.80	0.39–1.63	0.802
TT	7 (11.5)	9 (8.0)	1.40	0.40–4.91	0.602
rs9457827					
CC	54 (88.5)	99 (86.8)	(ref)	–	–
CT	6 (9.8)	14 (12.3)	0.75	0.24–2.40	0.633
TT[a]	1 (1.6)	1 (0.9)	–	–	–
rs909253					
AA	28 (48.3)	51 (45.5)	(ref)	–	–
AG	23 (39.7)	49 (43.8)	0.80	0.38–1.69	0.563
GG	7 (12.1)	12 (10.7)	1.46	0.47–4.49	0.510

CCS=childhood cancer survivors; AMH=anti-Müllerian hormone (µg/L), OR=odds ratio; CI=confidence interval; MAF=minor allele frequency; AAD score=alkylating agent dose score; RT=radiotherapy. Multivariate analysis adjusted for age at AMH measurement, AAD score and abdominal radiotherapy. [a]Analysis was not performed because of small number of patients (<5 per subgroup).

After correction for multiple testing, women with the CT genotype of rs1172822 had a significantly increased risk for a low serum AMH level (OR: 3.15, 95% CI: 1.35–7.32, p=0.008), while there was a trend towards a higher risk of a low serum AMH level in women with the TT genotype (OR: 3.45, 95% CI: 1.06–11.27, p=0.04) (Table III) after correction for the age at AMH measurement, AAD score and

abdominal irradiation. None of the other SNPs was associated with an increased risk for a low AMH. Using a carrier model, the T-allele of rs1172822 showed a borderline significant association with continuous serum AMH levels (β: -0.16, 95% CI: -0.62 to -0.06, p=0.017, Table IV). This association may not be as significant as the analysis of the subgroups owing to the small number of patients (n=21) homozygous for the minor allele (T). Therefore, a linear regression of AMH levels and the three genotypes (CC, CT, TT) has been performed. A significant negative association between AMH and the heterozygote CT genotype of rs1172822 has been observed (β: -0.63, 95% CI: -1.02 to 0.24, p=0.002), while no significant association between the TT genotype and AMH has been found (β: -0.47, 95% CI: -1.06 to 0.13, p=0.123).

Table IV | Distribution of allelic frequencies associated with age at menopause.

SNP (minor allele)	Beta (Log AMH)	95% CI	p
rs7333181 (A)	0.06	-0.22 to 0.51	0.431
Age AMH	-0.24	-0.08 to -0.02	0.001
AAD score	-0.22	-0.36 to -0.08	0.002
RT abdomen (Gy)	-0.34	-0.11 to -0.05	< 0.0001
rs1172822 (T)	-0.16	-0.62 to -0.06	0.017
Age AMH	-0.23	-0.08 to -0.02	0.001
AAD score	-0.19	-0.32 to -0.06	0.006
RT abdomen (Gy)	-0.34	-0.11 to -0.05	< 0.0001
rs236114 (T)	0.07	-0.15 to 0.49	0.291
Age AMH	-0.24	-0.08 to -0.02	0.001
AAD score	-0.22	-0.35 to -0.08	0.002
RT abdomen (Gy)	-0.36	-0.11 to -0.05	< 0.0001
rs271924 (T)	0.05	-0.17 to 0.39	0.445
Age AMH	-0.23	-0.08 to -0.02	0.001
AAD score	-0.21	-0.35 to -0.08	0.002
RT abdomen (Gy)	-0.34	-0.11 to -0.05	< 0.0001
rs9457827 (T)	-0.05	-0.65 to 0.31	0.483
Age AMH	-0.23	-0.08 to -0.02	0.001
AAD score	-0.20	-0.34 to -0.07	0.004
RT abdomen (Gy)	-0.34	-0.11 to -0.05	< 0.0001
rs909253 (G)	-0.01	-0.30 to 0.25	0.862
Age AMH	-0.21	-0.07 to -0.02	0.004
AAD score	-0.17	-0.32 to -0.04	0.014
RT abdomen (Gy)	-0.35	-0.11 to -0.05	< 0.0001

CI=confidence interval; AAD score=alkylating agent dose score; RT=radiotherapy; AMH=anti-Müllerian hormone.

We evaluated whether rs1172822 was associated with the predicted age at menopause using two menopause prediction models [18,19]. The rs1172822 polymorphism was negatively associated with predicted age at menopause using the Dutch population model (β: -0.17, 95% CI: -1.18 to -0.02, p=0.044) after adjustment for AAD score and abdominal radiotherapy. However, the effect of treatment with alkylating agents or pelvic radiotherapy seemed to be larger than the effect of the polymorphism rs1172822 on the predicted age at menopause (Table V). When we predicted the age at menopause based on the Iranian population model, we did not find a significant correlation with rs1172822 (β: -0.15, 95% CI: -5.94 to 0.42, p=0.088) (Table V). When performing analyses without adjustment for confounders, the rs1172822 was borderline significant associated with predicted age at menopause in the Dutch population model (β: -0.64, 95% CI: -1.29 to 0.01, p=0.054) and was still not associated with the age in the Iranian population model (β: -2.99, 95% CI -6.12 to 0.15, p=0.062).

Table V | Predicted age at menopause and *BRSK1* polymorphism.

	OR	95% CI	p
Method of Broer *et al* (Dutch population) [18]			
rs1172822 (T)	-0.17	-1.18 to -0.02	**0.044**
AAD score	-0.22	-0.56 to -0.08	0.010
Abdominal RT (Gy)	-0.27	-0.18 to -0.05	0.001
Method of Tehrani *et al* (Iranian population) [19]			
rs1172822 (T)	-0.15	-5.94 to 0.42	0.088
AAD score	-0.16	-2.56 to 0.04	0.058
Abdominal RT (Gy)	-0.17	-0.74 to 0.01	0.055

OR=odds ratio; CI=confidence interval; AAD=alkylating agent dose; RT=radiotherapy.

DISCUSSION

We evaluated the importance of SNPs, which had previously been shown to be associated with the age at natural menopause in healthy women, to the ovarian reserve in female CCS. We found that the CT genotype of rs1172822 of the *BRSK1* gene was negatively associated with serum AMH levels after correction for multiple testing. Moreover, the T-allele of rs1172822 was associated with the predicted age at menopause of the Dutch population model.

Serum AMH levels were used as a proxy for the ovarian reserve. As most of our patients were in the premenopausal age range, we needed a marker that does not fluctuate during the menstrual cycle and is representative for the ovarian reserve. AMH is strongly correlated with the antral follicle count and is relatively constant during and between menstrual cycles, in contrast to FSH [23-26]. Although some studies did report variations during the cycle [27-30], the differences were small in amplitude and similar to the reported minor variability between cycles, which suggests that AMH production is independent of gonadotropins. Therefore, AMH is a useful serum marker for the ovarian reserve.

Moreover, two studies showed that serum AMH levels can be used to predict the age at menopause [18,19], and therefore serum AMH levels were used in our study. An AMH level of 1 µg/L has been used as a cut-off value to identify a decreased ovarian reserve. Several studies have tried to identify a cut-off value for AMH to classify decreased fertility, poor response after IVF and non-pregnancy, and these studies have been reviewed [31]. The mean of these AMH cut-off values is approximately 1 µg/L and we used this value as it is the best available estimate. However, we also performed our quantitative analyses with AMH levels and found a borderline significant association with the minor allele (T) of rs1172822. This association may not be as significant as the analysis of the subgroups owing to the small number of patients (n=21) homozygous for the minor allele (T). Therefore, a linear regression of AMH levels and the three genotypes (CC, CT, TT) has been performed. A highly significant negative association between AMH and the heterozygote CT genotype of rs1172822 has been observed (β: -0.63, 95% CI: -1.02 to -0.24, p=0.002), which supports the negative association between rs1172822 and AMH levels.

A low serum AMH level in adult female CCS was associated with the CT genotype of rs1172822, which is consistent with previous findings in healthy women in which this polymorphism was associated with a decrease in the age at menopause of 4 months per T-allele [11,12]. Rs1172822 is a polymorphism in the BR serine/ threonine kinase 1 gene (*BRSK1*), which is located on chromosome 19q13.4, intron 17 and encodes an AMP-activated protein kinase (AMPK)-related kinase. It is highly expressed in the human forebrain [32] and moderately expressed in mammalian ovaries [33]. Interestingly, the downstream targets of *BRSK1* are several members of the family of AMPK-related kinases phosphorylate tau, a microtubule-associated protein that regulates the stability of the microtubule network [32]. This includes the maternal embryonic leucine zipper kinase (MELK2; chromosome 9) that is highly expressed in spermatogonia and oocytes [34,35]. *BRSK1* is specifically activated by phosphorylation, together with 12 other AMPKs, including MELK, through the serine/threonine protein kinase 11, named STK11 or LKB1 [36]. LKB1 (19p13.3) is considered a master regulator of cell polarity (by regulating cytoskeletal dynamics) and, being the only identified protein with this activity, it is expressed in mouse oocytes [37]. Mutation of LKB1 affects epithelial, neuronal and oocyte polarity, thereby influencing cell growth. More recently, *BRSK1* was found to be essential for centriole duplication and therefore plays an important role in cell-cycle progression [38]. *BRSK1* is therefore important for cells to function normally. Recent hypotheses concerning an association between genome-stability genes and ageing have been proposed: recent studies link specific deficiencies in genome maintenance to symptoms of premature ageing, which are likely related to a tissue-specific spectrum of DNA lesions caused by the unique metabolic profile of each particular organ or tissue [39]. This may explain the involvement of *BRSK1* in the ovarian ageing process, possibly resulting in reduced AMH levels.

In the present study, only one of the six previously identified polymorphisms in the healthy population was associated with AMH levels in adult female CCS. These findings suggest that the effects of gonadotoxic treatment exceed the influence of these genetic polymorphisms. However, most of these SNPs have a low MAF (Table I). The lower the MAF, the higher the number of survivors needed to achieve sufficient power to observe an influence on AMH levels. Therefore, final conclusions regarding these associations cannot be drawn given the relatively small effect size

of those polymorphisms and the large effect of alkylating agents and pelvic radiotherapy on the ovarian reserve in a small sample of CCS. Our observations should be investigated further in a larger sample.

Rs1172822 is significantly associated with the predicted age at menopause of the Dutch population model by Broer *et al* [18]. This is in line with a previous report that the rs1172822 polymorphism is associated with the age at menopause in the healthy population [11]. Still, the effect of treatment with alkylating agents or pelvic radiotherapy seemed to be stronger than the effect of the rs1172822 polymorphism on the predicted age at menopause. We found no association between rs1172822 and the predicted age at menopause using the Iranian population model: the different genetic background of the women in the Iranian population model is most likely the cause. Indeed, the rs1172822 MAF in Asian subjects is ten-fold lower than in the European population [40].

We realize that our pilot study only shows preliminary data. To confirm our findings and enlarge the study, a nationwide GWA project on the ovarian reserve in female CCS should be performed, including a replication cohort.

In conclusion, we showed that the CT genotype of rs1172822 of the *BRSK1* gene in female adult CCS is associated with a decreased serum AMH level and lower predicted age at menopause, based on a Dutch population model. These findings support the idea that previously identified polymorphisms which are associated with age at menopause in healthy women may also have an effect on the onset of menopause in female CCS. This pilot study appears to show a new aspect of the influence of genetic variants on the ovarian reserve following treatment of childhood cancer and should be investigated further in a nationwide GWA study. Eventually, this information can help us to improve counselling on fertility preservation prior to cancer treatment, based on genetic factors in individual patients.

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Supplementary Table I | Tertile distribution of alkylating agents in cumulative dose.

Alkylating Agent	First tertile	Second tertile	Third tertile
Oral cyclophosphamide, mg/m^2	1–4,800	4,801–8,800	8,801–45,990
Parenteral cyclophosphamide, mg/m^2	1–2,000	2,001–4,600	4,601–21,300
Ifosfamide, mg/m^2	1–12,000	12,001–45,000	45,001–102,000
Busulfan, mg/m^2	1–320	321–436	437–940
Lomustine, mg/^{2}	1–450	451–600	601–1,050
Melphalan, mg/m^2	1–138	139–180	181–320
Mitoxine, mg/m^2	1–42	43–60	61–160
Procarbazine, mg/m^2	1–3,500	3,501–5,600	5,601–19,200
Thiotepa, mg/m^2	1–392	393–800	801–1,200

CHAPTER 7

Recommendations for primary ovarian insufficiency surveillance for female childhood cancer survivors: a report from the international late effects of childhood cancer guideline harmonization group

W van Dorp, MM Hudson, LC Kremer, RL Mulder, MM van den Heuvel-Eibrink, MH van den Berg, JL Levine, E van Dulmen-den Broeder, N di Iorgi, WH Wallace & R Haupt; on behalf of the IGHG and PCSF female gonadal toxicity working group

In preparation

Abstract

Female childhood cancer survivors treated with alkylating agents and/or ovarian irradiation have an increased risk of primary ovarian insufficiency (POI). Clinical practice guidelines are necessary to warrant that survivors receive optimum care, and thereby improve optimal treatment of late effects of cancer treatment. However, surveillance recommendations vary among the existing long-term follow-up guidelines, resulting in a hampered implementation of screening. The present guideline offers international harmonized POI surveillance recommendations for female childhood cancer survivors treated up to age 18 years. Evidence-based methods were applied to develop the international harmonized recommendations. The recommendations were formulated by an international multidisciplinary guideline panel and categorized according to a 4-level colour grading schema adapted from existing level of evidence criteria. The harmonized POI surveillance recommendations are based on a transparent process and are intended to facilitate care for childhood cancer survivors.

Introduction

Currently, long-term survival of childhood cancer exceeds 80% for some cancer types following advances in the treatment regimens over the last decades [1-3]. Consequently, due to the increasing numbers of survivors, it has become increasingly apparent that a significant proportion of these survivors experience chronic health sequelae resulting from cancer, its treatment or both [4-6]. Of particular concern is the substantially elevated risk of gonadal dysfunction among female survivors, especially after treatment with alkylating agents and/or radiation to fields that include the ovaries [7]. Among female survivors, the risk of non-surgical primary ovarian insufficiency (POI) is increased as compared to their siblings, with a cumulative incidence of ~8% by the age of 40 years [8]. POI is associated with infertility, but also with serious other sequelae due to the deprivation of oestrogen levels, such as osteoporosis, cardiovascular disorders, impaired well-being and compromised sexual health [9]. Because of these risks, it has been acknowledged that survivors at risk and health care providers need guidance for awareness of cancer- and treatment-related health risks, and may benefit from long-term surveillance for POI in order to treat patients appropriately.

Development of clinical practice guidelines for long-term POI surveillance is warranted [10-13]. Already, a number of clinical practice guidelines have been developed by various North American and European groups to facilitate early detection and treatment of POI [14-18]. These guidelines differ with regards to definition of at risk populations, surveillance modality and frequency, and recommendations for interventions. As a result of the guideline differences, the implementation of screening may be hampered across a wide spectrum of clinical settings. Recognizing the importance of a global consensus, an international effort was organized to harmonize existing late effects screening recommendations for survivors of childhood cancer [19]. The current effort of the International Late Effects of Childhood Cancer Guideline Harmonization Group represents the summary of the evidence of- and recommendations for POI surveillance in childhood cancer survivors treated up to age 18 years.

Methods

Detailed information of the international guideline harmonization effort and methodology has been provided elsewhere [19]. The POI surveillance recommendations were prepared by representatives from the North American Children's Oncology Group (COG) [14], the Dutch Childhood Oncology Group (DCOG) [15], the Scottish Intercollegiate Guidelines Network (SIGN) [16] and the United Kingdom Children's Cancer and Leukaemia Group (UKCCLG) [17]. The current effort covers guidelines that were developed following systematic evaluation of the quality of the late effects literature, linking cancer treatment with adverse outcomes compared with expert opinions. The expert members represented all relevant disciplines, ranging from the paediatric oncology/haematology, radiotherapy, gynaecology and endocrinology, and survivorship care providers to guideline methodologists and epidemiologists in their efforts.

First, we evaluated concordances and discordances across the COG, DCOG, SIGN and UKCCLG guidelines [14-17]. In order to achieve consensus, clinical questions were formulated within the expert team to address areas of discordance for POI surveillance. Systematic electronic literature searches were performed in March 2013, and evidence summaries were generated to answer the relevant clinical questions. When evidence was lacking for childhood cancer survivors, we carefully extrapolated evidence from other populations, if applicable. In the case of concordance, we extracted and evaluated the evidence cited by the guidelines.

Considering the heterogeneity in definitions used to describe ovarian dysfunction, we proposed standardized definitions which were integrated into our literature review and final formulation of recommendations. Childhood cancer survivors were defined as individuals treated for cancer up to age 18 years, regardless of current age. POI was defined as a clinical situation developing in any adult female up to 40 years of age, characterized by: (1) absence of menses for at least 4 months, and (2) two elevated serum follicle-stimulating hormone (FSH) levels in the menopausal range (cut-off depends on the used assay) [20]. We defined the hard outcome POI as 'having both criteria', while the surrogate outcome was defined as having at least one of the two POI criteria (amenorrhoea, elevated serum FSH levels). Delayed puberty was defined as the absence of initiation of puberty (Tanner stage 2 breast development) in girls 13 years or older. Amenorrhoea was defined as the absence of menstrual cycles for at least 4 months [21]. Irregular menstrual cycles were defined as cycles <21 days or >35 days [22]. Ovarian radiation was defined as radiotherapy involving the ovaries (lumbar, sacral, whole spine, flank/hemi-abdomen extending below iliac crest, whole abdomen, inverted Y, pelvic, vaginal, bladder, iliac, total body irradiation (TBI)).

As agreed by the International Guideline Harmonization Group, the quality of the evidence and strength of the recommendations were determined according to a revised version of the Grading of Recommendations Assessment Development and Evaluation (GRADE) criteria and the Applying classification of recommendations and level of evidence criteria of the American Heart Association (ACC/AHA) [23,24]. The quality of the evidence was graded as previously published [19]. Briefly, it was defined as: **A**, high level of evidence (i.e. consistent evidence from well performed and high quality studies or systematic reviews with a low risk of bias, and direct, consistent and precise results); **B**, moderate to low level of evidence (i.e. evidence from studies or systematic reviews with few important limitations); and **C**, very low level of evidence (i.e. evidence from studies with serious flaws, only expert opinion or standards of care). The strength of the recommendations was categorized using the 4-color schema adapted from the ACC/AHA [23]. Green represents a strong recommendation (high quality evidence), using anchor terms such as 'is recommended', and with a low degree of uncertainty. Yellow (moderate quality evidence) and orange (weak quality evidence) represent moderate level recommendations, using terms such as 'is reasonable' and 'may be reasonable', respectively. Red is used to recommend against a particular intervention, with harms outweighing benefits. Final recommendations were based on this scientific knowledge combined with other considerations such as clinical judgments, decisions about thresholds and costs. The yellow/orange recommendations represent a higher degree of uncertainty, meaning that other factors, such as the clinical expertise, patient preferences, relevant risk factors and costs, need to be considered in the final decision-making process for the individual patient [25]. The international POI

surveillance recommendations will be critically appraised by independent external experts in the field and patient representatives. The recommendations will be updated within two years.

Search strategy and selection criteria

In order to identify all available evidence concerning the risk of POI after childhood cancer, specific risk groups, and the treatment of POI, we performed multiple searches in MEDLINE (through PubMed), and the Cochrane Central Library of Controlled Trials (CENTRAL) using the following main search terms "childhood", "adolescent", "neoplasm", "female", "survivor", "alkylating", "antimetabolites", "platin", "radiation", "primary ovarian insufficiency", "FSH", "oestradiol", "AMH", "antral follicle count", "puberty induction", "hormone replacement therapy", "final height", "sexual development", "bone density", cardiovascular diseases", "secondary neoplasms" from 1993 until March 2013 (search strategies are provided in Appendix I). If not included initially, cross-references picked up during the review procedure were also selected. In addition, references supporting the existing recommendations were extensively discussed with experts in the field to determine if there was any additional evidence. Only papers published in English were reviewed. The final selection of studies was determined on the basis of relevance to the broad scope of this guideline.

Results

Concordances and discordances among the POI surveillance recommendations of the COG, DCOG, SIGN and UKCCLG are provided in Table I. There was concordance across guidelines for the following statements: (1) the use of Tanner stage as surveillance modality in childhood cancer survivors, (2) to refer childhood cancer survivors to a specialist in case abnormalities were identified, (3) to consider hormone replacement therapy in childhood cancer survivors with POI. For concordant areas, we extracted the evidence cited by the guidelines and determined the levels of evidence.

As illustrated in Table I, there were also areas of discordance that required more detailed investigation of the available literature. The evidence summaries for the following discordant areas are presented in Appendix II: POI risk after alkylating agents, antimetabolites, platinum compounds, ovarian radiation; POI risk by alkylating agents dose, antimetabolites dose, platinum compounds dose, ovarian radiation dose; POI risk by age at treatment; antral follicle counts (AFC) and anti-Müllerian hormone (AMH) for surveillance of POI; FSH, oestradiol, AFC and AMH for prediction of POI; frequency of screening in survivors; risk of deterioration in POI risk during follow-up.

The conclusions of the evidence and the final recommendations are summarized in Table II and Table III, respectively. The rationale for the grading of evidence and subsequent recommendations are provided below, categorized as per clinical question.

Table I | Concordances and discordances among POI surveillance recommendations.

	North American Children's Oncology Group	Dutch Childhood Oncology Group	UK Children's Cancer and Leukaemia Group	Scottish Intercollegiate Guidelines Network	Concordant/ Discordant
Who needs surveillance?					
At risk		All survivors			
Alkylating agents	Yes	Not specified	Yes	Yes	Discordant
Carboplatin, cisplatin	Yes	Not specified	Yes	Not specified	Discordant
Non-classical alkylators*	Yes	Not specified	Yes	Not specified	Discordant
RT involving ovaries**	Yes	Not specified	Yes	Yes	Discordant
RT involving brain***	Yes	Not specified	Yes	Yes	Discordant
Oophorectomy	Yes	Not specified	Not specified	Not specified	Discordant
High risk	Higher doses alkylating agents	Not specified	Not specified	Not specified	Discordant
	Combination alkylating agents				
	Alkylating agents + RT ovaries/ RT brain				
	≥40 Gy RT involving brain				
	Pre-pubertal: ≥10 Gy RT ovaries				
	Pubertal: ≥5 Gy RT ovaries				
	≥18 Gy RT involving brain: precocious puberty				
	Unilateral oophorectomy				
Highest risk	MOPP >3 cycles	Not specified	>24 Gy cranial RT delayed puberty	≥30 Gy cranial RT delayed puberty	Discordant
	Busulfan >600 mg/m^2		<24 Gy cranial RT precocious puberty	<30 Gy cranial RT precocious puberty	
	Cyclophosphamide >7.5 g/m^2 Cyclophosphamide for HCT			High-dose busulfan	
	Alkylating agents + pelvic RT/TBI				
	Pre-pubertal: ≥15 Gy RT ovaries				
	Pubertal: ≥10 Gy RT ovaries				
	Unilateral oophorectomy + pelvic RT /alkylating agents/TBI				
	Bilateral oophorectomy				

Table I | *Continued.*

	North American Children's Oncology Group	Dutch Childhood Oncology Group	UK Children's Cancer and Leukaemia Group	Scottish Intercollegiate Guidelines Network	Concordant/ Discordant
What surveillance modality should be used?					
Tanner staging	Yes	Yes	Yes	Yes	Concordant
Height	Yes (testing precocious puberty)	As general recommendation	Yes	As general recommendation	Discordant
Weight	Yes (testing precocious puberty)	As general recommendation	As general recommendation	As general recommendation	Discordant
Menstrual/pregnancy history	Yes	Yes	Yes	Not specified	Discordant
Inspection external genitals	No	Yes	Not specified	Not specified	Discordant
FSH	Yes	Yes	Not specified	Not specified	Discordant
LH	Yes	Yes	Not specified	Not specified	Discordant
Oestradiol	Yes	Yes	Not specified	Not specified	Discordant
At what frequency should surveillance be performed?					
Tanner staging (until sexually mature)	Every 1 yr	Every visit (all survivors)	Every 0.5 yr (all survivors) 3-4 times a year (if cranial RT)	3-4 times a year (if cranial RT)	Discordant
Height	Yearly until sexually mature (testing precocious puberty)	Not applicable	Every 0.5 yr (all survivors, until growth spurt established) 3-4 times a year (if cranial RT)	Not applicable	Discordant
Weight	Yearly until sexually mature (testing precocious puberty)	Not applicable	Not applicable	Not applicable	Not applicable
Menstrual/pregnancy history	Every 1 yr	Every visit (all survivors)	When appropriate	Not applicable	Discordant

Table I | *Continued*

	North American Children's Oncology Group	Dutch Childhood Oncology Group	UK Children's Cancer and Leukaemia Group	Scottish Intercollegiate Guidelines Network	Concordant/ Discordant
At what frequency should surveillance be performed? *(Continued)*					
Inspection external genitals	Not applicable	Every visit (all survivors, when sexually mature)	Not applicable	Not applicable	Not applicable
FSH	Baseline at age 13 yr and as clinically indicated	As clinically indicated	Not applicable	Not applicable	Partly concordant
LH	Baseline at age 13 yr and as clinically indicated	As clinically indicated	Not applicable	Not applicable	Partly concordant
Oestradiol	Baseline at age 13 yr and as clinically indicated	As clinically indicated	Not applicable	Not applicable	Partly concordant
What should be done when abnormalities are identified?					
Refer to specialist	Yes	Yes	Yes	Yes	Concordant
Consider hormonal therapy	Yes	Yes	Yes	Yes	Concordant
Consider assisted reproductive technology	Yes	Not specified	Yes	Yes	Discordant

UK=United Kingdom; RT=radiotherapy; MOPP=mustargen, oncovin, procarbazine, prednisone; HCT=haematopoietic cell transplantation; TBI=total body irradiation; yr=year; FSH=follicle-stimulating hormone; LH=luteinizing hormone.

Table II | Conclusions of evidence for POI surveillance for female childhood cancer survivors.

Who needs POI surveillance?	
POI risk in childhood cancer survivors	
– Increased risk after alkylating agents and procarbazine	Level A
– Increased risk after increasing doses alkylating agents and procarbazine	Level A
– Alkylating threshold dose	No evidence
– Increased risk after ovarian irradiation	Level A
– Increased risk after increasing doses ovarian radiation	Level A
– Ovarian radiation threshold dose	No evidence
– Increasing risk after alkylating agents + ovarian radiation compared to either treatment in same dose alone	Level B
– Increased risk after treatment at older age	Level B
– Risk after platinum compounds	No evidence
– Risk after antimetabolites	No evidence
– Increased risk of hypogonadotropic-hypogonadism after radiation to the field that includes the hypothalamic-pituitary axis	Level C
What surveillance modalities should be used?	
Diagnostic value clinical exam, endocrine measurements, ovarian ultrasound in childhood cancer survivors	
○ Diagnostic value for weight, height, menstrual cycle history, Tanner stage	Level A
– FSH and oestradiol	Level B
– Anti-Müllerian hormone	No evidence
– Antral Follicle Count	No evidence
Prognostic value endocrine measurements, ovarian ultrasound in childhood cancer survivors	
– FSH and oestradiol	No evidence
– Anti-Müllerian hormone	No evidence
– Antral Follicle Count	No evidence
At what frequency and for how low should surveillance for POI be performed?	
– The risk of deterioration in gonadal function continues to increase with longer follow-up	Level A
What should be done when abnormalities are found?	
○ Refer to specialist	Level A
○ Consider hormonal therapy (hormone replacement therapy)	Level A

Level A=high level of evidence; Level B=moderate/low level of evidence; Level C=very low level of evidence.

○ Evidence extracted from the existing guidelines: COG [14], DCOG [15], SIGN [16], and UKCCLG [17]

– Evidence as presented in the evidence summaries (Appendix II).

Table III | Harmonized recommendations for POI surveillance for childhood cancer survivors.

General recommendation
Survivors treated with gonadotoxic chemotherapy* and/or ovarian radiation* and their providers should be aware of the risk of primary ovarian insufficiency and its implications for future fertility.
Who needs POI surveillance and what surveillance modality should be done?
Monitoring of growth and pubertal development (height, weight, Tanner stage) ***is recommended*** for pre-pubertal females treated with gonadotoxic chemotherapy* and/or ovarian radiation*[#].
Counselling regarding the risk of primary ovarian insufficiency and its implications for future fertility ***is recommended*** for post-pubertal females treated with gonadotoxic chemotherapy* and/or ovarian radiation*.
Laboratory evaluation with FSH and oestradiol ***is recommended*** for pre-pubertal females who fail to initiate or progress through puberty[@].
Laboratory evaluation with FSH and oestradiol ***is recommended*** for post-pubertal females who present with menstrual cycle dysfunction suggesting primary ovarian insufficiency or who desire assessment about potential for future fertility. Hormone replacement therapy should be discontinued prior to laboratory evaluation when applicable[$].
Laboratory evaluation with AMH ***may be reasonable*** for female survivors 25 years or older who present with menstrual cycle dysfunction suggesting primary ovarian insufficiency or who desire assessment about potential for future fertility.
What should be done when abnormalities are identified?
Referral to paediatric endocrinology/gynaecology ***is recommended*** for pre-pubertal females who fail to initiate or progress through puberty[@].
Referral to gynaecology/(reproductive) endocrinology ***is recommended*** for post-pubertal females who present with menstrual cycle dysfunction suggesting primary ovarian insufficiency.
Treatment with sex steroid replacement therapy (transdermal or oral oestrogens plus progestin) ***is recommended*** in pre-pubertal and post-pubertal females diagnosed with primary ovarian insufficiency.
What should be done when potential for future fertility is questioned?
Referral to gynaecology/(reproductive) endocrinology and laboratory evaluation with FSH and oestradiol ***is recommended*** for post-pubertal females treated with gonadotoxic chemotherapy* and/or ovarian irradiation* without signs and symptoms of primary ovarian insufficiency who desire assessment about potential for future fertility.

*Gonadotoxic chemotherapy: alkylating agents and procarbazine; Ovarian radiation: radiation treatment fields involving the ovaries (lumbar, sacral, whole spine, flank/hemiabdomen extending below iliac crest, whole abdomen, inverted Y, pelvic, vaginal, bladder, iliac, TLI, TBI); [#]Expert opinion: at least annually, with increasing frequency as clinically indicated based on growth and pubertal progress; [@]Expert opinion: at least for girls of 11 years of age and older; [$]Expert opinion: this assessment should be performed after ending oral contraceptive pill use, preferably after four months without oral contraceptive pills (based on: amenorrhoea=no menstrual cycle for at least four months).

Who needs POI surveillance?

Evidence in childhood cancer survivors

There is evidence from large cohort studies that the risk of POI in female childhood cancer survivors treated with gonadotoxic chemotherapy and/or ovarian radiation is increased [8,26-34]. Importantly, there appears to be no clear cut-off for a safe alkylating agent dose. It is well known that women

exposed to higher alkylating agents doses have an increased risk of POI [8,27,29], but previous studies have scored alkylating agent doses differently, which resulted in the inability to compare doses and define a cut-off score.

Similarly, the working group agreed that there is an increasing risk of POI with increasing ovarian radiation doses, but a clear cut-off for a safe radiation dose could not be defined. Ovarian radiation at an older age confers a greater dose-related risk, with increased risk resulting from a smaller pre-existent primordial follicle pool [26,31]. Mathematic modelling based on data on the rate of oocyte decline suggests that the sterilizing dose is 20.3 Gy in infants, 18.4 Gy at age 10 years, and 16.5 Gy at age 20 years [31]. Moreover, treatment with a combination of ovarian radiation and alkylating agents increases the risk of POI compared to either treatment in same dose alone [8,27].

While some studies have reported no influence of age at treatment [8,33,34], others have found an increased risk of POI in individuals treated at a younger age [28,32]. As described above, the increased risk seems to result from a smaller primordial pool at older age. As a result of the inconsistency of the literature, no recommendations could be made regarding surveillance intensity by age at exposure. Evidence suggests that survivors treated with radiation to fields that includes the hypothalamic-pituitary-axis, the risk hypogonadotropic-hypogonadism is increased [35]. Since this guideline focusses on POI surveillance, no recommendations for POI surveillance have been formulated in childhood cancer survivors treated with radiation to fields that includes the hypothalamic-pituitary-axis. In addition, due to lack of data, no recommendations could be made regarding surveillance after treatment with antimetabolites and platinum compounds.

Recommendations

Based upon the evidence and consensus, we recommend awareness of the risk of POI and its complications for future fertility after completion of therapy in female childhood cancer survivors treated with gonadotoxic chemotherapy and/or ovarian radiation **(strong recommendation)**. Subsequently, we recommend intensive surveillance for females treated with gonadotoxic chemotherapy and/or ovarian radiation **(strong recommendation)**.

What surveillance modality should be used?

Evidence in childhood cancer survivors

There is agreement across the existing guidelines on the use of detailed history and physical examination with specific attention for failure to initiate or progress through puberty and POI symptoms. Importantly, since POI presents with amenorrhoea, standard laboratory evaluation is not recommended as the primary surveillance in female survivors treated with gonadotoxic chemotherapy and/or ovarian radiation. FSH and oestradiol are important for diagnosing POI since these markers are part of the definition itself [21,36]. Antral follicle count (AFC) by transvaginal ultrasound is the most established method for assessing ovarian reserve in adult women, but is not part of the current clinical criteria of POI [37,38]. In survivors of childhood cancer, the additive value of AFC on the current clinical criteria of POI has not been studied. Anti-Müllerian hormone (AMH) correlates well with AFC [39-41], and would be more convenient for young survivors and easier to use in daily practice. Unlike FSH and oestradiol, AMH remains relatively constant during

and between menstrual cycles [36,42]. In addition, from the age of 25 years onwards, AMH correlates inversely with age, implying that AMH is applicable as marker of ovarian reserve in women of this age and older [43,44]. There is a wide range of AMH levels in healthy young adult women, but very low AMH levels are indicative of ovarian failure. In survivors of childhood cancer, AMH has been frequently used as marker of ovarian reserve [33,38,45], but, to our knowledge, no studies have been performed in which AMH has been used as marker of POI. Still, AMH might be useful to distinguish women with POI who have little to no follicles left from those who are at risk for POI, but still have a reasonably sized follicle pool (AFC ≥3) [36]. Furthermore, AMH could be useful to assess the follicle pool at baseline prior to ovarian senescence [36]. AMH may be of additive value for childhood cancer survivors treated with alkylating agents and/or ovarian radiation.

Recommendations

Detailed history and physical examination with specific attention for POI symptoms, e.g. amenorrhoea, but also irregular cycles (as first sign of the development of POI) and failure to initiate or progress through puberty, is recommended during routine follow-up in all childhood cancer survivors treated with gonadotoxic chemotherapy and/or ovarian radiation **(strong recommendation)**. If abnormalities are found during physical examination, laboratory examination is recommended in pre-pubertal females who fail to initiate or progress through puberty, as part of the current clinical criteria of POI **(strong recommendation)**. In post-pubertal females who present with menstrual cycle dysfunction suggesting POI (irregular menstrual cycles), or who desire assessment about potential future fertility, laboratory evaluation with FSH and oestradiol is recommended **(strong recommendation)**. Based on expert opinion, this assessment should be performed after discontinuing hormone replacement or oral contraceptive therapy (preferably after four months without hormone substitution) when applicable. Based on the evidence in the general population, laboratory evaluation with AMH may be reasonable for female survivors 25 years or older who present with menstrual cycle dysfunction suggesting POI **(moderate recommendation)**.

At what frequency and for how long should surveillance be performed?

Evidence in childhood cancer survivors

In the general population, in girls puberty starts between the age of 8 to 10 years on average [46,47]. The variability is importantly influenced by ethnic differences [46]. By the age of 11 years, most girls have a Tanner stage 2 breast development. To date, there is no evidence describing the frequency of surveillance in female childhood cancer survivors. As data to support changes in POI risk during the fertile life span is lacking, no recommendations could be made for how long surveillance should be performed. However, POI surveillance can obviously be discontinued when the average menopausal age of 50−51 years has been reached [48,49].

Recommendations

Due to lack of data in childhood cancer survivor cohorts, recommendations regarding initiation and frequency of surveillance are largely based on consensus. In order to identify girls with delayed or arrested puberty because of gonadal failure, monitoring of pubertal development is recommended

for pre-pubertal females treated with alkylating agents and/ or ovarian radiation **(strong recommendation)**. Based on expert opinion, this surveillance should be performed at least annually, with increasing frequency as clinically indicated based on growth and pubertal progress. Moreover, for girls of 11 years of age and older, laboratory evaluation is recommended in pre-pubertal females who fail to initiate or progress through puberty.

What should be done when abnormalities are identified?

Evidence in childhood cancer survivors

Because of the health problems induced by oestrogen deprivation in patients with POI, sex steroid replacement therapy is used to replace oestrogens and establishes normal long-term bone health and cardiovascular health [50,51]. Progesterone therapy is also needed to avoid an unopposed oestrogen effect and maintain endometrial health in women with a uterus.

Sex steroid replacement therapy differs for survivors who are pre-pubertal and those who experience gonadal failure after menarche. Timing and tempo of oestrogen substitution in the pre-pubertal patient is crucial to ensure normal pubertal development and an acceptable final height, which should be managed by a provider with expertise in paediatric endocrine development.

Sex steroid replacement therapy can be provided as oral as well as transdermal preparation [52]. Oral sex steroid replacement therapy is the standard method used to normalize oestrogen levels, in order to attain normal long-term bone- and cardiovascular health. Childhood cancer survivor studies comparing sex steroid replacement therapy methods are lacking. Transdermal sex steroid replacement therapy in Turner patients seems to have a comparable or even an increased positive impact on final height, bone and cardiovascular health compared with oral sex steroid replacement therapy [53-56]. However, it is not possible to extent these data to childhood cancer survivors as Turner syndrome patients have often been treated with growth hormone during childhood, in contrast to childhood cancer survivors, which may also affect bone mineral density and body composition [57]. Moreover, it is impossible to generalize treatment benefits for all patients with POI disregarding aetiology and timing.

Recommendations

The recommendations outlined in the current paper are for primary surveillance and do not address all the investigative steps necessary for the diagnosis of POI. As such, paediatric endocrinology/ gynaecology consultation is recommended for pre-pubertal females who fail to initiate or progress through puberty **(strong recommendation)**. In addition, gynaecology/endocrinology/reproductive endocrinology consultation is recommended for post-pubertal females who present with menstrual cycle dysfunction suggesting POI **(strong recommendation)**.

Evidence is insufficient to recommend the optimal sex steroid replacement therapy method for childhood cancer survivors with POI. Nevertheless, treatment with sex hormone replacement therapy (either oral or transdermal oestrogens plus progestin) is recommended in pre-pubertal and post-pubertal females diagnosed with POI **(strong recommendation)**.

What should be done when potential for future fertility is questioned?

Evidence in childhood cancer survivors

Direct measurement of the ovarian reserve is not possible, since markers of oocyte quality and methods to directly determine the number of primordial follicles have yet to be identified [36]. However, the size of the primordial follicle pool is reflected by the number of early, growing follicles. AMH is a product of these growing ovarian follicles, and can be used as an indirect marker of the ovarian reserve in women aged 25 years onwards [43,44]. Subsequently, there is evidence for reduced AMH levels in childhood cancer survivors treated with alkylating agents, procarbazine and/or ovarian radiation [38,58-61].

Recommendations

Although POI presents with amenorrhoea, survivors with regular menstrual cycles treated with gonadotoxic therapy are still at risk of a decreased ovarian reserve, and may therefore be at risk of reduced fertility. Referral to gynaecology/endocrinology/reproductive endocrinology including laboratory evaluation with FSH and oestradiol is recommended for post-pubertal females treated with alkylating agents, procarbazine and/or ovarian radiation without signs and symptoms of POI who desire assessment about potential for future fertility **(strong recommendation)**. Based on the evidence in the general population, laboratory evaluation with AMH may be reasonable for female survivors 25 years and older as a measure of ovarian reserve **(moderate recommendation)**.

Discussion

Because of the rapid growing childhood cancer survivor population, chronic health sequelae that can significantly impact the overall quality and quantity of survival have become apparent, and thus require our attention [4,62]. In female childhood cancer survivors, gonadal dysfunction is one of the major long-term side effects of treatment. Damage to the ovaries causes follicle loss and may therefore result in POI, which results in infertility, but is also associated with osteoporosis and cardiovascular disorders [9]. We present the international harmonized primary ovarian insufficiency surveillance recommendations for childhood cancer survivors treated up to age 18 years. The subsequent recommendations are derived from knowledge gained from extensive scientific review of the available literature, strict standards used to grade the supporting evidence and additional expert consensus. These recommendations are intended to positively influence health outcomes, and to facilitate consistent international follow-up care for female childhood cancer survivors.

It is obviously clear that female childhood cancer survivors treated with gonadotoxic chemotherapy and/or ovarian radiation are at increased risk of POI. Although there is evidence that a higher treatment dose is associated with an increased risk of POI, there is virtually no information on a safe cut-off dose. This might at least partly be explained by the complexity of factors influencing ovarian reserve. A maximum of 6–7 million oogonia is attained by 16–20 weeks gestation, and decreases from that stage onwards [63-67]. During the reproductive lifespan, only 400 to 500 will be selected to ovulate, and the primary follicles will eventually be depleted to a point at menopause when only

a few hundred remain [68]. Because of this primordial pool depletion during life, age at treatment and its corresponding primordial pool size seem important for the determination of a safe treatment dose. Important limitations of previous studies on the impact of alkylating agents- and ovarian radiation dosages were the use of different scoring models, resulting in the impossibility to compare study results. Therefore, we recommend in future studies to use generally approved methods to comprehensively calculate treatment modalities and doses.

Elaborating on the latter, some survivors may develop POI at relatively low gonadotoxic treatment doses while others do not appear to be effected, suggesting that other factors, such as age at treatment, individual susceptibility, dose, combination, but also genetic variation may play a role in the determination of the pre-treatment primordial follicle pool. In both the general female population [69-72] as well as in female childhood cancer survivors [45], polymorphisms have been identified that were associated with (post-treatment) ovarian reserve, even independently from administrated gonadotoxic cancer treatment. Genetic variation, when further established, could advance our understanding of the pathogenesis of treatment-related POI, and may improve patient-tailored counselling and surveillance throughout life. In general, it is of importance to realize that every chemotherapeutic agent is potentially gonadotoxic, depending on age at treatment, individual susceptibility and genetic background, dose and combination.

There is agreement across the COG, DCOG, SIGN, and UKCCLG guidelines that POI surveillance at late effects clinics should include weight, height, Tanner stage, and, if applicable, menstrual history. In case of clinical symptoms of POI, such as irregular menstrual cycles, we recommend laboratory evaluation with FSH and oestradiol. It is noteworthy that these markers should be measured during the early follicular phase in survivors who are still having a menstrual cycle and do not use oral contraceptive pills (OCP), since these markers vary throughout the cycle and during OCP use. Less is known about the diagnostic and predictive value of antral follicle count and AMH levels for diagnosing POI in childhood cancer survivors. Both are not yet included in the definition of POI, but seem of importance in predicting the decrease of ovarian reserve and finally POI over time, since FSH is a relatively late marker of gonadal dysfunction since it starts to increase above normal ranges after severe loss of ovarian reserve [73].

There is agreement across the COG, DCOG, SIGN, and UKCCLG guidelines that, if abnormalities are found during POI surveillance, one should be referred to a specialist; if the survivor is pre-pubertal to a paediatric endocrinologist/gynaecologist, and in case of a post-pubertal survivor to a gynaecologist/(reproductive) endocrinologist. When POI has been diagnosed, sex steroid replacement therapy is recommended. Less evidence is available on the effect of various sex steroid replacement therapy methods on bone and cardiovascular health in childhood cancer survivors. In Turner patients, oral sex steroid replacement therapy seems to have a larger effect on bone- and cardiovascular health compared to oral sex steroid replacement therapy, but studies in childhood cancer survivors are needed to study this phenomenon since Turner patients used growth hormone influencing bone health. In addition, although it is known that POI reduces the risk of radiation-associated breast cancer [74,75], the effect of sex steroid replacement therapy on breast cancer risk in childhood cancer survivors is unknown.

We recommend counselling childhood cancer survivors who received gonadotoxic chemotherapy and/or ovarian radiation regarding their risk of infertility by medical specialists experienced in fertility after childhood cancer. Although AFC by transvaginal ultrasound is the most established method for assessing ovarian reserve in adult women [37,38], AMH correlates well with it and is superior to basal FSH, oestradiol, and inhibin B in healthy women [76,77]. AMH can be easily determined in post-pubertal women, since its serum levels are stable during and between menstrual cycles, in contrast to the cyclic fluctuations observed in serum FSH levels [78]. In children, AMH levels rise during infancy, whereas AMH levels show only minor fluctuations during childhood and adolescence, finally decreasing from the age of 25 years onwards. In addition, the negative AMH–FSH correlation in pre-pubertal girls supports the notion that AMH is a quantitative marker of ovarian follicles even in young girls [79]. Moreover, AMH has been found to be highly predictive for the onset of menopause [73,80,81]. Recent studies have shown that several years prior to menopausal transition, serum AMH levels were low or undetectable and were more predictive of the onset of menopause compared to estimates based on chronological age (82-84). Nomograms predicting the age at menopause have been successfully reported [82,83]. AMH levels have been reported to be decreased in both adult and paediatric survivors of childhood cancer after gonadotoxic therapy [38,58-61], but also in adult and paediatric patients with newly diagnosed cancer before therapy has started [84,85]. AMH shows promise to be useful as a predictor of ovarian reserve and timing of onset of menopause in adult patients with cancer, but studies in childhood cancer survivors are needed to identify the predictive value of AMH on POI and fertility by correlation with AFC, pregnancy outcome and time to pregnancy. Therefore, AMH may be reasonable in women of 25 years and older as measure of ovarian reserve.

An important result of the harmonization process is the demonstration of a research agenda, based upon the identification of key gaps in knowledge (Table IV). The agenda may serve as the impetus for collaborative research aimed at improving POI surveillance and care of at risk childhood cancer survivors. According to our findings, future studies should focus on the identification of threshold doses of both alkylating agents and ovarian radiation, and the influence of other determinants such as genetic variation, which should finally be integrated in a risk model. Furthermore, the prognostic and predictive value of AFC and AMH on POI after childhood cancer is unclear. Longitudinally studies are recommended to identify the effect of both markers.

The POI surveillance harmonization endeavour was strengthened by our evidence-based approach, reliance on standardized definitions for outcomes of interest, transparent presentation of the quality of the available evidence and the strength of recommendations, and the multidisciplinary approach necessary to derive a consensus for screening. Importantly, we have identified key gaps in knowledge regarding safe alkylating agent and ovarian radiation dose and surveillance frequency and modalities. These gaps should be approached in a systematic comprehensive manner preferably by international guided efforts and collaborative opportunities under the umbrella of international groups such as SIOP and Pancare [86,87]. The implementation of the harmonized recommendations will be an ongoing process. Countries, with or without existing long-term follow-up guidelines for childhood cancer survivors, can use the current harmonized guidelines as basis for an evidence-based national policy. The current international harmonization effort was developed to increase collaborative research to ultimately optimize the quality of care for, and minimize the burden of disease of childhood cancer survivors.

Table IV | Gaps in knowledge and future directions for research.

- Safe alkylating agents dose with regards to the risk of POI in childhood cancer survivors.
- Safe ovarian radiation dose with regards to the risk of POI in childhood cancer survivors.
- Diagnostic value of AMH to detect POI in female childhood cancer survivors.
- Value of AMH to predict POI in female childhood cancer survivors.
- Diagnostic value of AFC to detect POI in female childhood cancer survivors.
- Value of AFC to predict POI in female childhood cancer survivors.
- Value of FSH and oestradiol to predict POI in female childhood cancer survivors.
- Lifetime risk of POI in childhood cancer survivors treated with alkylating agents and/or ovarian radiation.
- Potential recovery of ovarian dysfunction in female childhood cancer survivors treated with alkylating agents and/or ovarian radiation.
- Utility of FSH, oestradiol, AFC and AMH on predicting fertility in female childhood cancer survivors.
- Efficacy of oral versus transdermal oestrogen replacement for puberty induction on final height and sexual development in female childhood cancer survivors with gonadal failure.
- Efficacy of oral versus transdermal hormone replacement therapy on bone health, cardiovascular health, and mental health in female childhood cancer survivors with POI.
- Risk of secondary malignancies in female childhood cancer survivors treated with HRT.
- Risk of secondary malignancies in female childhood cancer survivors using HRT and treated with breast irrradiaton.
- Examination of the role of genetic susceptibility on subsequent POI risk in survivors treated with alkylating agents or ovarian radiation.

POI=primary ovarian insufficiency; AMH=anti-Müllerian hormone; AFC=antral follicle count; FSH=follicle-stimulating hormone; HRT=hormone replacement therapy.

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86. http://www.pancare.eu.

87. http://www.siop-online.org/.

Appendix I | Search strategies

POI risk after treatment with alkylating agents, antimetabolites, platinum compounds (humans, search from March 1993 to March 2013):

1. (leukemia OR leukemi* OR leukaemi* OR (childhood ALL) OR AML OR lymphoma OR lymphom* OR hodgkin OR hodgkin* OR T-cell OR B-cell OR non-hodgkin OR sarcoma OR sarcom* OR sarcoma, Ewing's OR Ewing* OR osteosarcoma OR osteosarcom* OR wilms tumor OR wilms* OR nephroblastom* OR neuroblastoma OR neuroblastom* OR rhabdomyosarcoma OR rhabdomyosarcom* OR teratoma OR teratom* OR hepatoma OR hepatom* OR hepatoblastoma OR hepatoblastom* OR PNET OR medulloblastoma OR medulloblastom* OR PNET* OR neuroectodermal tumors, primitive OR retinoblastoma OR retinoblastom* OR meningioma OR meningiom* OR glioma OR gliom*) OR (pediatric oncology OR paediatric oncology) OR (childhood cancer OR childhood tumor OR childhood tumors)) OR (brain tumor* OR brain tumour* OR brain neoplasms OR central nervous system neoplasm OR central nervous system neoplasms OR central nervous system tumor* OR central nervous system tumour* OR brain cancer* OR brain neoplasm* OR intracranial neoplasm*) OR (leukemia, lymphocytic, acute) OR (leukemia, lymphocytic, acute*)
2. infant OR infan* OR newborn OR newborn* OR new-born* OR baby OR baby* OR babies OR neonat* OR perinat* OR postnat* OR child OR child* OR schoolchild* OR schoolchild OR school child OR school child* OR kid OR kids OR toddler* OR adolescent OR adoles* OR teen* OR boy* OR girl* OR minors OR minors* OR underag* OR under ag* OR juvenil* OR youth* OR kindergar* OR puberty OR puber* OR pubescen* OR prepubescen* OR prepuberty* OR pediatrics OR pediatric* OR paediatric* OR peadiatric* OR schools OR nursery school* OR preschool* OR pre school* OR primary school* OR secondary school* OR elementary school* OR elementary school OR high school* OR highschool* OR school age OR schoolage OR school age* OR schoolage* OR infancy OR schools, nursery OR infant, newborn OR young adult[mh] OR adult[mh] OR young adult OR young woman OR young women OR female
3. female[tiab] OR females OR girl OR girls OR girlfriend OR girlhood
4. Survivor OR survivors OR Long-Term Survivors OR Long Term Survivors OR Long-Term Survivor OR Survivor, Long-Term OR Survivors, Long-Term OR survivo* OR surviving
5. Antineoplastic agents, alkylating* OR antineoplastic alkylating agents OR alkylating agents, antineoplastic OR antineoplastic drugs, alkylating OR antineoplastics, alkylating OR alkylating antineoplastic drugs OR alkylating drugs, antineoplastic OR antineoplastic alkylating drugs OR drugs, antineoplastic alkylating OR alkylating antineoplastic agents OR alkylating antineoplastics OR Alkylating Agents OR alkylating agent* OR busulphan OR Carmustine OR Chlorambucil OR ifosfamide OR iphosphamide OR cyclophosphamide OR Lomustine OR Chlorethazine OR Procarbazine OR Melphalan OR Carboplatin OR Cisplatin OR cytosine OR temozolomide OR dacarbazine OR Fludarabine monophosphate* OR Methotrexate
6. premature menopause* OR early menopause* OR menopausal status* OR ovarian failure* OR premature ovarian failure* OR acute ovarian failure* OR imminent ovarian failure* OR ovarian insufficiency* OR ovarian function* OR ovarian damage* OR Gonadotropin-Resistant Ovary Syndrome* OR Female Genital Diseases* OR Female infertility* OR primary ovarian insufficiency* OR gonadotoxicity OR gonado toxicity* OR gonadal damage* OR hypergonadotropic amenorrhoea* OR gonad dysfunction* OR gonadal function* OR gonadal effects* OR ovarian reserve* OR gonadal hormone deficiency*

POI risk after treatment with ovarian radiation, and hypogonadotropic-hypogonadism risk after treatment with cranial radiation (humans, search from March 1993 to March 2013):

1. (leukemia OR leukemi* OR leukaemi* OR (childhood ALL) OR AML OR lymphoma OR lymphom* OR hodgkin OR hodgkin* OR T-cell OR B-cell OR non-hodgkin OR sarcoma OR sarcom* OR sarcoma, Ewing's OR Ewing* OR osteosarcoma OR osteosarcom* OR wilms tumor OR wilms* OR nephroblastom* OR neuroblastoma OR neuroblastom* OR rhabdomyosarcoma OR rhabdomyosarcom* OR teratoma OR teratom* OR hepatoma OR hepatom* OR hepatoblastoma OR hepatoblastom* OR PNET OR medulloblastoma OR medulloblastom* OR PNET* OR neuroectodermal tumors, primitive OR retinoblastoma OR retinoblastom* OR meningioma OR meningiom* OR glioma OR gliom*) OR (pediatric oncology OR paediatric oncology) OR (childhood cancer OR childhood tumor OR childhood tumors)) OR (brain tumor* OR brain tumour* OR brain neoplasms OR central nervous system neoplasm OR central nervous system neoplasms OR central nervous system tumor* OR central nervous system tumour* OR brain cancer* OR brain neoplasm* OR intracranial neoplasm*) OR (leukemia, lymphocytic, acute) OR (leukemia, lymphocytic, acute*)
2. female[tiab] OR females OR girl OR girls OR girlfriend OR girlhood
3. (Ovary OR Pelvis OR Lesser Pelvis OR Abdomen OR Spine OR Lumbosacral Region OR Urinary Bladder OR Vagina OR Ilium OR Hypothalamus OR Hypothalamus, Middle OR Hypothalamus, Anterior, OR Hypothalamus Posterior OR Pituitary Gland, Posterior OR Skull OR Orbit OR Orbits OR Eye OR Ear OR Nasopharynx) AND Radiotherapy OR Cranial Irradiation OR Whole-Body Irradiation
4. premature menopause* OR early menopause* OR menopausal status* OR ovarian failure* OR premature ovarian failure* OR acute ovarian failure* OR imminent ovarian failure* OR ovarian insufficiency* OR ovarian function* OR ovarian damage* OR Gonadotropin-Resistant Ovary Syndrome* OR Female Genital Diseases* OR Female infertility* OR primary ovarian insufficiency* OR gonadotoxicity OR gonado toxicity* OR gonadal damage* OR hypergonadotropic amenorrhoea* OR gonad dysfunction* OR gonadal function* OR gonadal effects* OR ovarian reserve* OR gonadal hormone deficiency* OR hypogonadism OR hypogonadotropic hypogonadism* OR hypogonadism

Diagnostic value of AFC and AMH, and predictive value of FSH, estradiol, AFC, AMH for POI (humans, search from March 1993 to March 2013):

1. (leukemia OR leukemi* OR leukaemi* OR (childhood ALL) OR AML OR lymphoma OR lymphom* OR hodgkin OR hodgkin* OR T-cell OR B-cell OR non-hodgkin OR sarcoma OR sarcom* OR sarcoma, Ewing's OR Ewing* OR osteosarcoma OR osteosarcom* OR wilms tumor OR wilms* OR nephroblastom* OR neuroblastoma OR neuroblastom* OR rhabdomyosarcoma OR rhabdomyosarcom* OR teratoma OR teratom* OR hepatoma OR hepatom* OR hepatoblastoma OR hepatoblastom* OR PNET OR medulloblastoma OR medulloblastom* OR PNET* OR neuroectodermal tumors, primitive OR retinoblastoma OR retinoblastom* OR meningioma OR meningiom* OR glioma OR gliom*) OR (pediatric oncology OR paediatric oncology) OR (childhood cancer OR childhood tumor OR childhood tumors)) OR (brain tumor* OR brain tumour* OR brain neoplasms OR central nervous system neoplasm OR central nervous system neoplasms OR central nervous system tumor* OR central nervous system tumour* OR brain cancer* OR brain neoplasm* OR intracranial neoplasm*) OR (leukemia, lymphocytic, acute) OR (leukemia, lymphocytic, acute*)
2. female[tiab] OR females OR girl OR girls OR girlfriend OR girlhood
3. Follicle Stimulating Hormone, Human* OR Follicle Stimulating Hormone* OR Gonadotrophs OR Estradiol OR Estrogen OR Anti-Mullerian Hormone* OR Ovarian Follicle* OR Antral Follicle Count* OR AFC
4. premature menopause OR early menopause OR menopausal status OR ovarian failure OR premature ovarian failure OR acute ovarian failure OR imminent ovarian failure OR ovarian insufficiency OR ovarian function OR ovarian damage OR Gonadotropin-Resistant Ovary Syndrome OR Female Genital Diseases OR Female infertility OR primary ovarian insufficiency OR gonadotoxicity OR gonado toxicity OR gonadal damage OR hypergonadotropic amenorrhoea OR gonad dysfunction OR gonadal function OR gonadal effects OR ovarian reserve OR gonadal hormone deficiency

POI treatment before puberty (humans, search from March 1993 to March 2013):

1. (leukemia OR leukemi* OR leukaemi* OR (childhood ALL) OR AML OR lymphoma OR lymphom* OR hodgkin OR hodgkin* OR T-cell OR B-cell OR non-hodgkin OR sarcoma OR sarcom* OR sarcoma, Ewing's OR Ewing* OR osteosarcoma OR osteosarcom* OR wilms tumor OR wilms* OR nephroblastom* OR neuroblastoma OR neuroblastom* OR rhabdomyosarcoma OR rhabdomyosarcom* OR teratoma OR teratom* OR hepatoma OR hepatom* OR hepatoblastoma OR hepatoblastom* OR PNET OR medulloblastoma OR medulloblastom* OR PNET* OR neuroectodermal tumors, primitive OR retinoblastoma OR retinoblastom* OR meningioma OR meningiom* OR glioma OR gliom*) OR (pediatric oncology OR paediatric oncology) OR (childhood cancer OR childhood tumor OR childhood tumors)) OR (brain tumor* OR brain tumour* OR brain neoplasms OR central nervous system neoplasm OR central nervous system neoplasms OR central nervous system tumor* OR central nervous system tumour* OR brain cancer* OR brain neoplasm* OR intracranial neoplasm*) OR (leukemia, lymphocytic, acute) OR (leukemia, lymphocytic, acute*)
2. female[tiab] OR females OR girl OR girls OR girlfriend OR girlhood
3. Puberty, delayed* OR puberty induction* OR puberty OR estrogen OR estradiol OR Hormone Replacement Therapy* OR Estrogen Replacement Therapy*
4. Final height* OR body height* OR sexual development* OR disorders of sex development*

POI treatment comparison: oral versus transdermal (humans, search from March 1993 to March 2013):

1. (premature menopause OR early menopause OR menopausal status OR ovarian failure OR premature ovarian failure OR acute ovarian failure OR imminent ovarian failure OR ovarian insufficiency OR ovarian function OR ovarian damage OR Gonadotropin-Resistant Ovary Syndrome OR Female Genital Diseases OR Female infertility OR primary ovarian insufficiency OR gonadotoxicity OR gonado toxicity OR gonadal damage OR hypergonadotropic amenorrhoea OR gonad dysfunction OR gonadal function OR gonadal effects OR ovarian reserve OR gonadal hormone deficiency)
2. estrogen OR estradiol OR Hormone Replacement Therapy* OR Estrogen Replacement Therapy*
3. Bone density* OR bone and Bones* OR blood coagulation* OR coagulation protein disorders* OR blood coagulation disorders* OR thrombosis OR venous thrombosis* OR cardiovascular diseases* OR mental health* OR personal satisfaction* OR quality of life

Risk of secondary malignancies after POI treatment (humans, search from March 1993 to March 2013):

1. (leukemia OR leukemi* OR leukaemi* OR (childhood ALL) OR AML OR lymphoma OR lymphom* OR hodgkin OR hodgkin* OR T-cell OR B-cell OR non-hodgkin OR sarcoma OR sarcom* OR sarcoma, Ewing's OR Ewing* OR osteosarcoma OR osteosarcom* OR wilms tumor OR wilms* OR nephroblastom* OR neuroblastoma OR neuroblastom* OR rhabdomyosarcoma OR rhabdomyosarcom* OR teratoma OR teratom* OR hepatoma OR hepatom* OR hepatoblastoma OR hepatoblastom* OR PNET OR medulloblastoma OR medulloblastom* OR PNET* OR neuroectodermal tumors, primitive OR retinoblastoma OR retinoblastom* OR meningioma OR meningiom* OR glioma OR gliom*) OR (pediatric oncology OR paediatric oncology) OR (childhood cancer OR childhood tumor OR childhood tumors)) OR (brain tumor* OR brain tumour* OR brain neoplasms OR central nervous system neoplasm OR central nervous system neoplasms OR central nervous system tumor* OR central nervous system tumour* OR brain cancer* OR brain neoplasm* OR intracranial neoplasm*) OR (leukemia, lymphocytic, acute) OR (leukemia, lymphocytic, acute
2. female[tiab] OR females OR girl OR girls OR girlfriend OR girlhood OR woman OR women
3. estrogen OR estradiol OR Hormone Replacement Therapy* OR Estrogen Replacement
4. Neoplasms, Second Primary*, Secondary malignancy* OR secondary tumor* OR secondary

Appendix II | Evidence summaries for discordant areas among the existing POI surveillance recommendations.

Who needs surveillance?

Overbeek et al. Chemotherapy-related late adverse effects on ovarian function in female survivors of childhood cancer and cancer in the reproductive age (Cochrane review)				
Study design **Treatment era** **Years of follow-up**	**Participants**	**Treatment**	**Main outcomes**	**Additional remarks**
Systematic review Diagnosis 1945-2008 Follow-up: Range 7-43 years (not stated if this is follow-up from diagnosis or treatment)	N=24 studies, (6–405 patients per study) **Childhood cancer survivors:** N=9 studies, n=395 participants; median age at diagnosis 4.1–12.9 yrs, age at follow-up 12.6–32.3 yrs. **Hodgkin lymphoma:** N=10 studies; n=1,301 Hodgkin lymphoma participants; median age at diagnosis 8–37 yrs, median age at follow-up 21–44 yrs. **Acute myeloid leukaemia:** N=2 studies; n=23 participants; median age at diagnosis: 10.3–30.9 yrs, median age at FU 15.4 yrs (other study not reported).	All patients in 24 included studies exposed to chemotherapy, 13 studies reported on risk factors (using multivariate analyses) **Alkylating agents:** In 22/24 studies chemotherapy regimens contained alkylating agents (12 multivariate). **Antimetabolites (methotrexate):** In 4/24 studies chemotherapy regimens contained methotrexate (1 multivariate). **Platinum compounds:** In 0/24 studies chemotherapy regimens contained platinum compounds. **Radiotherapy:** In 6 of 24 studies patients were treated with radiation to the field that includes the ovaries (6 multivariate). In 3 of 24 studies patients were treated with radiation to the field that includes the hypothalamic-pituitary axis (1 multivariate).	**Prevalence of amenorrhea CCS (15 studies):** **0–19.1%** **Prevalence of high FSH (12 studies on FSH levels):** 21.1–74.4% No pooling of data because of unexplained heterogeneity. Risk factors for ovarian dysfunction/ premature menopause with multivariate analyses (13 studies): alkylating agents (Busulfan versus no Busulfan: OR 3.7) (dose dependent), older age at diagnosis (no specific age reported), Hodgkin's disease (no effect measures reported). In one multivariate study, methotrexate was associated with increased prevalence of iatrogenic ovarian failure.	Risk of bias in the included studies: – Selection bias: 66% – Attrition bias: 39% – Detection bias: unclear (no study reported blinding of investigator) – Confounding bias: 46% – Reporting bias: 42% *Note:* both univariate and multivariable analyses have been performed. We only reported risk factors in multivariate analyses, and only included variables: amenorrhea, FSH, oestradiol, and only included studies on adult CCS.
Conclusions				
Evidence for clinical question 1 (risk of POI for **alkylating agents**):				
In a systematic review 2 of 5 studies reporting on risk factors in female CCS, alkylating agents were significantly associated with reduced ovarian function as compared to survivors treated without alkylating agents in multivariate analyses. This association was dose-dependent (1/1 study).				
Evidence for clinical question 2 (risk of POI for **antimetabolites**):				
In a systematic review 1 of the studies (Lantinga et al, 2006) reporting on risk factors in female survivors of childhood cancer, antimetabolites (MTX) were significantly associated with reduced risk of POI as compared to survivors treated without antimetabolites in multivariate analyses.				
Evidence for clinical question 3 (risk of POI for **platinum compounds**):				
Effect of platinum compounds not evaluated in this study.				
Evidence for clinical question 4 (influence of **age at treatment** on risk of POI):				
In a systematic review 1 of 3 studies reporting on risk factors in female CCS, older age at diagnosis (no specific age reported) was significantly associated with reduced ovarian function as compared to survivors who were younger at diagnosis in multivariate analyses. In 2 of 3 studies reporting on risk factors in female CCS, no association between age at diagnosis and reduced ovarian reserve was observed in multivariate analyses.				
Evidence for clinical question 5 (risk of POI for **radiation to the field that includes the ovaries**):				
In a systematic review, 2 of 2 studies reporting on risk factors in female CCS also treated with chemotherapy, radiation to the field that includes the ovaries was significantly associated with reduced ovarian function as compared to survivors treated without radiation to the field that includes the ovaries in multivariate analyses. (OR 19.7 (95% CI 5.2-72.8), OR 10.95 (95% CI 0.98-212.23, p=0.05, OR 13.47, CI 1.987-90.485), other effect measures not reported).				
Evidence for clinical question 6 (risk of hypogonadotropic-hypogonadism for **radiation to the field that includes the HP axis**):				
In a systematic review, 1 of the studies (Martin et al, 2009) reporting on risk factors in female CCS also treated with chemotherapy, radiation to the subdiaphragmatic region and brain irradiation (as combined factor) were significantly associated with ovarian insufficiency as compared to survivors treated without radiation to the subdiaphragmatic region and brain in multivariate analyses.				

* **List of abbreviations.** N=number; yrs=years; CCS=childhood cancer survivors; FSH=follicle-stimulating hormone; POI=primary ovarian insufficiency; HP axis=hypothalamic-pituitary axis.

***Chemaitilly** et al.* Acute ovarian failure in the childhood cancer survivor study. J Clin Endocrinol Metab. 2006;91(5):1723–8.				
Study design **Treatment era** **Years of follow-up**	**Participants**	**Treatment**	**Main outcomes**	**Additional remarks**
Retrospective, multicentre, survey: self-report 1970–1986 Follow-up: 0–5 years after diagnosis (median unknown)	3,390 CCS with and without AOF <21 yrs at diagnosis Exclusion criteria: cranial radiotherapy >30 Gy, tumour in area of hypothalamus or pituitary, bilateral oophorectomy, incomplete radiation records	**Alkylating agents:** 1,684/3,390 (49.7%) **Antimetabolites:** Included, but percentage not reported. **Platinum compounds:** Included, but percentage not reported. **Abdominal/pelvic radiotherapy:** 832/3,390 (24.5%) **Alkylating agents (AA)+ abdominal/pelvic radiotherapy (RT):** 393/3,390 (11.6%) Cumulative doses of RT to ovaries were calculated and grouped as follows: <100, 100–999, 1000–1999, and 2000cGy+	**Outcome definition:** Self-reported: primary amenorrhoea or secondary amenorrhoea <5 years after cancer diagnosis. **Prevalence of AOF:** 215/ 3,390 (6.3%) **Univariate analysis age at diagnosis:** ≥12 yrs: OR 1.8 (1.4–2.4) **Multivariate analyses:** Age at diagnosis 0–12 yr: – procarbazine: OR 3.2 (1.3–7.3) – cyclophosphamide: OR 1.2 (0.7–2.1) – ovarian irradiation (cGy) 1–99: OR 3.7 (1.6–10.2) 100–999: OR 9.0 (3.4–26.5) 1000–1999: OR 55.3 (22.3–157.8) ≥2000: OR 950.1 (352.9–3043.2) Age at diagnosis 13–20 yr: – proacarbazine: OR 2.6 (1.4–4.7) – cyclophosphamide: OR 4.9 (2.8–9.2) – ovarian irradiation (cGy) 1–99: OR 2.9 (1.2–8.3) 100–999: OR 17.2 (6.8–49.5) 1000–1999: OR 90.9 (29.1–323.5) ≥2000: OR 171.2 (55.8–609.8)	Limitation: AOF defined as no spontaneous menses within 5 yr after cancer diagnosis and never spontaneous menses (all self–reported). Median follow-up >2.5 yr (only stated: follow-up 0–5 years)

Conclusions
Evidence for clinical question 1 (risk of POI for alkylating agents):
In female CCS, cyclophosphamide was significantly associated with AOF in children diagnosed with cancer between 13–20 years of age as compared to survivors treated without cyclophosphamide (OR 4.9 95%CI 2.8–9.2) in multivariate analyses. In female CCS, cyclophosphamide was non–significantly associated with AOF in children diagnosed with cancer between 0–12 years of age as compared to survivors treated without cyclophosphamide (OR 1.2 95% CI 0.7–2.1) in multivariate analyses. In female CCS procarbazine was significantly associated with AOF in children diagnosed with cancer between 0–12 years of age as compared to survivors treated without procarbazine (OR 3.2 (1.3–7.3) in multivariate analyses. In female CCS procarbazine was significantly associated with AOF in children diagnosed with cancer between 13–20 years of age as compared to survivors treated without procarbazine (OR 2.6 (1.4–4.7) in multivariate analyses.
Evidence for clinical question 2–3 (risk of POI for antimetabolites or platinum compounds):
Effects not evaluated in this study.
Evidence for clinical question 4 (influence of age at treatment on risk of POI):
In female CCS, age at diagnosis between 13–20 yr was significantly associated with AOF as compared to children aged 0–12 at diagnosis (OR 1.8 (1.4–2.4) in univariate analyses.
Evidence for clinical question 5 (risk of POI for radiation to the field that includes the ovaries):
In female CCS, radiation to the ovaries (1–2000 cGy) was significantly associated with AOF as compared to survivors treated without radiation to the ovaries in multivariate analyses. In female CCS, higher doses of radiation to the ovaries were significantly associated with a higher risk of AOF (0–12 yr: 1–99cGy: OR 3.7 (1.6–10.2); 100–999: OR 9.0 (3.4–26.5); 1000–1999: OR 55.3 (22.3–157.8); ≥2000: OR 950.1 (352.9–3043.2); 13–20 yr: 1–99 cGy: OR 2.9 (1.2–8.3); 100–999: OR 17.2 (6.8–49.5); 1000–1999: OR 90.9 (29.1–323.5); ≥2000: OR 171.2 (55.8–609.8)) in multivariate analysis.
Evidence for clinical question 6 (risk of hypogonadotropic–hypogonadism for radiation to the field that includes the hypothalamic–pituitary axis):
Effect of radiation to fields that includes the hypothalamic–pituitary axis not evaluated in this study.

* **List of abbreviations.** Yrs=years; CCS=childhood cancer survivors; AOF=acute ovarian failure; OR=odds ratio.

Jadoul et al 2011. Clinical and biologic evaluation of ovarian function in women treated by bone marrow transplantation for various indications during childhood or adolescence. Fertil Steril 2011;96(1):126–133.

Study design Treatment era Years of follow-up	Participants	Treatment	Main outcomes	Additional remarks
Cross-sectional, single-centre study Treatment era not reported Years of FU from BMT: Mean (range) 15.5y (3.3–33.7) FU from diagnosis not reported	35 (of 59 eligible) females >16 yrs who underwent BMT at age , <19 yrs, in complete remission for ≥3 yrs 23 (66%) diagnosed with a malignancy, 12 (34%) diagnosed with a benign disease. 66% pre-menarcheal at BMT Mean age at BMT (range): 9.8 +/–5.2y (1.2–19.0) Mean age at study: 25.3+/–7.2y (16.6–46.4) Mean years of follow up from BMT: 15.5+/–5.5y (3.3–33.7)	**Alkylating agents:** 35 (100%): busulfan + cyclophosphamide, busulfan + melfalan, cyclophosphamide only, melfalan only **Antimetabolites:** 0 (0.0%) **Platinum compounds:** 0 (0.0%) **Radiotherapy involving ovaries:** 18 (51.4%) TBI (4–12Gy) **BMT:** 19 (54%) allogeneic; 16 (46%) autologous All patients with malignancy had appropriate previous CT for their disease.	**Outcome definition:** Absence of pubertal development or progression and secondary amenorrhea, confirmed by menopausal FSH levels. **Whole Cohort:** 16/35 (45.7%) persistent ovarian function, but 85% low AMH levels (<1.2 ug/L). **Persistent ovarian function:** BMT for malignancy 8/23 (35%) v BMT benign disease 8/12 (67%) (p=0.07). After 10y post-BMT 5/21 (24%) v 7/12 respectively, significant (p=0.047). **Clinically proven ovarian failure and hormone measurement:** Prevalence POI post-BMT: 21 (60.0%) (immediate 19, subsequently 2) 35 (100%) low oestradiol and high FSH 35 (100%) low AMH 0.16–1.03 microg/L (median 0.5). AMH 0.25–2.83 microg/L (median 0.90) No significant difference in AMH levels between patients treated for a malignant disease and those transplanted for a benign pathology. **Persistence of ovarian function by treatment (not analysed for malignant disease separately):** TBI + alkylating agents:4/18 (22%) Alkylating agents only: 12/17 (71%) (p<0.005); this remained significant at 10 yrs post-BMT (p=0.01) Multivariate regression analysis: independent negative effect of TBI on ovarian failure (p=0.014) AMH levels and pregnancy N/S difference (other variables within the model: not reported). **Age and menarcheal status:** Multivariate regression analysis: independent protective effect of young age at BMT (p=0.004). 100% girls >10y at BMT with TBI had irreversible premature ovarian failure vs. 40% girls <10y at BMT spontaneous puberty (other variables in the model not reported). **Age at evaluation and time since BMT:** Not significant (p-value not reported).	Authors' Conclusion: – After BMT ovarian function is impaired in the majority of women even without clinical signs of ovarian failure (as judged by AMH) – This impairment is mainly related to older age at BMT (>10y) and TBI *Comments* – Multivariate analyses: only p–values shown. – Fractionation of TBI not stated. Single fraction TBI generally has greater adverse effect on ovarian function. – Previous cranial or other radiation not stated. – Small numbers but all had hormonal assessment – No separate analyses for group with BMT for malignant disease We only reported risk factors in multivariate analyses
Conclusions				
Evidence for clinical question 1 (risk of POI for **alkylating agents**):				
Effect of alkylating agents not evaluated in multivariate analyses in this study.				

Evidence for clinical question 2–3 (risk of POI for **antimetabolites or platinum compounds**):
Effect of antimetabolites not evaluated in this study.
Evidence for clinical question 4 (influence of **age at treatment** on risk of POI):
In female CCS treated with a bone marrow transplantation (conditioning: TBI and alkylating agents), older age at transplantation is significantly associated with ovarian failure/ POI in multivariate analysis (p=0.004) .
Evidence for clinical question 5 (risk of POI for **radiation to the field that includes the ovaries**):
In female CCS treated with a bone marrow transplantation (conditioning: TBI and/or alkylating agents), TBI was significantly associated with ovarian failure in multivariate analyses (p=0.014).
Evidence for clinical question 6 (risk of hypogonadotropic–hypogonadism for **radiation to the field that includes the hypothalamic–pituitary axis**):
Effect of TBI on hypogonadotropic–hypogonadism not evaluated in this study.

* **List of abbreviations.** Yrs=years; FU=follow up; TBI=total body irradiation; BMT=bone marrow transplantation; FSH=follicle-stimulating hormone; AMH=anti-Müllerian hormone; CT=chemotherapy; v=versus; POI=primary ovarian insufficiency; N/S=not significant.

Sklar et al 2006. Premature Menopause in Survivors of Childhood Cancer: A Report from the Childhood Cancer Survivor Study. J Natl Cancer Inst 2006;98(13):890–6.

Study design Treatment era Years of follow-up	Participants	Treatment	Main outcomes	Additional remarks
Retrospective, multicentre survey: self–report 1970 – 1989 Years of FU: not stated. Follow up study: 2000–01 →FU from diagnosis: 14–30 yrs	2,819 female CCS from total cohort of 6,079 females alive, >18y of age at Nov 2000. Median age at diagnosis 7 yrs (range 0–20 yrs), median age at study 29 yrs (range 18–50 yrs). Diagnoses N (%): Leukaemia 1,025(36); HL 404 (14); tumours of bone 324(11); kidney 297(11); brain 137(5); sarcomas 271(10); NBl 154(5). Exclusion criteria (n=1,801): diagnosis associated with ovarian dysfunction, primary amenorrhoea, menses ceased <5y from diagnosis, AOF (6%), questionnaire completed by other than participant. Controls: N=1,065 siblings Sibling subset of CCSS cohort with spontaneous menstruation Median age: not reported.	Surgery only: 287 (10%) CT only: 287 (10%) RT only: 2 (<1%) CT+RT: 487 (17%) Surgery+CT: 573 (20%) Surgery+RT: 238 (8%) Surgery+CT+RT: 942 (33%) SCT: 32 (1%) No information reported on specific chemotherapy agents. Radiation dosimetry for each ovary separately was calculated from institution records (quantified by a single dosimetrist) RT doses grouped as No RT, 1–99,100–999, or 1000 or >cGy Chemotherapy: 7 broad classes of CT drugs from treatment records. Total exposure to (AA) was calculated for an individual by the overall AA score of 0,1,2 or 3 according to the dose distribution tertiles (none, lower, middle or upper)	**Outcome definition:** Self-reported: if subjects had not experienced a spontaneous menses for >6 months and other causes, e.g. pregnancy, use of agents such as injectable progesterone and GnRH – a have been excluded. **Premature menopause (<40yrs):** – 15% (RR 1.05, 95% CI: 1.04–1.07, p<0.001) compared to siblings. – Surgical PM ns different in CCS and siblings (RR 0.8, 95% CI: 0.52–1.23). – Non–surgical PM: 8% in CCS, 0.8% in siblings (RR 13.21, 95% CI: 3.26–53.51, p<0.001) **Risk–factors non–surgical PM in multivariate analyses:** Attained age RR 1.15, 95% CI: 1.09– 1.21, p< 0.001 RT dose to ovary: – RT 1–99 cGy: RR 4.30, 95% CI: 1.20– 15.47, p=0.04) – RT 100–999 cGy: RR 5.70, 95% CI: 1.12–28.99, p=0.04 – RT ≥1000 cGy: RR 109.59, 95% CI: 28.15–426.70, p<0.001 AA score: – AA score 1–2: RR 2.3, 95% CI:1.08–4.90, p=0.03, – AA score 3 RR 5.78, 95% CI: 2.9–11.55, p<0.001 HL (minimum ovarian RT): – No ovarian RT: RR 9.18, 95% CI: 1.52–55.24, p=0.02 – 1–99 cGy: RR 12.26, 95% CI: 3.41–44.14, p<0.001 – 100–999 cGy: RR 11.41, 95% CI: 2.75–47.26, p<0.001 – ≥1000 cGy: RR 6.74, 95% CI: 0.63–71.74, p=0.11 (Age at diagnosis not associated, data not shown). Interactions: – Cum. risk of non–surgical PM in CCS treated with AA + abdominopelvic RT: 30%. – HL and RT dose to the ovary: Non–surgical PM: 9.18 fold higher (95%C: 1.52 –55.24, p=0.02) compared to no RT in HL. – Compared with other cancer – Diagnoses: Ovarian RT in other diagnoses than HL: ≥1000cGy RR 109.59, 95% CI: 28.15–426.70, p=<0.001	**Authors Conclusion** Risk for non–surgical PM 8% in CCS compared to 0.8% in siblings. Risk factors for non-surgical PM: attained age, HL, exposure to increasing doses of AA and/ or RT to ovaries (any dose). The highest risk of non-surgical PM was associated with treatment including abdominopelvic RT and AA **Comments** Excellent large study with some limitations: – Self-reported – Large number of participants were excluded (1801 from 4620) – Among non-menopausal women 20% of survivors and 24% siblings were taking OC (however after exclusion of these subjects results were almost identical to entire cohort)
Conclusions				
Evidence for clinical question 1 (risk of POI for alkylating agents):				

In female CCS, alkylating agents were significantly associated with premature menopause as compared to survivors treated without alkylating agents in multivariate analyses. In female CCS, higher doses of alkylating agents were significantly associated with a higher risk of premature menopause as compared to survivors treated without alkylating agents in multivariate analyses (AA score 1–2: RR 2.30, 95% CI 1.08–4.90; AA score 3: RR 5.78, 95% CI 2.90–11.55).
Evidence for clinical question 2–3 (risk of POI for antimetabolites or platinum compounds):
Effect of antimetabolites not evaluated in this study,
Evidence for clinical question 4 (influence of age at treatment on risk of POI):
In female CCS, age at diagnosis was not associated with premature menopause in multivariate analyses (effect measure not reported).
Evidence for clinical question 5 (risk of POI for radiation to the field that includes the ovaries):
In female CCS, radiotherapy to the ovaries was significantly associated with premature menopause compared to survivors treated without radiation to the ovaries in multivariate analysis. In female CCS, higher doses of radiation to the ovaries were significantly associated with a higher risk of premature menopause (1–99 cGy: RR 4.30, 95% CI 1.20–15.47; 100–999 cGy: RR 5.70, 95% CI 1.12–28.99; ≥1000 cGy: RR 109.59, 95% CI 28.15–426.70). In female survivors of Hodgkin lymphoma, radiotherapy to the ovaries was significantly associated with a higher risk of premature menopause compared to Hodgkin lymphoma survivors treated without radiation to the ovaries in multivariate analysis, and increased with dosage (no ovarian RT but diagnosed with HL compared to other diagnoses: RR 9.18, 95% CI 1.52–55.24; 1–99 cGy: RR 12.26, 95% CI 3.41–44.14; 100–999 cGy: RR 11.41, 95% CI 2.75–47.26; ≥1000 cGy: RR 6.74, 95% CI 0.63–71.74).
Evidence for clinical question 6 (risk of hypogonadotropic–hypogonadism for radiation to the field that includes the hypothalamic–pituitary axis):
Effect of radiation to fields that includes the hypothalamic–pituitary axis not evaluated in this study.

* **List of abbreviations.** FU=follow-up; yrs=years; CCS=childhood cancer survivors; n=number; HL-hodgkin lymphoma; Nbl=neuroblastoma; AOF=acute ovarian failure; CT=chemotherapy; RT=radiotherapy; SCT=stem cell transplantation; PM=premature menopause; AA=Alkylating agents; GnRH-a=gonadotrophin-releasing hormone analogue.

Chiarelli et al. Early menopause and Infertility in Females after Treatment for Childhood Cancer diagnosed in 1964–1988 in Ontario, Canada. Am J Epidemiol 1999;150(3):245–54.				
Study design **Treatment era** **Years of follow-up**	**Participants**	**Treatment**	**Main outcomes**	**Additional remarks**
Retrospective cohort study 1964–1988 Follow-up >5 yrs after diagnosis: – 5–10yrs: 25.2% – 11–15yrs: 24.9% – 16–20yrs: 26.3% – 21–30yrs: 23.6%	719 from total cohort of 1,581 female childhood cancers survivors. Median age: 28 yr (range 18–49). Excluded: sterilising surgery	Alkylating agents: 150 (21%). Antimetabolites: not reported. Platinum compounds: not reported. Radiotherapy involving ovaries: 154 (21%) Alkylating agents + radiotherapy involving ovaries: 71 (10%). RT: <20 Gy, 20–35 Gy, >35 Gy abdominal pelvic. CT: AA (number of Alkylating agents, number of months). AA<: 1–13 low, 14–21 medium, >22 high risk.	Outcome definition: Menopausal status based on Telephone questionnaire: "Have you stopped having periods?" "Have you ever used hormonal supplement pills?" 63 women (8.8%) menopausal after treatment (29 (46%) surgical menopause). **Risk of menopause in multivariate analyses:** Alkylating agents and abdominal-pelvic RT vs. non-sterilizing surgery: RR 2.58 (95% CI 1.14–5.80) Alkylating agents vs. non–sterilizing surgery: RR 0.77 (95% CI 0.30–1.97) Other treatments vs. non–sterilizing surgery: RR 0.75 (95% CI 0.34–1.65) Abdominal–pelvic RT vs. non–sterilizing surgery: – <2000 cGy: RR 1.02 (95% CI 0.29–3.59) – 2000–3499 cGy: RR 1.36 (95% CI: 0.57–3.25) – ≥3500 cGy: RR 3.27 (95% CI: 1.57–6.81) Alkylating agent score vs. non–sterilizing surgery: – 1–13: RR 1.13 (95% C: 0.41–3.09) – 14–21: RR 1.90 (95% CI: 0.52–6.92) – ≥21: RR 3.08 (95% CI: 1.15–8.21)	Subset not representative for cohort. Based on telephone questionnaire No controls Were patients treated with BMT or TBI included?
Conclusions				
Evidence for clinical question 1 (risk of POI for **alkylating agents**):				
In female CCS, high doses of alkylating agents were significantly associated with menopause compared to survivors treated with non-sterilizing surgery in multivariate analyses (AA-score ≥21: RR 3.08, 95% CI: 1.15–8.21). In female CCS, low to median doses of alkylating agents were non-significantly associated with menopause compared to survivors treated with non-sterilizing surgery only in multivariate analyses (AA-score 1–13: RR 1.13, 95% CI: 0.41–3.09).				
Evidence for clinical question 2–4 (risk of POI for **antimetabolites, platinum compounds or age at treatment**):				
Effect of antimetabolites not evaluated in this study.				
Evidence for clinical question 5 (risk of POI for **radiation to the field that includes the ovaries**):				
In female CCS, high doses abdominal-pelvic radiotherapy (≥3500 cGy) were significantly associated with menopause compared to survivors treated with non-sterilizing surgery in multivariate analyses (RR 3.27, 95% CI: 1.57–6.81). In female CCS, <2000 cGy to 3499 cGy abdominal-pelvic radiotherapy were non-significantly associated with menopause compared to survivors treated with non-sterilizing surgery, in multivariate analyses (<2000 cGy: RR 1.02, 95% CI: 0.29– 3.59; 2000–3499 cGy: RR 1.36, 95% CI: 0.57–3.25). In female CCS, alkylating agents combined with abdominal-pelvic radiotherapy was significantly associated with menopause compared to survivors treated with non-sterilizing surgery in multivariate analyses (RR 2.58, 95% CI: 1.14–5.80).				
Evidence for clinical question 6 (risk of hypogonadotropic-hypogonadism for **radiation to the field that includes the hypothalamic-pituitary axis**):				
Effect of radiation to fields that includes the hypothalamic-pituitary axis not evaluated in this study.				

* **List of abbreviations.** Yrs=years; CT=chemotherapy; AA=alkylating agents; RT=radiotherapy; BMT=bone marrow transplantation; TBI=total body irradiation.

Wallace et al. The radiosensitivity of the human oocyte. Hum Reprod 2003;18(1):117–121.				
Study design **Treatment era** **Years of follow-up**	**Participants**	**Treatment**	**Main outcomes**	**Additional remarks**
Retrospective study Cohort 1: Treatment era not reported, Scotland Cohort 2: treatment 1966–1975, UK Years of follow-up not reported.	Two cohorts (n=27): Cohort 1 (n=8) Median age: 17.1 yr (15.4–21.5), leukaemia, Scotland Cohort 2 (n=19): intra-abdominal tumour	Cohort 1: CT+TBI, 14.4 Gy in 8 fractions over 2 days, leukaemia (1st or second remission), median 11.5 yrs (4.9–15.1), no shields to the ovaries. Cohort 2: whole abdominal RT (30 Gy, 16–26 fractions), surgery and CT. median age at treatment 4 yrs (1.3–13.1), 8 pts no CT, remaining 11 vincristine/adriamycin/actinomycin D.	Cohort 1: POI 6/8 Cohort 2: POI 18/19 Based on Faddy–Gosden mathematical model : LD50 (Dose of radiation required to destroy 50% of the oocytes)=1.99 Gy.	Not based on exact radiation dose received by each ovary, homogeneous, small sample.
Conclusions				
Evidence for clinical question 1–4 (risk of POI for **alkylating agents, antimetabolites, platinum compounds, age at treatment**):				
Effect of alkylating agents not evaluated in this study.				
Evidence for clinical question 5 (risk of POI for **radiation to the field that includes the ovaries**):				
In female survivors of childhood cancer, a dose of 1.99 Gy to the abdomen is required to destroy 50% of the oocytes based on the Faddy–Gosden mathematical model.				
Evidence for clinical question 6 (risk of hypogonadotropic–hypogonadism for **radiation to the field that includes the hypothalamic–pituitary axis**):				
Effect of radiation to fields that includes the hypothalamic–pituitary axis not evaluated in this study.				

* **List of abbreviations.** N=number; yr(s)=year(s); CT=chemotherapy; TBI=total body irradiation; RT=radiotherapy; POI=primary ovarian insufficiency.

***Wallace** et al.* Predicting age of ovarian failure after radiation to the field that includes the ovaries. Int J Radiation Oncology Biol Phys 2005;62(3):738–744.				
Study design **Treatment era** **Years of follow-up**	**Participants**	**Treatment**	**Main outcomes**	**Additional remarks**
Retrospective study Cohorts previously described in Wallace *et al*, 2003 Cohort 1: Treatment era not reported, Scotland Cohort 2: treatment 1966–1975, UK Years of follow-up not reported.	Two cohorts (n=27): Cohort 1 (n=8) Median age at treatment 11.5 yrs (4.9–15.1), median age at assessment: 17.1 (15.4–21.5), leukaemia, Scotland Cohort 2 (n=19): intra–abdominal tumour, median age at treatment 4 yrs (1.3–13.1), median age at follow-up not stated.	Cohort 1: CT +TBI, 14.4 Gy in 8 fractions over 2 days, leukaemia (1st or second remission), no shields to the ovaries. Cohort 2: whole abdominal RT (30 Gy, 16–26 fractions), surgery and CT, 8 pts no CT, remaining 11 vincristine/adriamycin/actinomycin D.	Cohort 1: POI 6/8 (reported previously) Cohort 2: POI 18/19 (reported previously) Based on Faddy-Gosden mathematical model (estimation): Effective sterilizing dose (POI occurs immediately after treatment in 97.5% of patients): At birth: 20.3 Gy; at 10 yrs: 18.4 Gy; at 20 yrs: 16.5 Gy; at 30 yrs: 14.3 Gy Dy/day x=−y[0.0595 + 3.716 / (11.780 + y)] X=age, y(x)=population at age x, y(0)=population at birth Surviving % oocyte population=log(10) g(z)=2–0.15z. Z= dose (Gy) Using average oocyte population at x(chron) –> calculating age at menopause. 95% CI=(age at menopause) ± (1.96xSD) (see table for age at POI below)	Small sample n=27, mathematical model. – estimation – cohort 1: TBI, cohort 2: abdominal irradiation (comparable?)
Conclusions				
Evidence for clinical question 1–3 (risk of POI for **alkylating agents, antimetabolites, platinum compounds**):				
Effect of alkylating agents not evaluated in this study.				
Evidence for clinical question 4 (influence of **age at treatment** on risk of POI):				
In female CCS, increasing age at treatment is associated with a higher risk for POI.				
Evidence for clinical question 5 (risk of POI for **radiation to the field that includes the ovaries**):				
In female CCS, a dose of 20.3 Gy to the ovaries at birth is associated with POI in 97.5% of the patients, based on the Faddy–Gosden mathematical model. In female CCS, a dose of 18.4 Gy to the ovaries at 10 years of age is associated with POI in 97.5% of the patients, based on the Faddy–Gosden mathematical model. In female CCS, a dose of 16.5 Gy to the ovaries at 20 years of age is associated with POI in 97.5% of the patients, based on the Faddy–Gosden mathematical model.				
Evidence for clinical question 6 (risk of hypogonadotropic–hypogonadism for **radiation to the field that includes the hypothalamic–pituitary axis**):				
Effect of radiation to fields that includes the hypothalamic–pituitary axis not evaluated in this study.				

* **List of abbreviations.** N=number; yr(s)=year(s); CT=chemotherapy; TBI=total body irradiation; RT=radiotherapy; POI=primary ovarian insufficiency.

What surveillance modality should be used and at what frequency should surveillance be performed?

- What is the diagnostic value of AMH to detect POI in female childhood cancer survivors?
- What is the diagnostic value of (transvaginal vs abdominal) ovarian ultrasound (antral follicle count) to detect POI in female childhood cancer survivors?
- Does the risk of POI increase after a certain follow-up time in female childhood cancer survivors?
- What is the predictive value of FSH/oestradiol/AMH/AFC to predict POI in female childhood cancer survivors?
- Does the risk of POI change as the length of follow-up time increases in female childhood cancer survivors?
- Is recovery possible after chemotherapy or radiation in female childhood cancer survivors?
- What is the value of FSH/oestradiol/AMH/antral follicle count to predict fertility in female childhood cancer survivors?

No studies identified in childhood cancer survivors or in other populations.

What should be done when abnormalities are identified?

Nabhan et al. Conjugated oral versus transdermal estrogen replacement in girls with Turner syndrome: a pilot comparative study. J Clin Endocrinol Metab 2009.

Study design Treatment era Years of follow-up	Participants	Intervention	Main outcomes	Additional remarks
Randomized trial 2009 Follow-up: 1 year	Pre-pubertal GH-treated (treatment for at least 6 months) girls with Turner Syndrome N=12 Age at inclusion: 14.0 ± 1.7 yrs (range 11.3–17.1) Follow-up: 1 year	**Group A:** TDE2: 0.025 mg patch twice a week for 6 months followed by a 0.0375 mg patch twice a week for the second 6 months **Group B:** Conjugated oral oestrogen: 0.3 mg every day for the first 6 months followed by 0.3 mg alternating with 0.625 mg every day for the second 6 months	Bone health TD E2 resulted in greater increase in group A compared to group B in: – spine bone mineral content (9.0±0.9 vs. 5.8±0.9 g, P=0.04) – spine BMD (0.12±0.01 vs. 0.06±0.01 g/cm^2, P<=0.004) – spine BMD Zscore (0.7±0.1 vs. 0.3±0.1, P=0.03) Puberty induction TD E2 resulted in greater increase in group A compared to group B in: – uterine length (4.13 vs. 1.98 cm, P<0.003) – uterine volume (22.2 vs. 4.0 ml, P<0.02) No effect of karyotype (P=0.18 for length; P=0.54 for volume) or duration of GH therapy (P=0.45 for length; P=0.21 for volume) on uterine growth was observed. – At study end, 66% in group A had a mature uterus vs. 0% in group B. – Breast Tanner stage increased with a trend toward greater breast development in group A (P=0.09) (effect measure not reported). No significant differences in growth velocity (5.9±0.8 in TD vs 4.7±0.8 in conjugated oral group, p=0.44). CVD surrogates No significant differences in other parameters examined were seen (high–density lipo-protein: 41±3 vs 60±3 mg/dl, p=0.49), low–density lipoprotein (82±9 vs 81±9, p=0.53), triglycerides (121±13 vs 69±13, p=0.47), BMI SD score, fat mass, percent fat mass, fat free mass (effect measures not reported).	– Small number of patients – Girls with Turner syndrome comparable to CCS? – All on GH treatment – Bias: not blinded.

Conclusions

Evidence for clinical question 14 (**effect of different methods of puberty induction in children on final height and sexual development**):

In pre-pubertal girls diagnosed with Turner syndrome and treated with growth hormone for at least 6 months, one year treatment with transdermal HRT was not significantly associated with a difference in growth velocity compared to one year oral conjugated oestrogens (5.9±0.8 in transdermal HRT vs 4.7±0.8 in conjugated oral group, p=0.44).
In pre-pubertal girls diagnosed with Turner syndrome and treated with growth hormone for at least 6 months, one year treatment with transdermal HRT was significantly associated with a faster uterine maturation compared to one year oral oestrogens (uterine length 4.13 versus 1.98 cm, p<0.003, and uterine volume 22.2 versus 4.0 ml, p<0.02).

Evidence for clinical question 15 (**effect of different methods of HRT in young women with POI on bone health, cardiovascular health and mental health**):

In pre-pubertal girls diagnosed with Turner syndrome and treated with growth hormone for at least 6 months, one year treatment with transdermal HRT was significantly associated with an increased spine bone mass compared to one year oral oestrogens (BMD 0.12±0.01 vs. 0.06±0.01 g/cm^2, P<=0.004; BMC 9.0± 0.9 vs. 5.8±0.9 g, P=0.04).
In pre-pubertal girls diagnosed with Turner syndrome and treated with growth hormone for at least 6 months, one year treatment with transdermal HRT was not significantly associated with a difference in CVD surrogate markers (high-density lipoprotein, low-density lipoprotein, triglyceride) compared to one year oral oestrogens.

* **List of abbreviations.** N=number; GH=growth hormone; yr(s)=year(s); TD=transdermal; E2=oestradiol; BMD=bone mineral density; BMI=body mass index; SD=standard deviation; HRT=hormone replacement therapy; CVD=cardiovascular disease; CCS=childhood cancer survivors.

Crofton et al. Physiological versus standard sex steroid replacement in young women with premature ovarian failure: effects on bone mass acquisition and turnover. Clin Endocrinol (Oxf) 2010;73(6):707–714.				
Study design **Treatment era** **Years of follow-up**	**Participants**	**Intervention**	**Main outcomes**	**Additional remarks**
Open-label randomized controlled trial 2002–2006 Follow-up: 1 year per treatment (1+1=2 yrs)	N=18 POF: Turner n=7, post-chemotherapy n=4, Idiopathic n=7. Median age at start: 25 years Compared to total group, Turner girls mean age 23 years versus 29 years.	**Treatment plan:** 12 months therapy A + 2 months wash out, and 12 months therapy B (or first B then A). **Treatment A:** Transdermal oestradiol 100 ug week 1 and 150 ug week 2–4 + progesterone (200 mg vaginal pessaries or 10 mg orally) twice daily for week 3–4 **Treatment B:** Ethinyl oestradiol 30 ug + norethisterone 1.5 mg die for weeks 1–3 followed by 1 week pill free.	**Lumbar spine BMD z score (CI):** +0.17 (0.07–0.27) +0.07 (-0.03–0.18) P<0.01 Treatment pSSR (A) vs. sHRT (B) in the same subject: lumber spine BMD z score increases by 0.09 (–0.06 – +0.25) but P=0.2 **Femoral neck BMD z score (CI)** + 0.12 (–0.05 – + 0.29) + 0.11 (–0.04 – + 0.25) Not significant **Total hip BMD z score (CI)** –0.04 (–0.16 – +0.08) + 0.03 (–0.08 – + 0.13) Not significant **Bone markers (BALP, PINP, CrossLaps)** Increase in ALP and PINP and decrease of CrossLaps Decrease of all markers Significant at 3, 6 and 12 months for ALP and PINP Only significant at 3 and 6 for CrossLaps FSH and LH were similarly suppressed (P>0.05)	– Small number of patients, but crossover design overcomes this slightly. – More girls in the study had onset of POF pre puberty rather than post puberty – Heterogeneous diagnoses / patient population – No information on results by type of participant – High dropout rate (48%), more in the first phase of treatment (suggested a lack of tolerance of the research protocol rather than the intervention) – Short follow up period to show changes in DEXA (12 months) – Use of 30ug COCP as sHRT probably higher than 'standard treatment' since a number of 20ug pills available. But still used due to breakthrough bleeding. But questionnaire to choose the most commonly prescribed method of SSR in UK – Patch reaction / intolerance accounted for 3 drop outs
Conclusions				
Evidence for clinical question 14 (**effect of different methods for puberty induction in children on final height and sexual development**):				
Effect on final height and sexual development not evaluated in this study.				
Evidence for clinical question 15 (**effect of different methods of HRT in young women with POI on bone health, cardiovascular health and mental health**):				
In women diagnosed with POI (including 4 cases with POI after childhood cancer treatment), one year treatment with transdermal HRT was significantly associated with an increased lumbar spine bone mineral density compared to one year oral conjugated oestrogens (BMD z score +0.17; CI: 0.07–0.27 vs. +0.07; CI: –0.03 – 0.18 , P<0.01). In women diagnosed with POI (including 4 cases with POI after childhood cancer treatment), one year treatment with transdermal HRT was not significantly associated with a difference in femoral neck bone mineral density compared to oral conjugated oestrogens (BMD z score pSSR: +0.12 (–0.05 – + 0.29), sHRT: +0.11 (–0.04– +0.25, NS). In women diagnosed with POI (including 4 cases with POI after childhood cancer treatment), one year treatment with transdermal HRT was not significantly associated with a difference in total hip bone mineral density compared to oral conjugated oestrogens (BMD z score pSSR: –0.04 (–0.16– + 0.08), sHRT: +0.03 (–0.08–+0.13), NS).				

* **List of abbreviations.** N=number; POF=premature ovarian failure; BMD=bone mineral density; BALP=bone alkaline phosphatase; PINP=type I collagen N-terminal propeptide; CrossLaps=bone resorption ; COCP=combined oral contraceptive pill=sHRT: standard hormone replacement therapy; pSSR=physiological sex hormone replacement; FSH=follicle-stimulating hormone; LH=lunteinizing hormone.

Langrish et al. Cardiovascular effects of physiological and standard sex steroid replacement regimens in premature ovarian failure. Hypertension 2009; 53(5):805–811.				
Study design **Treatment era** **Years of follow-up**	**Participants**	**Intervention**	**Main outcomes**	**Additional remarks**
Open-label randomized controlled trial 2002–2006 Follow-up: 1 year per treatment (1+1=2 yrs)	N=18 POF: Turner n=7, post–chemotherapy n=4, Idiopathic n=7. Median age at start: 25 years Compared to total group, Turner girls mean age 23 years versus 29 years.	**Treatment plan:** 12 months therapy A + 2 months wash out, and 12 months therapy B (or first B then A). **Treatment A:** Transdermal oestradiol 100 ug week 1 and 150 ug week 2–4 + progesterone (200 mg vaginal pessaries or 10 mg orally) twice daily for week 3–4 **Treatment B:** Ethinyl oestradiol 30 ug + norethisterone 1.5 mg die for weeks 1–3 followed by 1 week pill free.	**24–hour systolic and diastolic BP:** Treatment A associated with lower BP systolic and diastolic at 3 ($P<0.05$), 6 ($P<0.05$) and 12 months ($P<0.01$) compared to treatment B. At 12 months systolic 7.3 mmHg (95% CI: 2.5–12.0) and diastolic 7.4 mmHg (95% CI: 3.9–11.0) lower. Treatment A associated with lower plasma angiotensin II ($P=0.007$) and serum creatinine concentrations ($P=0.015$) compared to treatment B (no effect measures reported). No significant difference in aldosterone concentrations between treatment arms. No significant difference in PRA (p=0.180), BMI (p=0.146), serum urea nitrogen (p=0.063), sodium (p=0.402), and potassium concentrations (p=0.895). FSH and LH were similarly suppressed ($P>0.05$).	– Small number of patients, but crossover design overcomes this slightly – More girls in the study had onset of POF pre puberty rather than post puberty – Heterogeneous population – No information on results by type of participant – High dropout rate (48%) – Short follow up period. Using surrogate markers of CVD. – Use of 30ug COCP as sHRT probably higher than 'standard treatment' since a number of 20ug pills available. But still used due to breakthrough bleeding. But questionnaire to choose the most commonly prescribed method of SSR in UK – Patch reaction / intolerance accounted for 3 drop outs – All participants were normotensive
Conclusions				
Evidence for clinical question 14 (**effect of different methods for puberty induction in children on final height and sexual development**):				
Effect on final height and sexual development not evaluated in this study.				
Evidence for clinical question 15 (**effect of different methods of HRT in young women with POI on bone health, cardiovascular health and mental health**):				
In women diagnosed with POI (including 4 cases with POI after childhood cancer treatment), one year treatment with transdermal HRT was significantly associated with a decrease in blood pressure (mean 24 hours systolic blood pressure benefit 7.3 mmHg; CI: 2.5–12 mm Hg and mean 24 hours diastolic blood pressure benefit 7.4 mmHg; CI 3.9–11 mm Hg), angiotensinogen II (effect measures not reported, p=0.007) and serum creatinine concentrations (effect measures not reported, p=0.015) compared to oral conjugated oestrogens.				

* **List of abbreviations.** Yrs=years; n=number; POF=premature ovarian failure; BP=blood pressure; PRA=plasma renin activity; BMI=body mass index; FSH=follicle-stimulating hormone; LH=luteinizing hormone; COCP=combined oral contraceptive pill; sHRT=standard hormone replacement therapy; pSSR=physiological sex hormone replacement.

Torres-Santiago L et al. Metabolic effects of oral versus transdermal 17β–estradiol (e2): a randomized clinical trial in girls with turner syndrome. J Clin Endocrinol Metab. 2013;98(7):2716–24.				
Study design **Treatment era** **Years of follow-up**	**Participants**	**Intervention**	**Main outcomes**	**Additional remarks**
Randomized trial Follow-up: 1 year	Nearly final height previously GH-treated (treatment discontinued for at least 6 months/1.5 yrs) girls with Turner Syndrome N=40/41 (1 early drop out) Age at inclusion: 16.7±1.7 yrs (range 13–20) Follow-up: 1 year	**Group A:** TD 17BE2: starting dose 0.0375 mg patch twice a week **Group B:** oral 17B–E2: starting dose 0.5 mg daily E2 doses were titrated between visits in the first 3 months in order to achieve E2 levels within the normal range **Group A:** TD 17BE2: 0.05, 0.075 or 0.1 mg patch twice a week **Group B:** oral 17B–E2: 1, 1.5 or 2 mg daily Average dose: **Group A:** TD 17BE2: 0.1 mg patch twice a week **Group B:** oral 17B–E2: 2 mg daily	CVD surrogates No significant differences between Group A) and B) in: – weight gain after 6 (+0.6±0.6 vs +0.9±0.6 kg, P=.68) – and 12 months (+1.9±0.6 vs +1.1±0.6 kg, P=.37) – BMI gain after 6 (+0.22±0.28 vs +0.26±0.28 kg/m^2, P=.97) and 12 months (+0.65±0.38 vs +0.075±0.38 kg, P=.2); – free fat mass gain after 6 (+1.0±0.37 vs +1.03±0.37 kg, P=.96) and 12 months (+1.67±0.4 vs +1.03±0.37 kg, P=.54) – percent fat mass decrease after 6 (-1.63±0.47% vs -0.54±0.47%, P=.14) and 12 months (-0.64±0.56% vs -0.14±0.56 kg, P=.5); – abdominal fat mass decrease after 6 (-2.2±0.65% vs -1.1±0.63%, P=.39) and 12 months (-0.9±0.8% vs -0.35±0.8%, P=.39). No significant differences in other biochemical surrogates examined were seen (high-density lipoprotein, low-density lipoprotein, triglycerides insulin, fasting glucose, HsCRP) (effects to be calculated as a mixed model was used) nor in substrate oxidation rates (Lipid oxidation /REE) **Bone health** Increases but not significantly different between Group A) and B) in: – total body BMD Z-score (+0.29±0.09 *vs.*+*0.22*±0.09, *P*=0.23) – spine BMD Z-score (0.20±0.07 *vs.* 0.19±0.07, *P*=0.91) No significant differences in osteocalcin between Group A) and B)	– Small number of patients, but sufficient to satisfy the power calculation based on the main outcome, i.e differential effect on body composition. – Girls with Turner syndrome comparable to CCS? – not clear the method of randomization – Bias: not blinded. – Short follow up period. Using surrogate markers of CVD.

Conclusions
Evidence for clinical question 14 (**effect of different methods of puberty induction in children on final height and sexual development**):
Effect of different methods of puberty induction in children on final height and sexual development not studied.
Evidence for clinical question 15 (**effect of different methods of HRT in young women with POI on bone health, cardiovascular health and mental health**):
In young women diagnosed with Turner syndrome and previously treated with growth hormone, one year treatment with transdermal HRT or oral therapy exerted similar beneficial effects on total body and spine BMD Z–score (+0.29±0.09 vs.+0.22±0.09, P=0.23 and 0.20±0.07 vs. 0.19±0.07, P=0.91, respectively). In young women diagnosed with Turner syndrome and previously treated with growth hormone, one year treatment with transdermal HRT or oral therapy exerted similar beneficial effects on cardiovascular surrogates as free fat mass gain after 6 (+1.0±0.37 vs +1.03±0.37 kg, P=.96) and 12 months (+1.67±0.4 vs +1.03±0.37 kg, P=.54), percent fat mass decrease after 6 (-1.63±0.47% vs -0.54±0.47%, P=.14) and 12 months (-0.64±0.56% vs -0.14±0.56 kg, P=.5) and abdominal fat mass decrease after 6 (–2.2±0.65% vs -1.1±0.63%, P=.39) and 12 months (-0.9±0.8% vs -0.35±0.8%, P=.39).

* **List of abbreviations.** GH=growth hormone; n=number; E2=oestradiol; CVD=cardiovascular disease; BMI=body mass index; HsCRP=highly sensitive c-reactive protein; REE=resting energy expenditure ; BMD=bone mineral density; CCS=childhood cancer survivors.

Does HRT in post pubertal female childhood cancer survivors increase the risk of secondary malignancies compared to not HRT users? If yes, is there a difference between females treated with RT involving the breast compared to those not exposed? (if not, statement based on existing guidelines)

No studies identified in childhood, adolescent and young adult cancer survivors or in other populations.

CHAPTER 8

Treatment and pregnancy outcome of oocyte donation in a Dutch single-centre cohort

W van Dorp, JM Rietveld, JSE Laven, MM van den Heuvel-Eibrink, CWPM Hukkelhoven, I Schipper

Submitted

CHAPTER 9

Obesity independently influences gonadal function in very long-term adult male survivors of childhood cancer

W van Dorp & K Blijdorp, JSE Laven, R Pieters, FH de Jong, SMF Pluijm, AJ van der Lely, MM van den Heuvel-Eibrink, SJCMM Neggers

Submitted

Abstract

Background: Although obesity is associated with gonadal dysfunction in the general population, gonadotoxic treatment might diminish the impact of obesity in childhood cancer survivors (CCS). We aimed to evaluate whether altered body composition is associated with gonadal dysfunction in male CCS, independently of gonadotoxic cancer treatment.

Methods: Data of 351 male CCS who visited the late effects clinic were analysed. Median age at diagnosis was 5.9 years (0−17.8) and median age at follow-up was 25.6 years (18.0−45.8). Main outcome measures were total and free testosterone, sex hormone-binding globulin (SHBG), inhibin B and FSH. Potential determinants were BMI, waist circumference, waist-hip ratio and body composition measures, determined by dual energy X-ray absorptiometry.

Results: Free testosterone was significantly decreased in survivors with obesity (BMI ≥30 kg/m^2) (β −1.8, 95% CI -3.2;-0.3, p=0.015), high fat percentage (β -1.2, 95% CI: -2.0;-0.4, p=0.004), and high waist circumference (>102 cm) (β -2.0, 95% CI: -3.7;-0.3, p=0.020). Survivors with high fat percentage (≥25%) had significantly lower inhibin B and inhibin B/FSH ratios compared to survivors with normal total fat percentage after adjustment for confounders (inhibin B: β -25, p=0.047; inhibin B / FSH ratio: β -34%, p=0.041).

Conclusion: Obesity is associated with an impaired gonadal function in male survivors of childhood cancer, independent of the irreversible effect of the previous cancer treatment. Lifestyle counselling is recommended in CCS to partly improve gonadal function, but longitudinal studies are needed to confirm the positive effect of weight loss.

Introduction

Today, 70–80% of children with cancer reach 5 years survival thanks to the increased survival of childhood cancer [1]. Because of the increasing number of survivors, the late effects of cancer treatment became more apparent. It has been shown that almost 75% of the survivors have one or more late treatment effects, while almost 60% have two or more late effects. This means that a remarkable number of survivors suffer from a combination of late treatment effects [2,3]. Two of the major and well-described late effects are obesity and gonadal dysfunction.

In the general population, obesity prevalence has increased dramatically [4]. It has harmful effects on human health by increasing the risk of diabetes, hypertension, heart disease, stroke, and cancer [5]. In the general male population, obesity is associated with gonadal dysfunction and infertility [6,7]. Although contradictory results have been reported, most studies found a negative association between obesity or underweight and sperm quality or quantity [6,8,9]. Obesity is known to be an important long-term effect of treatment for childhood cancer and occurs in 9–50% of long-term survivors, mainly depending on former treatment [10].

Gonadal dysfunction has been observed in both male and female childhood cancer survivors [11,12]. Especially former treatment with total body irradiation, abdominal irradiation and alkylating agents have previously been reported to be gonadotoxic, as represented by a low inhibin B level [12], which is considered a reliable first screening marker for spermatogenesis [13]. Inhibin B is produced by the Sertoli cells and has an inhibitory effect on the pituitary FSH-secretion [14]. Its production depends on the interaction of Sertoli cells with the germinal epithelium in the seminiferous tubules. Therefore, a low level of inhibin B is a reflection of dysfunction of the tubular compartment of the testis. In men, obesity is associated with decreased sperm quantity, motility and morphology [6,8,15], which might be caused by insulin resistance-related damage to the seminiferous tubules, affecting Sertoli cell function. Reduced testosterone levels might be explained by increased aromatization of androgens in the adipose tissue leading to higher circulating oestradiol levels [7,16,17]. Additionally, low testosterone levels are thought to be the result of decreased sex hormone-binding globulin (SHBG) binding activity caused by increased insulin levels. Additionally, leptin and other adipocyte-derived hormones might directly affect Leydig cell function [7].

Although it is known that obesity is associated with impaired gonadal function in the general male population, this association might be different in male survivors of childhood cancer since it seems likely that the cancer treatment had such a detrimental impact that effects of obesity would be absent. We hypothesized that obesity independently influences gonadal dysfunction in cancer survivors. Therefore, we aimed to determine the association between body composition and gonadal function, independently of former gonadotoxic cancer treatment in a large representative single-centre cohort of very long-term adult male childhood cancer survivors.

Methods

Patients

A retrospective single-centre cohort study was performed among male survivors who visited our outpatient clinic for long-term childhood cancer survivors. Inclusion criteria were: age ≥18 years, male sex, history of childhood cancer, cancer diagnosis between 1964 and 2005, and determination of BMI and reproductive hormones at the same moment at least 5 years after cessation of cancer treatment. Survivors treated with testosterone and/or growth hormone therapy were excluded from this analysis. Informed consent from every patient that visited the outpatient clinic was obtained according to standards of the Institutional Review Board (IRB).

Outcome measures

Peripheral serum samples were stored at -20° C until analysis. Inhibin B was used as a surrogate marker for gonadal function, since the correlation between inhibin B and sperm concentration has been reported to be strong in two publications about subsets of our cohort of childhood cancer survivors (r=0.54, r=0.76, respectively, p<0.001) [12,13]. Inhibin B levels were measured using kits purchased from Serotec (Oxford, United Kingdom). Within-assay and between-assay coefficients of variation (CV) were <9%, and <15%, respectively. Serum follicle-stimulating hormone (FSH), luteinizing hormone (LH) and SHBG were determined with the Immulite assay (Siemens DPC, Los Angeles, CA). Within-assay and between-assay CV were <6% and <9%, <5% and 11%, and 4% and 5%, for FSH, LH and SHBG, respectively. Serum total testosterone levels were determined using coated-tube radioimmunoassays (Siemens DPC). Intra-assay and inter-assay variation coefficients were 3% and 4.5%. The reference values of LH, FSH, inhibin B, SHBG and total testosterone for male adults in our institute are 1.5−8.0 (U/L), 2.0−7.0 U/L, 150−400 ng/L, 10−70 nmol/L and 10−30 nmol/L, respectively [12,18]. Free testosterone was calculated from total testosterone and SHBG [19].

Obesity variables

Follow-up data of the most recent visit included measurement of the following variables: weight, height, body mass index (BMI) calculated from height and weight [20], and waist-hip ratio (WHR), as measured by waist circumference divided by hip circumference [21]. Lean body mass (kg) and percentage of body fat were measured in a subset of survivors by dual energy X-ray absorptiometry (DXA, Lunar Prodigy, GE Healthcare, Madison, WI, USA). Visceral fat percentage was calculated from intra-abdominal fat (kg) and total fat (kg), as measured by DXA [22]. Visceral fat percentage, waist and WHR were not analysed in the subset of survivors treated with abdominal radiotherapy, because of the inferior value as markers for obesity in this subgroup [23]. Obesity was defined as BMI ≥30, total fat percentage ≥25% or waist circumference >102 cm [24,25].

Potential confounders

Data concerning treatment protocols, disease and patient characteristics were retrieved from the local database and were completed using the medical records where necessary. Follow-up time was defined as time since cessation of treatment. Among patients exposed to alkylating agents, the

alkylating agent dose (AAD) score was calculated by determining the drug dose tertile distribution in our entire cohort of survivors and adding the tertile scores (1, 2 or 3) for each of the alkylating agents given to a particular patient as previously described by Green *et al* [26,27]. An AAD score of zero was assigned to patients not exposed to any alkylating agent.

Statistics

To evaluate the differences in hormone levels between BMI categories, analysis of variance (ANOVA) was performed. To examine the associations between obesity variables and reproductive hormone levels, we used univariate as well as multiple linear regression analyses. In multiple linear regression models, age at follow-up, age at diagnosis, current smoking status (yes or no), and treatment factors (total body irradiation, abdominal radiotherapy and AAD score) were included as confounders, based on what is known from previous studies describing the influence of treatment factors on gonadal reserve [7,12,28]. The analyses were performed in several steps. First, BMI and body composition were entered as continuous variables. Additionally, BMI was divided in 4 categories: BMI ≥30 kg/m^2 (obese), BMI 25−30 kg/m^2 (overweight), BMI 18.5−25 kg/m^2 (normal weight) and BMI <18.5 kg/m^2 (underweight) (*The National Institute of Health Website*) and added to the model as dummy variables with normal weight as reference category. Total fat percentage and waist circumference were divided in 2 categories: total fat ≥25% (obese) versus total fat <25% (non-obese) and waist circumference >102 cm (obese) versus waist circumference ≤102 cm (non-obese). Associations were expressed as standardized regression coefficients because this measure allows direct comparison of the strengths of associations between different determinants. The distribution of inhibin B/FSH ratio was normalised by ^{10}log transformation to improve the plots of the residual analyses and expressed as percentage in- or decrease compared to the reference category. P-values <0.05 (two-tailed) were considered statistically significant. SPSS 20.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis.

Results

Survivors

Data on reproductive hormone levels in combination with BMI were available in 351 out of 562 male survivors (Table I) after exclusion of survivors treated with testosterone supplementation (n=10) and GH treatment (n=3). At follow-up, 11 used thyroxin and thyroid function had been stable for at least 2 years in all subjects. Median age at diagnosis was 5.9 years (0−17.8) and median age at follow-up was 25.6 years (18.0−45.8). Median follow-up time since cessation of treatment was 17.5 years (5.0−43.0). Baseline characteristics of included subjects and of all male survivors are depicted in Table I, to illustrate the representativeness of our study group. Compared to excluded survivors, age at diagnosis was significantly lower (p<0.001) and acute lymphoblastic leukaemia, Wilms tumour and neuroblastoma survivors were overrepresented, while brain tumour survivors were underrepresented in this cohort. In- and excluded survivors were comparable with regard to treatment factors, including dosages of abdominal irradiation, total body irradiation and AAD score.

Table I | Baseline characteristics of survivors included in this study.

	Included survivors n=351		All male survivors n=562	
Age at diagnosis (years)	5.9 (0−17.8)		7.0 (0–18.0)	
Age at follow up (years)	25.6 (18.0–45.8)		NA	
Follow up time (years)	17.5 (5.0–43.0)		NA	
Diagnosis n (%)				
ALL & T-NHL	113 (32)		153 (27)	
Acute myeloid leukaemia	12 (3)		19 (3)	
B-cell non-Hodgkin lymphoma	40 (11)		57 (10)	
Hodgkin lymphoma	34 (10)		60 (11)	
Bone tumour	18 (5)		27 (5)	
Wilms tumour	40 (11)		51 (9)	
Neuroblastoma	21 (6)		25 (4)	
Germ cell tumour	6 (2)		10 (2)	
Malignant mesenchymal tumour	28 (9)		48 (9)	
Brain tumour	19 (5)		78 (14)	
Other	20 (5)		34 (6)	
Therapy n (%)	**n (%)**	**TCD (Gy)**	**n (%)**	**TCD (Gy)**
Abdominal radiotherapy	24 (7)	30(15–64)	34 (6)	30 (11–64)
Total body irradiation	13 (4)	12 (6–20)	23 (4)	12 (6–19.5)
Cranial radiotherapy	31 (9)	25 (25–48)	65 (12)	25 (10–40)
Brain tumour irradiation (BRT)	28 (8)	36 (10–108)	56 (10)	40 (18–108)
AAD score				
0	139 (40)		260 (46)	
1	44 (13)		60 (11)	
2	58 (17)		72 (13)	
3	86 (25)		131 (23)	
4	14 (4)		20 (4)	
≥5	10 (3)		19 (3)	

Data are expressed as median (range) or frequencies (%). ALL=acute lymphoblastic leukaemia; T-NHL=T-cell non-Hodgkin lymphoma; AAD score=alkylating agent dose score; TCD=total cumulative dose; NA=not applicable.

Endocrine function

Median inhibin B of the total group was 139 ng/L (interquartile range (IQR) 66−221). Median inhibin B/FSH ratio was 30.8 (IQR 6.6−65.7). The spearman correlation coefficient (ρ) between inhibin B and inhibin B/FSH ratio was 0.93 ($p<0.001$). Median total testosterone was 15.7 nmol/L (IQR 12.9−19.0). Median free testosterone was 10.4 nmol/L (IQR 8.7−12.6) and median SHBG was 26.8 nmol/L (IQR 20.3-33.8). Twenty subjects (6%) were defined as obese by BMI (≥30 kg/m^2), 98

(28%) as overweight (BMI 25–30 kg/m^2), 14 (4%) as underweight (BMI <18.5 kg/m^2) and 219 (62%) had normal weight. In Table II, hormone levels are presented per BMI category. Subjects with BMI <18.5 appeared to have lower inhibin B levels (median 104 ng/L) than normal weight subjects (140 ng/L); however this difference did not reach significance. Obese subjects had significantly lower total and free testosterone levels than normal weight subjects (13.8 versus 16.2, p=0.010 and 9.4 versus 10.5, p=0.040). Overweight subjects had significantly lower SHBG levels than normal weight subjects (23.7 versus 28.3, p=0.001).

Table II | Median levels of inhibin B, inhibin B/FSH ratio, total & free testosterone and SHBG in the total group and different BMI categories.

	Total group n=351	BMI ≥30 n=20	BMI 25–30 n=98	BMI 18.5–25 n=219	BMI <18.5 n=14
inhibin B (ng/L)	139 (66–221)	125 (46–199)	137 (69–230)	140 (67–209)	104 (20–188)
FSH (U/L)	5.1 (3.1–11.3)	3.8 (2.9–9.0)	5.2 (3.2–10.1)	5.2 (3.2–11.9)	5.0 (3.8–22.6)
inhibin B/FSH ratio	30.8 (6.6–65.7)	31.9 (9.4–63.0)	29.2 (7.3–76.1)	32.3 (7.3–63.3)	25.4 (0.7–49.5)
Total testosterone (nmol/L)	15.7 (12.9–19.0)	13.8 (10.3–16.3)*	14.8 (12.3–18.5)	16.2 (13.2–19.7)	17.6 (13.7–19.8)
Free Testosterone (nmol/L)	10.4 (8.7–12.6)	9.4 (7.6–10.8)*	10.3 (8.8–12.9)	10.5 (8.7–12.7)	11.5 (8.4–12.9)
SHBG (nmol/L)	26.8 (20.3–33.8)	21.9 (15.0–29.3)	23.7 (18.0–30.2)*	28.3 (22.0–36.4)	35.8 (29.2–40.7)
LH (U/L)	3.7 (2.6–5.5)	3.9 (2.2–5.4)	3.5 (2.5–4.9)	3.8 (2.7–5.7)	3.1 (1.5–7.0)

Data are expressed as median (interquartile ranges). BMI=body mass index, expressed as kg/m^2; SHBG=sex hormone-binding globulin; FSH=follicle stimulating hormone; LH=luteinizing hormone. *P<0.05 as compared to survivors with BMI 18.5–25 kg/m^2 (analysis of variance – ANOVA).

Influence of obesity and body composition on free testosterone

In univariate analysis, free testosterone was significantly inversely associated with BMI, total fat percentage, waist and WHR (Table III). After adjustment for age at follow-up, age at diagnosis, current smoking status, TBI, abdominal radiotherapy and AAD score, free testosterone was significantly inversely associated with BMI (β -0.1, p=0.029), total fat percentage (β -0.08, p=0.002), waist circumference (β -0.06, p=0.001) and WHR (β -0.1, p=0.002), but not with visceral fat percentage. Survivors with obesity, defined by BMI ≥30 (β -1.8, p=0.015), total fat percentage ≥25% (β -1.2, p=0.004) or waist circumference >102 cm (β -2.0, p=0.020) had significantly lower free testosterone than survivors without obesity. For total testosterone, similar, but stronger associations were observed (Supplementary Table I).

Influence of obesity and body composition on SHBG

SHBG levels were significantly inversely associated with BMI and all measures of body composition in univariate and multivariate analysis: BMI, including age at follow-up, age at diagnosis, current smoking status, TBI, abdominal radiotherapy and AAD score: BMI (β –1.0, p<0.001), total fat percentage (β -0.5, p<0.001), lean body mass (β -0.3, p=0.003), waist circumference (β -0.3, p<0.001),

WHR (β -0.4, p<0.001) and visceral fat percentage (β -3.2, p<0.001). Survivors with obesity, defined by BMI ≥30 (β -7.6, p=0.003), total fat percentage ≥25% (β -6.6, p<0.001) or waist circumference >102 cm (β -7.6, p<0.001) had significantly lower SHBG than survivors without obesity (Table IV).

Table III | The influence of body mass index (BMI) and measures of body composition on free testosterone.

	Free testosterone n=341 (224)[a]					
	Univariate model			Multivariate model		
	β	95% CI	p	β	95% CI	p
Body mass index	-0.15	-0.23; -0.06	0.001	-0.1	-0.2; -0.01	0.029
Obesity (BMI ≥ 30)	-2.0	-3.5; -0.6	0.006	-1.8	-3.2; -0.3	0.015
Overweight (BMI 25-30)	-0.4	-1.1; 0.4	0.364	0.04	-0.7; 0.8	0.927
Underweight (BMI <18.5)	-0.2	-2.0; 1.7	0.872	-0.7	-2.4; 1.1	0.462
Total fat percentage	-0.11	-0.16; -0.06	<0.001	-0.08	-0.13; -0.03	0.002
Total fat percentage ≥25%	-1.6	-2.4; -0.8	<0.001	-1.2	-2.0; -0.4	0.004
Lean body mass (kg)	0.01	-0.04; 0.07	0.624	-0.01	-0.06; 0.05	0.861
Waist circumference (cm)[b]	-0.08	-0.12; -0.05	<0.001	-0.06	-0.10; -0.02	0.001
Waist >102 cm[b]	-2.6	-4.4; -0.9	0.003	-2.0	-3.7;-0.3	0.020
Waist – hip ratio[b]	-0.15	-0.21; -0.09	<0.001	-0.1	-0.17; -0.04	0.002
Visceral fat percentage[b]	-0.21	-0.50; 0.08	0.159	0.12	-0.21; 0.45	0.479

CI=confidence interval; BMI=body mass index; [a]number of available DXA scans; [b]survivors treated with abdominal radiotherapy are excluded from this analysis (n=21). Univariate and multivariate linear regression analyses. Model was adjusted for age at follow-up, age at diagnosis, current smoking status, total body irradiation, abdominal radiotherapy and alkylating agent dose score.

Influence of obesity and body composition on inhibin B

Inhibin B was significantly inversely associated with total fat percentage and WHR and positively associated with lean body mass in univariate analysis (Table V). After adjustment for age at follow-up, age at diagnosis, current smoking status, TBI, abdominal radiotherapy and AAD score, inhibin B was independently and inversely associated with total fat percentage (β -1.9, p=0.010) and WHR (β -2.1, p=0.042), but not with lean body mass. Survivors with obesity, defined by total fat percentage ≥25% (β -25, p=0.047) had significantly lower inhibin B than survivors without obesity.

In univariate analysis, inhibin B/FSH ratios were significantly inversely associated with total fat percentage and WHR and positively associated with lean body mass (Supplementary Table II). After adjustment for confounders, the inhibin B/FSH ratio was significantly inversely associated with total fat percentage (β (%) -2.8, p=0.018), but not with WHR or lean body mass. Survivors with obesity, defined by total fat percentage ≥25% (β (%) -34, p=0.041) had significantly lower inhibin B/FSH ratio than survivors without obesity.

Table IV | The influence of body mass index and measures of body composition on sex hormone-binding globulin.

	SHBG n=344 (225)[a]					
	Univariate model			Multivariate model		
	β	95% CI	p	β	95% CI	p
Body mass index	-0.9	-1.2; -0.6	<0.001	-1.0	-1.3; -0.7	<0.001
Obesity (BMI ≥30)	-6.3	-11.4; -1.2	0.016	-7.6	-12.5; -2.6	0.003
Overweight (BMI 25–30)	-5.2	-7.9; -2.5	<0.001	-5.7	-8.4;-3.1	<0.001
Underweight (BMI <18.5)	4.2	-2.3;10.6	0.205	3.6	-2.6; 9.9	0.254
Total fat percentage	-0.5	-0.6; -0.3	<0.001	-0.5	-0.7; -0.3	<0.001
Total fat percentage ≥25%	-6.7	-9.7; -3.7	<0.001	-6.6	-9.6; -3.5	<0.001
Lean body mass (kg)	-0.3	-0.5; -0.1	0.004	-0.3	-0.5; -0.1	0.003
Waist circumference (cm) [b]	-0.3	-0.4; -0.2	<0.001	-0.3	-0.5; -0.2	<0.001
Waist >102 cm[b]	-5.7	12.0; 0.6	0.076	-7.6	-13.6; -1.5	0.014
Waist – hip ratio[b]	-0.3	-0.5; -0.1	0.004	-0.4	-0.7; -0.2	<0.001
Visceral fat percentage[b]	-1.9	-3.0; -0.9	<0.001	-3.2	-4.3; -2.1	<0.001

CI=confidence interval; BMI=body mass index; SHBG=sex hormone-binding globulin; [a]number of available DXA scans; [b]survivors treated with abdominal radiotherapy are excluded from this analysis (n=21). Univariate and multivariate linear regression analyses. Model was adjusted for age at follow-up, age at diagnosis, current smoking status, total body irradiation, abdominal radiotherapy and alkylating agent dose score.

Table V | The influence of body mass index (BMI) and measures of body composition on serum inhibin B.

	inhibin B n=340 (226)[a]					
	Univariate model			Multivariate model		
	β	95% CI	p	β	95% CI	p
Body mass index	-0.2	-3.0; 2.6	0.863	-1.3	-4.0; 1.3	0.317
Obesity (BMI ≥30)	-7.2	-54;39	0.761	-10.3	-52; 31.0	0.626
Overweight (BMI 25–30)	5.0	-20; 30	0.689	0.4	-22; 23	0.971
Underweight (BMI <18.5)	-3.7	-61; 53	0.897	2.8	-48; 54	0.914
Total fat percentage	-2.2	-3.7; -0.6	0.006	-1.9	-3.3; -0.5	0.010
Total fat percentage ≥25%	-30	-57; -4	0.026	-25	-49; -0.4	0.047
Lean body mass (kg)	2.3	0.5; 4.0	0.011	0.1	-1.6; 1.8	0.882
Waist circumference (cm)[b]	-1.0	-2.1; 0.2	0.106	-1.0	-2.1; 0.1	0.087
Waist >102 cm[b]	-44	-101; 14	0.135	-44	-97; 8	0.096
Waist – hip ratio[b]	-2.1	-4.1; -0.2	0.034	-2.1	-4.1; -0.07	0.042
Visceral fat percentage[b]	-5.2	-14.1; 3.8	0.254	-0.6	-9.8; 8.7	0.905

CI=confidence interval; BMI=body mass index; [a]number of available DXA scans; [b]survivors treated with abdominal radiotherapy are excluded from this analysis (n=21). Univariate and multivariate linear regression analyses. Model was adjusted for age at follow-up, age at diagnosis, current smoking status, total body irradiation, abdominal radiotherapy and alkylating agent dose score.

Discussion

Although it is known that obesity is associated with impaired gonadal function in the general male population, this association might be different in male survivors of childhood cancer since it seems likely that the cancer treatment had such a detrimental impact that effects of obesity would be absent. However, our study observed that obesity itself has an additive negative effect on gonadal function in survivors of childhood cancer, independent of previous gonadotoxic cancer treatment and therefore lifestyle intervention might be beneficial.

In the current study, an inverse association between BMI and testosterone was found which is in line with previous reports in the general population [6,7,15-17]. However, most previous studies are based on BMI as marker for obesity, while it was recently shown that BMI is an inaccurate measure for total body fat since obesity was underestimated in 39% of the general population and in 52% of childhood cancer survivors [29,30]. Waist circumference, representing the amount of intra-abdominal fat, is a slightly better marker, but the most accurate method for total body fat measurement is dual energy X-ray absorptiometry (DXA) [31]. In the present study, we were able to include data of total body fat measured by DXA in a substantial subset of the survivors and found that obesity, represented by total fat percentage, was an independent risk factor for low total and free testosterone.

Although some studies reported no or a different association, a negative association between BMI and inhibin B was reported several times in the general population [7,8,32]. The current study clearly describes an independent relationship between obesity, represented by high total fat percentage or high waist hip-ratio, and low inhibin B levels in male survivors of childhood cancer. Additionally, low inhibin B levels were observed in underweight subjects. However, no significant relation between underweight and inhibin B levels was found, probably due to the small number of subjects in this BMI category.

We hypothesize that obesity negatively affects gonadal function in male survivors of childhood cancer. This may be due to reduced androgen levels, which can be induced by higher aromatase activity in overweight and obese men. P450 aromatase cytochrome converts testosterone to oestradiol, is expressed at high levels in white adipose tissue and is responsible for the key step in the biosynthesis of oestrogens [31,33]. Unfortunately, oestradiol levels were not available in our male survivors, but we showed that testosterone levels were low in obese men, which supports this hypothesis. Both LH and FSH remained normal, which is probably because of the phenomenon that plasma oestradiol levels substantially suppress LH levels in men [34].

Recently, in female childhood cancer survivors, we also found that obesity and insulin resistance are associated with a reduced ovarian function as reflected by decreased anti-Müllerian hormone (AMH) levels and reduced follicle counts [35]. In previous studies regarding men, a negative or no association between obesity and insulin resistance with gonadal function has been found. A previous study among male survivors of adult cancer showed that total and free testosterone levels were significantly negatively associated with insulin levels [36]. Unfortunately, no data of fasting insulin were available in our cases. However, it is well known that insulin resistance is a major determinant of decreased SHBG levels, by its inhibition of SHBG production in the liver [37]. We

found a strong relationship between SHBG and obesity, which is probably secondary to insulin resistance. The negative influence of obesity on gonadal function might thus be secondary to insulin resistance, which relation has previously been investigated in animal studies. Hyperglycemic conditions in diabetic rats affected Sertoli cell function directly, due to atrophy of the seminiferous tubules, followed by depletion of germ cells, or indirectly due to damage to the Leydig cells affecting testosterone dependent sperm production [38,39]. In the present study we hypothesized that obesity negatively influences male reproductive capacity, based on knowledge from previous studies in the general population. However, due to the cross-sectional design we cannot exclude that in childhood cancer survivors hypogonadism may be a determinant rather than a consequence of obesity, but based on literature, the first hypothesis seems to be more likely. We were not able to include sperm analyses in the survey. Nevertheless, the correlation between inhibin B values and sperm analyses was reported to be strong (r=0.54, r=0.76, respectively, $p<0.001$) [12,13], indicating that it is justified to use inhibin B as a surrogate marker for gonadal function. Moreover, we measured testosterone to provide complete information on reproductive hormone status, and also found a significant negative correlation between total fat percentage and free testosterone.

In conclusion, obesity itself is associated with an impaired gonadal function in male survivors of childhood cancer, independent of the effect of the previous cancer treatment. We recommend counselling male survivors on lifestyle in order to at least partly improve their gonadal function. However, longitudinal studies are needed to confirm the positive effect of weight loss on gonadal function.

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Supplementary Table I | Univariate and multivariate linear regression analyses illustrating the influence of body mass index and measures of body composition on total testosterone levels.

	Total testosterone n=351 (226)[a]					
	Univariate model			Multivariate model		
	β	95% CI	p	β	95% CI	p
Body mass index	-0.4	-0.5; -0.2	<0.001	-0.3	-0.5; -0.2	<0.001
Obesity (BMI ≥30 kg/m²)	-3.8	-6.1; -1.4	0.002	-3.7	-6.0; -1.4	0.002
Overweight (BMI 25–30 kg/m²)	-1.5	-2.7; -0.2	0.019	-1.1	-2.3; 0.1	0.075
Underweight (BMI <18.5 kg/m²)	0.5	-2.2; 3.3	0.713	0.05	-2.6; 2.7	0.972
Total fat percentage	-0.2	-0.3; -0.2	<0.001	-0.2	-0.3; -0.1	<0.001
Total fat percentage ≥25%	-3.4	-4.7; -2.1	<0.001	-2.9	-4.2; -1.6	<0.001
Lean body mass (kg)	-0.04	-0.1; 0.1	0.441	-0.1	-0.2; 0.03	0.169
Waist circumference (cm) [b]	-0.2	-0.2; -0.1	<0.001	-0.1	-0.2; -0.1	<0.001
Waist circumference >102 cm[b]	-4.5	-7.4; -1.6	0.003	-4.1	-6.9; -1.2	0.005
Waist – hip ratio[b]	-0.3	-0.4; -0.2	<0.001	-0.2	-0.3; -0.1	<0.001
Visceral fat percentage[b]	-0.6	-1.0; -0.1	0.020	-0.4	-0.9; 0.2	0.198

CI=confidence interval; BMI=body mass index; [a]number of available DXA scans; [b]survivors treated with abdominal radiotherapy are excluded from this analysis (n=21). Model was adjusted for age at follow-up, age at diagnosis, current smoking status, total body irradiation, abdominal radiotherapy and alkylating agent dose score.

Supplementary Table II | Univariate and multivariate linear regression analyses illustrating the influence of obesity on inhibin B/FSH ratio.

	Inhibin B/FSH ratio n=341 (226)[a]					
	Univariate model			Multivariate model		
	β (%)	95% CI	p	β (%)	95% CI	p
Body mass index	2.1	-3.2; 7.7	0.440	0.3	-4.2; 4.9	0.912
Obesity (BMI ≥30 kg/m²)	2.9	-57; 149	0.949	1.5	-50; 106	0.968
Overweight (BMI 25–30 kg/m²)	4.6	-35; 67	0.852	-1.2	-33; 45	0.951
Underweight (BMI <18.5 kg/m²)	-50	-83; 46	0.202	-45	-77; 30	0.171
Total fat percentage	-4.0	-6.8; -1.2	0.006	-2.8	-5.0; -0.5	0.018
Total fat percentage ≥25%	-46	-67; -12	0.014	-34	-55; -1.7	0.041
Lean body mass (kg)	5.1	1.7; 8.5	0.003	-0.4	-3.1; 2.3	0.753
Waist circumference (cm) [b]	-1.9	-3.9; 0.2	0.076	-1.5	-3.3; 0.4	0.113
Waist circumference >102 cm[b]	-56	-84; 24	0.122	-53	-80; 12	0.088
Waist – hip ratio[b]	-3.2	-5.2; -0.2	0.037	-2.2	-4.4; 0.9	0.141
Visceral fat percentage[b]	-13	-26; 2	0.091	-3	-16; 13	0.711

FSH=follicle-stimulating hormone; CI=confidence interval; BMI=body mass index; [a]number of available DXA scans; [b]Survivors treated with abdominal radiotherapy are excluded from this analysis (n=21). Model was adjusted for age at follow-up, age at diagnosis, current smoking status, total body irradiation, abdominal radiotherapy and alkylating agent dose score.

CHAPTER 10

Gonadal function recovery in very long-term male survivors of childhood cancer

W van Dorp & IMM van der Geest, JSE Laven, WCJ Hop,
SJCMM Neggers, ACH de Vries, R Pieters, MM van den Heuvel-Eibrink

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ABSTRACT

Background: Although gonadal toxicity has been reported, no data are available on recovery of gonadal function in very long-term survivors of childhood cancer. Inhibin B is a novel reliable serum marker which has been shown to be of value in childhood cancer survivor studies to identify risk groups for impaired gonadal function, but consecutive long-term follow-up studies using serum inhibin B as a marker are not available.

Objective: To evaluate possible recovery of gonadal dysfunction over time in adult male survivors of childhood cancer.

Methods: In this retrospective study, adult male long-term childhood cancer survivors (n=201) who visited our outpatient late effects clinic were included and we used inhibin B as a surrogate marker for gonadal function.

Results: Median age at diagnosis was 5.9 years (range 0.0–17.5) and discontinuation of treatment was reached at a median age of 8.2 years (range 0.0–20.8). Inhibin B levels were first measured after a median follow-up time of 15.7 years (range 3.0–37.0). Median interval between the first (T1) and second measurement (T2) was 3.3 years (range 0.7–11.3). Median inhibin B level was 127 ng/L (range 5–366) at T1 and 155 ng/L (range 10–507) at T2. The prediction model suggests that inhibin B levels do not normalise in survivors with a very low inhibin B level at T1.

Conclusions: Our results suggest that recovery of gonadal function is possible even long after discontinuation of treatment. However, this recovery does not seem to occur in survivors who already reached critically low inhibin B levels after discontinuation of treatment.

INTRODUCTION

Due to improved survival rates [1], long-term side effects of cancer treatment, such as gonadal dysfunction, became increasingly important. Gonadal dysfunction can be caused by chemotherapy and radiotherapy [2-6]. Specific chemotherapeutics such as alkylating agents and cytosine arabinoside [7,8], total body irradiation (TBI) and local irradiation including the testis [9-11] were found to be highly gonadotoxic.

To assess gonadal function, performing semen analysis is the most direct way to establish a men's fertility potential. However, in adolescents as well as in young adults providing a semen sample for analysis might be difficult. Hence, the use of an alternative first screening method would be of value. Serum inhibin B levels are currently used to assess gonadal function as a first screening [12-16]. Inhibin B is directly produced by Sertoli cells in the seminiferous tubules [17]. Inhibin B levels are strongly associated with sperm quantity and thereby constituting a marker which might identify childhood cancer survivors (CCS) at risk of infertility [18-20].

In survivors of adult Hodgkin lymphoma, interestingly, after well-documented damage of spermatogenesis in some CCS, sperm quality can improve over time after long-term follow-up. Recovery of spermatogenesis has even been described at more than 10 years after treatment [2]. Even after high doses of cyclophosphamide, a slow recovery of spermatogenesis was described [3]. Recovery after low doses of alkylating agents in adulthood has been reported as well [4,5,21]. In survivors of childhood cancer however, only two small studies evaluated the follow-up of gonadal function. In 2 out of 12 survivors of Hodgkin lymphoma, recovery has been observed at 12–15 years following treatment, whereas a second study could not confirm this recovery in 19 survivors at 10 years after treatment [22,23]. To our knowledge, follow-up studies in other subsets of adult and childhood cancer survivors have not been performed.

We performed this study to evaluate recovery of spermatogenesis over time in a large single-centre cohort of male long-term CCS using inhibin B as a surrogate marker and using paired analyses.

MATERIALS AND METHODS

Subjects

A cross-sectional retrospective single-centre study was performed in our adult late-effects outpatient clinic for long-term CCS. Survivors were in continuous complete remission and older than 18 years. Male survivors, diagnosed between 1964 and 2005, who visited our late-effects outpatient clinic twice or more were included between 2004 and 2010. Informed consent was obtained from all participants.

Information on the type of disease, patient characteristics, treatment regimens and follow-up data were retrieved from the medical records. Recently, the alkylating agent dose (AAD) score was introduced to determine the effect of high-risk chemotherapy. We calculated this score by determining the drug dose tertile distribution in our entire cohort of survivors and adding the tertile scores (1–2–3) for each of the alkylating agents given to a particular patient as previously used in

childhood cancer survivors by Green *et al* [8,24]. An AAD score of zero was assigned to patients not exposed to alkylating agents. Radiotherapy at high risk for gonadal dysfunction was defined by TBI and testis irradiation.

Hormones

We obtained peripheral blood samples, which were stored at -20° Celsius until analysis. Inhibin B levels were measured using kits purchased from Serotec Ltd (Oxford, United Kingdom). Within-assay and between-assay coefficients of variation (CV) were <9% and <15%, respectively. Serum FSH and LH were determined with the Immulite assay (Diagnostic Products Corporation (DPC), Los Angeles, CA, United States of America). Within-assay and between-assay CV were <6% and <9%, and <5% and 11% for FSH and LH, respectively. Serum testosterone levels were determined using coated tube radioimmunoassays (DPC). Intra-assay and inter-assay variation coefficients were 3% and 4.5%. The reference values of LH, FSH and testosterone for male adults in our institute are 1.5–8.0 U/L, 2.0–7.0 U/L, 10.0–30.0 nmol/L, respectively [19]. Inhibin B levels between 150 and 400 ng/L were used as reference values; 150 ng/L provides the highest sensitivity and specificity as lower normal value for detecting low semen quality [16].

Data analysis

Survivor characteristics were expressed as median and range. Since the distribution was not normal, non-parametric paired tests were used. Wilcoxon signed-rank tests were used to analyse paired inhibin B levels in both the complete cohort of survivors, as well as in a subgroup excluding subjects with low LH or FSH at first assessment (T1) (n=194/201), as a proxy for hypogonadotropic hypogonadism. Results of both analyses were identical and only the analysis of all survivors was included in this paper. Logistic regression was used to evaluate the probability to reach an inhibin B level ≥150 ng/L at the second assessment (T2), allowing for age at diagnosis, first inhibin B level, age at T2, interval between discontinuation of treatment and T2, high-risk radiotherapy, AAD score and hypogonadism, represented by a low LH and/or FSH. Whether the effects of the variables AAD score and high-risk radiotherapy depended on the length of the interval between two assessments was evaluated using interaction terms. P-values <0.05 were considered statistically significant. Statistical analysis was performed using SPSS 17.0 software (SPSS, Chicago IL, USA).

RESULTS

Between 2004 and 2010, 217 out of 348 adult male survivors visited our outpatient clinic twice since the remaining 131 survivors only visited the outpatient clinic once. Out of these subjects, inhibin B levels were measured twice in 203 subjects. Two survivors were excluded because of testosterone substitution. Patient characteristics are presented in Table I. The correlation between serum inhibin B levels at T1 and T2 is presented in Supplementary Figure I.

Table I | Patient characteristics (n=201)

	Median (range)
Age at diagnosis (years)	6.0 (0.0–17.5)
Age at stop treatment (years)	8.3 (0.0–20.8)
Age at T1 (years)	22.6 (18.0–43.4)
Age at T2 (years)	26.9 (19.0–46.3)
Interval between T1 and T2 (years)	3.3 (0.7–11.3)
Interval after end of treatment to T1 (years)	15.7 (3.0–37.0)
Diagnosis	**Number (%)**
Acute lymphoblastic leukaemia	63 (31.3)
Renal tumour	28 (13.8)
Hodgkin lymphoma	24 (11.9)
T-cell non-Hodgkin lymphoma	16 (8.0)
B-cell non-Hodgkin lymphoma	13 (6.5)
Rhabdomyosarcoma	10 (5.0)
Brain tumour	8 (4.0)
Neuroblastoma	6 (3.0)
Osteosarcoma	6 (3.0)
Acute myeloid leukaemia	6 (3.0)
Ewing sarcoma	5 (2.5)
Other	16 (8.0)
Radiotherapy	
No irradiation	130 (64.7)
Cranial irradiation	19 (9.5)
Brain irradiation	15 (7.5)
Total body irradiation	6 (3.0)
Testis irradiation	1 (0.5)
Other	30 (14.9)
Chemotherapy	
AAD-score	
0	78 (38.8)
1	22 (10.9)
2	32 (15.9)
3	55 (27.4)
≥4	14 (7.0)

AAD score=Alkylating Agent Dose score; T1=first assessment; T2=second assessment.

A summary of the analyses of hormone levels is presented in Table II. Median inhibin B level at T1 was 127 ng/L (range 5–366), which is below the lower limit of 150 ng/L [16]. At T1, 61% (123/201) had a serum inhibin B level <150 ng/L. In survivors of B-cell non-Hodgkin lymphoma, neuroblastoma, osteosarcoma, rhabdomyosarcoma and Hodgkin lymphoma, median serum inhibin B levels were significantly below the normal limit of 150 ng/L. Especially in survivors of rhabdomyosarcoma (median 58 ng/L, range 10–226) and Hodgkin lymphoma (median 46 ng/L, range 10–274), inhibin B levels were dramatically low at T1. Only acute lymphoblastic leukaemia survivors (median 153 ng/L, range 9–296) and survivors of renal tumours (median 160 ng/L, range 13–366) had a median serum inhibin B level ≥150 ng/L (Figure I).

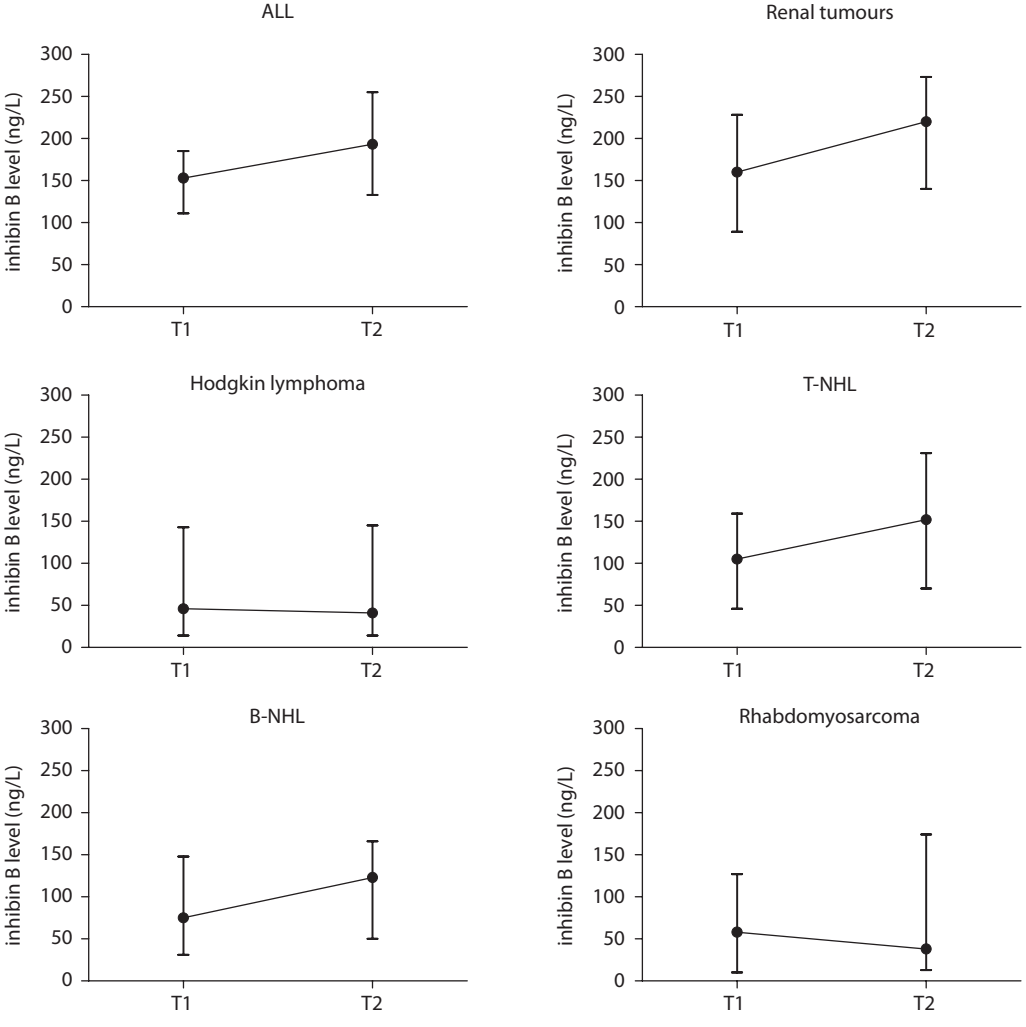

Figure I | Inhibin B levels per diagnosis. Median inhibin B levels and interquartile ranges per diagnosis at T1 (first assessment) and T2 (second assessment). ALL=acute lymphoblastic leukaemia; T-NHL=T-cell non-Hodgkin lymphoma; B-NHL=B-cell non-Hodgkin lymphoma. Other diagnoses were not presented due to the small number of survivors.

Table II | Hormone levels during follow-up of all survivors (n=201).

	T1	T2	Change	p
FSH (U/L)	4.6 (0.1–65.8)	5.0 (0.1–65.7)	0.3 (-16–14.5)	<0.001
LH (U/L)	3.4 (0.1–16.3)	3.9 (0.1–19.5)	0.5 (-6.6–8.4)	<0.001
Testosterone (nmol/L)	14.6 (2.8–32.7)	16.1 (5.7–36.9)	0.95 (-15.6–15.9)	0.012
Inhibin B (ng/L)	127 (5–366)	156 (10–507)	20 (-143–265)	<0.001

T1=first assessment; T2 second assessment; change T2-T1.

At second assessment (T2), the median inhibin B level in the total group of CCS was 156 ng/L (range 10–507), above the normal level. 47% (94/201) survivors had a serum inhibin B level <150 ng/L. Survivors of rhabdomyosarcoma (median 38 ng/L, range 10–342) and Hodgkin lymphoma (median 41 ng/L, range 10–269) still had dramatically low serum median inhibin B levels at T2, whereas acute lymphoblastic leukaemia survivors (median 193 ng/L, range 10–411) and renal tumour survivors (median 220 ng/L, range 10–371) had the highest serum inhibin B levels (Table III).

Paired analyses showed that the increase of inhibin B levels between T1 (127 ng/L) and T2 (156 ng/L) was significant (p<0.001). Except survivors of Hodgkin lymphoma (p=0.780) and survivors of rhabdomyosarcoma (p=0.688), all survivors showed a significant increase of serum inhibin B levels.

In survivors treated without irradiation, median inhibin B levels were nearby the normal range and these levels increased significantly to normal levels at T2 (median 165, range 10–507, p<0.001). In survivors treated with cranial irradiation, both inhibin B levels at T1 and T2 were within the normal range (T1: median 159, range 10–296; T2: median 188, range 12–411, p=0.038). In survivors treated with brain irradiation, the inhibin B levels at T1 and T2 were far below the normal range. Although the group size was too small to draw firm conclusions, the median inhibin B level of survivors treated with TBI remains far below the normal level (Table III).

We also analysed inhibin B levels in survivors according to AAD score. Survivors with an AAD score of 2 or higher had a median inhibin B level below the normal limit at T1, while survivors with an AAD score of 0 or 1 had normal median inhibin B levels (Table III). At T2, serum inhibin B levels in survivors with an AAD score ≥3 remained low, while the levels in survivors with an AAD score=2 recovered up to a normal level. Serum inhibin B levels in survivors who were not treated with alkylating agents or with an AAD score=1 remained within the normal range. Paired analyses showed that survivors treated without alkylating agents had a significant increase in median inhibin B levels. Only survivors with an AAD score=1 or an AAD score ≥4 showed no significant change over time. Survivors treated without alkylating agents or with an AAD score <3 reached normal median inhibin B levels.

Table III | Inhibin B in diagnostic and treatment subsets.

	n	Inhibin B First screening	Inhibin B Second screening	Change of inhibin B	p^
n	201	127 (5; 366)	156 (10; 507)	20 (-143; 265)	<0.001
Diagnosis					
Acute lymphoblastic leukaemia	63	153 (9; 296)	193 (10; 411)	38 (-143; 265)	<0.001
Renal tumour	28	160 (13; 366)	220 (10; 371)	31 (-100; 182)	0.005
Hodgkin lymphoma	24	46 (10; 274)	41 (10; 269)	0 (-118; 63)	0.780
T-cell non- Hodgkin lymphoma	16	105 (10; 225)	152 (23; 391)	44 (-44; 223)	0.003
B-cell non-Hodgkin lymphoma	13	75 (10; 225)	123 (10; 279)	21 (-70; 115)	0.012
Rhabdomyosarcoma	10	58 (10; 226)	38 (10; 342)	1 (-40; 116)	0.688
Brain tumour	8	105 (18; 184)	139 (39; 314)	29 (-29; 143)	*
Neuroblastoma	6	85 (21; 212)	90 (14; 345)	11 (-39; 133)	*
Ewing sarcoma	6	39 (15; 299)	65 (43; 336)	10 (-5; 50)	*
Osteosarcoma	6	86 (38; 339)	123 (24; 507)	35 (-19; 168)	*
Acute myeloid leukaemia	5	145 (10; 195)	126 (11; 287)	4 (-59; 109)	*
Other	16	109 (5; 206)	140 (10; 339)	43 (-34; 195)	
Radiotherapy					
No irradiation	130	137 (10; 366)	165 (10; 507)	26 (-118; 265)	<0.001
Cranial irradiation	19	159 (10; 296)	188 (12; 411)	38 (-103; 154)	0.038
Brain irradiation	15	88 (10; 171)	90 (11; 314)	21 (-143; 145)	0.050
Total body irradiation	6	16 (5; 35)	15 (10; 53)	3 (-11; 18)	*
Chemotherapy					
AAD score=0	78	152 (13; 366)	210 (10; 507)	40 (-100; 233)	<0.001
AAD score=1	22	161 (24; 279)	161 (21; 411)	21 (-143; 138)	0.144
AAD score=2	32	127 (9; 296)	154 (10; 392)	30 (-44; 265)	<0.001
AAD score=3	55	73 (10; 299)	112 (10; 336)	7 (-103; 143)	0.004
AAD score≥4	14	16 (5; 77)	15 (10; 57)	0 (-44; 15)	0.326

Data are presented as median (range). ^paired analysis (Wilcoxon signed rank test), *Analysis was not performed because of small number of patients. AAD score=Alkylating Agent Dose Score.

Using logistic regression, we found that the probability of recovery of serum inhibin B levels was significantly influenced by the first inhibin B level (p<0.001), but was not influenced by age at diagnosis (p=0.390), age at T2 (p=0.440), interval between discontinuation of treatment and T2 (p=0.368), high-risk radiotherapy (p=0.999), AAD score (p=0.297), hypogonadism (p=0.900) and the interaction terms (high-risk radiotherapy x interval between T1 and T2) (p=1.000) and (AAD score x interval between T1 and T2) (p=0.073). Figure II shows the estimated normalisation probabilities of inhibin B levels based on the levels at first screening. This figure shows that survivors with an inhibin B level at first assessment ≥105 ng/L have 50% chance to reach normal inhibin B levels, while this

probability of recovery is negligible when the first inhibin B level is below 60 ng/L. The latter group consists of survivors of Hodgkin lymphoma treated with alkylating agents (no radiotherapy) and survivors with an AAD score ≥3 (Supplementary Figures II and III).

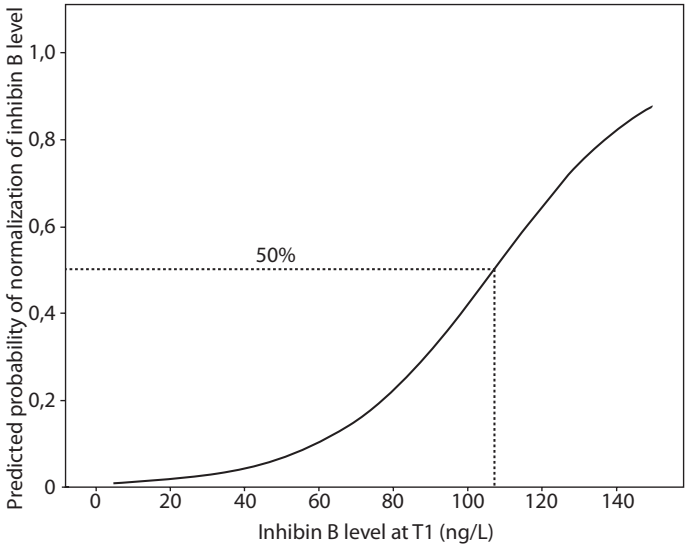

Figure II | Prediction of recovery. The prediction of the chance of reaching normal inhibin B levels based on inhibin B levels at T1. T1=first assessment of inhibin B.

DISCUSSION

As yet, large studies on recovery of gonadal function with a very long (>15 years) follow-up based on paired measurements have not been performed in male CCS. We showed that in general, inhibin B levels tend to increase over time in most CCS, suggesting that recovery of gonadal damage can occur long time after survivors discontinued their cancer treatment. However, this increase does not seem to occur in those survivors who already reached critically low serum inhibin B levels long after discontinuation of treatment.

Overall, longitudinal studies regarding the follow-up of gonadal dysfunction in survivors of childhood cancer are scarce. In survivors of childhood Hodgkin lymphoma, two smaller studies were published. In 17 and 19 survivors respectively, recovery after a median follow-up of 10 years in male long-term survivors of childhood Hodgkin lymphoma treated with alkylating agents did not occur, whether they received pelvic radiotherapy or not [22]. In contrast, in survivors of adult Hodgkin lymphoma, recovery of gonadal function after cessation of treatment occurred in a small subset treated with alkylating agents after 5–15 years follow-up [2,4].

We and others have shown that male survivors who have been treated with alkylating agents are at risk for infertility [8,19,25]. In the present study, we used the AAD score to determine the effect of high-risk chemotherapy in a specific survivor. Survivors with an AAD score ≥3 did not show recovery of inhibin B levels. Secondly, the chance of recovery of inhibin B did not depend on age at diagnosis in our study.

This study suggests that in the male after initial impairment of function, gonads can recover over time in subsets of CCS even after a very long follow-up time. Little is known about the mechanism of recovery after cancer treatment. We hypothesise that the normalisation of inhibin B, being a proxy for gonadal function, would be due to the slow recovery of spermatogenesis over time. Although this hypothesis was not investigated in long-term survivors, in mice and rats such a recovery process has been shown after Busulfan treatment. At day 96, spermatogenesis fully recovered in many seminiferous tubules and 80% of the rats regained various degrees of fertility at day 120 [26]. It was hypothesised that this recovery was the result of self-renewal of stem spermatogonia and suggested that the asymmetric division of type A spermatogonia and their close contact with the basal lamina and the Sertoli cells may be involved in regulating the number of stem spermatogonia. In mice, ageing does not affect the recovery of spermatogenesis after Busulfan [27], which could be similar to our finding that the probability of normalisation of inhibin B in human survivors was age-independent.

We were not able to include sperm analyses in paired analyses. Although the statement has been challenged [28], we and others have shown the reliability of inhibin B levels as a marker for male gonadal function [17,19,20]. Nevertheless, we recommend confirming our data in large longitudinal prospective cohorts in which serum inhibin B levels and sperm analyses were assessed. We have assumed that inhibin B is stable throughout adulthood. It is known that serum levels of inhibin B reach a peak during puberty [29-31], which seems to reflect the androgen-mediated differentiation of the Sertoli cells [32]. Thereafter, it is stable during life time [33]. Furthermore, serum inhibin B levels are relatively stable during daytime as shown in fertile men [34]. Therefore, we can conclude that inhibin B as a marker for spermatogenesis is stable enough to use in our follow-up study.

To our knowledge, we were the first to study the follow-up of gonadal function in male CCS, as measured by paired serum inhibin B levels as a proxy for spermatogenesis. We showed that in general, inhibin B levels are stable or increase over time, suggesting that recovery from gonadal damage may occur long after cessation of treatment for childhood cancer. However, this increase does not occur in survivors with critically low serum inhibin B levels more than 15 years after treatment, such as survivors treated with high dose alkylating agents or total body irradiation. Prospective longitudinal follow-up studies, using large nationwide cohorts and including sperm analyses, are necessary to confirm our results.

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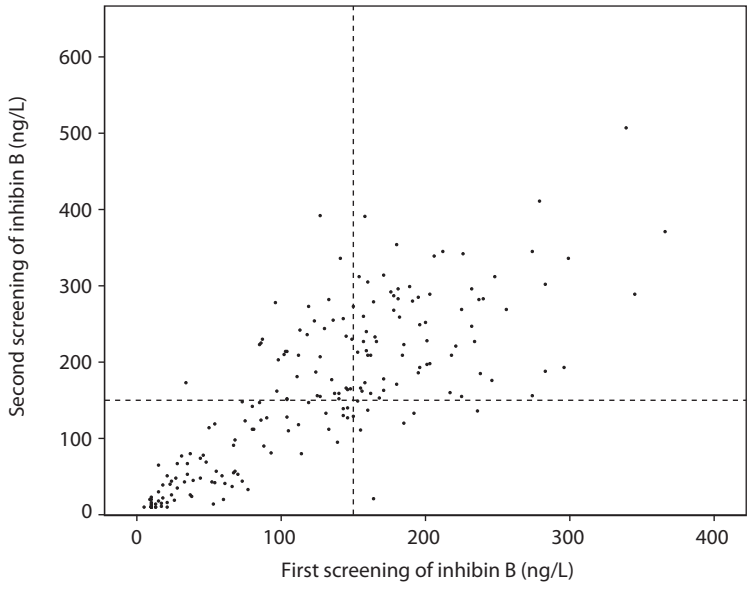
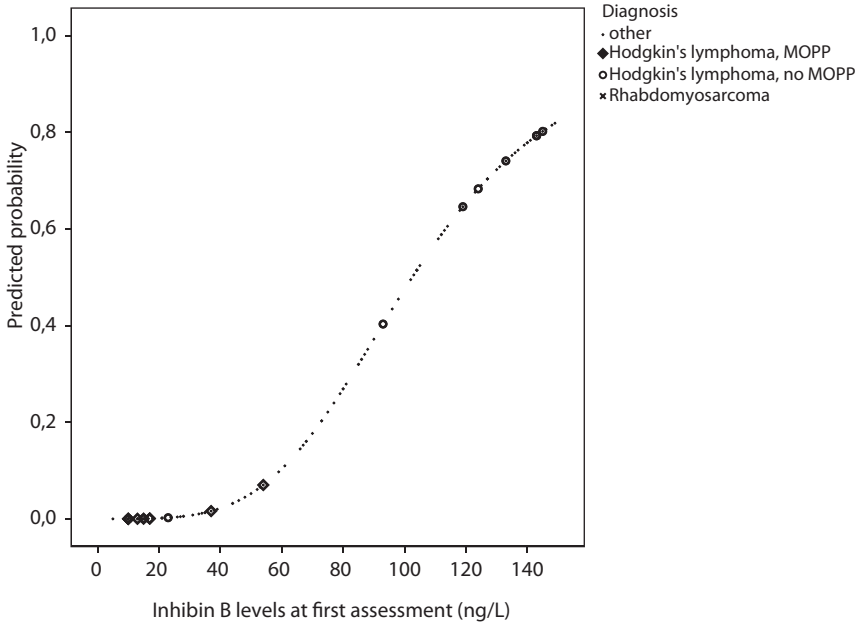
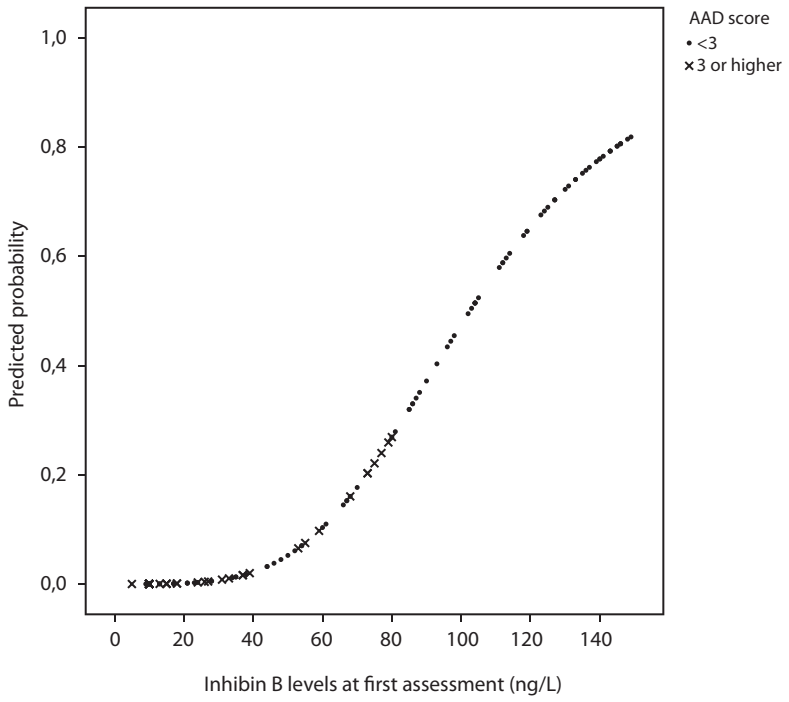


Supplementary Figure I | Correlation between inhibin B levels (ng/L) at first screening and inhibin B levels (ng/L) at second screening (r=0.819; p<0.001).



Supplementary Figure II | Prediction model of recovery probability of inhibin B based on first inhibin B level, showing high-risk subgroups based on diagnosis. MOPP=mustargen oncovin procarbazine prednisone.



Supplementary Figure III | Prediction model of recovery probability of inhibin B based on first inhibin B level, showing high-risk subgroups based on Alkylating Agent Dose (AAD) score.

CHAPTER 11

Electroejaculation as a method of fertility preservation in boys diagnosed with cancer: a single-centre experience and review of the literature

MC Adank, W van Dorp, M Smit, NJ van Casteren, JSE Laven,
R Pieters, MM van den Heuvel-Eibrink

Submitted

Abstract

Objective: To evaluate the feasibility of electroejaculation to perform semen cryopreservation in pubertal boys before gonadotoxic therapy, and to review literature on this topic.

Design: Retrospective cohort study and review of the literature.

Setting: Academic children's hospital.

Patient(s): Boys diagnosed with cancer to whom sperm cryopreservation was offered before start of gonadotoxic therapy.

Intervention(s): We studied the outcome of electroejaculation, including patients' characteristics, hormone levels, and pre-treatment semen parameters.

Main outcome measure(s): Semen cryopreservation.

Result(s): Pre-treatment semen samples were obtained by masturbation in 106/114 boys with cancer (median age 16.5 years, range 10.8−8.9), of which 78/106 were adequate for preservation. In 11 boys electroejaculation was offered, of which 3/11 appeared adequate for preservation. Reviewing all reported electroejaculation cases in children with cancer in the literature, 13/29 (45%) cases were successful. Testosterone levels were significantly higher in patients with successful sperm yield obtained by electroejaculation (testosterone: median 8.3 nmol/L (5.2−42.4) in successful versus median 1.7 nmol/L (0.01−17.9), p=0.006 in unsuccessful harvests).

Conclusion(s): Semen cryopreservation should be offered to all pubertal boys. If masturbation fails, electroejaculation can be considered as a useful option for semen cryopreservation, and leads to adequate material for cryopreservation in about half of the cases.

Introduction

The survival of childhood cancer has considerably increased over the last decades [1]. Because of this prolonged survival, long-term effects of treatment have become more apparent. One of these effects is infertility [2]. Treatment with high doses of alkylating agents, testicular irradiation with doses >1.2 Gy and total body irradiation prior to hematopoietic stem cell transplantation are risk factors for infertility later in life [3].

To preserve fertility, pre-treatment sperm cryopreservation can be offered to boys diagnosed with cancer to give these boys the chance to father their own offspring. However, especially young patients are frequently unable to produce sperm by masturbation. In adults diagnosed with cancer, neurological diseases and paraplegia, electroejaculation has been found to be a useful alternative [4,5]. In children diagnosed with cancer, only limited information on the feasibility and efficacy of electroejaculation is available [4-7]. Here, we provide an overview of semen cryopreservation after the introduction of electroejaculation in boys with cancer. Secondly, we present a narrative literature review on the outcome of electroejaculation in childhood cancer patients.

Materials and methods

Subjects

From January 1998 to March 2013 semen cryopreservation was offered to boys above the age of 10 years, with a Tanner stage ≥G2P2 before the start of their anticancer treatment at the Erasmus MC-Sophia Children's Hospital. The electroejaculation procedure was offered from 2003 onwards if sperm production by masturbation was not possible because of motor disabilities or early puberty (primarily), or if no ejaculation was obtained by masturbation after multiple attempts due to stress (secondary). For the current analysis, patients with previous gonadotoxic therapy, as well as patients diagnosed with a brain tumour were excluded. Patients were categorized in five diagnosis groups: leukaemia/non-Hodgkin lymphoma, Hodgkin lymphoma, sarcoma/Primitive Neuroectodermal Tumour (PNET), testicular tumours and other tumours.

The cohort of childhood cancer patients diagnosed from 1995 to 2005 was described previously [8].

Methods

Retrospectively, clinical data were retrieved from patient record files. Data were collected on age at diagnosis, type and stage of disease, therapeutic modalities, B-symptoms (fever for three consecutive days, drenching night sweats and weight loss exceeding 10% of body weight in six months), feasibility of ejaculation and masturbation at diagnosis, Tanner stage, testicular volume and reproductive hormone levels. Tanner stage was assessed clinically at diagnosis and classified as pre-pubertal (Tanner stage 1), mid-pubertal (Tanner stage 2–3), or late pubertal (Tanner stage 4–5) [9].

Endpoints were type and quality of ejaculate, semen volume, concentration, sperm count, morphology, progressive motility, pH, vitality, leukocytes (present or not), and number of round cells

as defined by the 5th WHO manual for semen analysis [10], and number of straws cryopreserved. Semen volume of 1.5 ml, total sperm number of $39x10^6$/ejaculate, sperm concentration of $15x10^6$/ml, total motility of 40%, progressive motility of 32%, vitality (live spermatozoa) of 58%, sperm morphology (normal forms) of 4% and pH ≥7.2 are considered normal values [10]. Oligospermia was defined as the total number or concentration of spermatozoa below the lower reference limit ($39x10^6$/ejaculate and $15x10^6$/ml, respectively). Azoospermia was defined as absence of spermatozoa in the ejaculate [10]. Regardless of meeting the WHO criteria, semen samples were defined to be adequate for cryopreservation if any motile spermatozoa were identified, since ultimately only few motile spermatozoa are needed for assisted reproduction techniques (ART). In order to compare our results with previous studies, and because none of the patients appeared to produce an adequate semen sample after an unsuccessful first attempt, the endpoint 'successful semen cryopreservation' was based on the first attempt.

The electroejaculation procedure was commenced under general anaesthesia as previous described by inserting a transrectal probe in contact with the prostate and seminal vesicles [4,5,7]. The procedure was combined with other procedures for oncological treatment that needed to be performed under general anaesthesia, such as insertion of a central venous access line.

Serum hormone levels

During the diagnostic phase, before start of anticancer therapy, peripheral blood samples were obtained for analysis of serum hormone levels. Inhibin B levels were measured using kits purchased from Serotec Ltd (Oxford, United Kingdom). Within-assay and between-assay coefficients of variation (CV) were <9%, and <15%, respectively. Serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were determined with the Immulite assay (Diagnostic Products Corporation (DPC), Los Angeles, CA, United States of America). Reference values of LH, FSH, inhibin B and testosterone are: 1.5−8.0 U/L, 2.0−7.0 U/L, 150−400 ng/L and 10.0−30.0 nmol/L respectively [11]. Within-assay and between-assay CV were <6% and <9%, and <5% and 11% for FSH and LH, respectively. Serum testosterone levels were determined using coated tube radioimmunoassays (DPC). Intra-assay and inter-assay variation coefficients were 3% and 4.5%.

Statistics

Statistical analyses were performed using IBM SPSS Statistics 20.0. Data were presented as median, range or percentages. Mann Whitney U non-parametric tests were used to compare the characteristics of patients with and without successful cryopreservation or electroejaculation. A p-value of <0.05 was considered statistically significant.

Literature review

A literature search on electroejaculation was conducted in July 2013 using Embase, PubMed, Medline Ovid SP, Cochrane, Web of Science and Google Scholar. The following key-words and their synonyms were used: male, neoplasms, child, fertility, sperm, electric stimulation and cryopreservation. Studies were eligible for selection if electroejaculation cryopreservation was described, patients were aged between 10−19 years at diagnosis, and the manuscript was published in a peer-reviewed scientific

journal written in the English or Dutch language. After removing duplicates, the authors screened titles and abstracts to select eligible studies. Full text papers were obtained of the selected abstracts, and were excluded if studies did not meet the inclusion criteria. If not included initially, cross-references picked up during the review procedure were also selected. The complete search strategy is available on request.

Results

Between 1998 and 2013, semen cryopreservation was offered to 114 boys diagnosed with cancer. Cryopreservation attempts by masturbation were reported in 106 boys with cancer (93%) prior to treatment, of which 78 (68%) patients had samples that were adequate for cryopreservation, 18 (16%) patients had immotile spermatozoa, or absence of spermatozoa. Ten patients were not able to produce an ejaculate by masturbation. In the attempt to achieve adequate banking, 61 patients had one attempt, 50 patients two, and three patients three attempts. None of the ten patients with an unsuccessful first attempt were able to produce an adequate consecutive semen sample at a new attempt. In order to compare our results with previous studies, and because none of the patients appeared to produce an adequate semen sample after an unsuccessful first attempt, the endpoint 'successful semen cryopreservation' was based on the first attempt.

Electroejaculation was offered to a selection of 11 patients (10%). Of these, 8 patients were offered electroejaculation primarily because of motor disabilities or early puberty (≥G2P2, but small testicular volume and no nocturnal ejaculations); 3 patients were offered electroejaculation secondary because they initially failed to produce adequate semen by masturbation after at least two attempts. Patient characteristics are listed in Table I. Of the 8 patients who received electroejaculation primarily, 2 (25%) produced a semen sample adequate for cryopreservation. In the selected 3 cases with secondary electroejaculation, 1 case produced an adequate semen sample.

Patients with adequate sperm yield retrieved by masturbation or electroejaculation were significantly older at time of diagnosis compared to patients without adequate sperm yield (median 16.6 years (10.8−18.9) versus median 16.0 years (12.0-18.3), p=0.02).

Levels of FSH, LH, inhibin B and testosterone from patients with adequate sperm yield retrieved by masturbation did not significantly differ from those of patients with no adequate sperm yield.

Literature review

The literature search on electroejaculation in children with cancer identified 1,979 articles. After removing duplicates, 1,112 reports were screened for title and abstract. Eighteen abstracts met the inclusion criteria and were retrieved in full text for further assessment. Of the selected studies, a cross-reference of related articles, references and citing articles was performed; this yielded no further manuscripts for inclusion (Supplemental Figure I). Ultimately, the literature search revealed four manuscripts (Table II). Including our 11 cases, in total 29 cases were evaluable.

Table I | Characteristics of boys referred for cryopreservation of semen.

		Cohort	Conservative cryopreservation	Electroejaculation		
		n=114	n=106	n=11	Successful (n=3)	Unsuccessful (n=8)
Age		16.3 (10.8–18.9)	16.5 (10.8–18.9)	13.7 (12.0–16.0)	13.8 (12.6–16.0)	13.3 (12.0–16.0)
Tanner	**G**	4 (2–5)	4.5 (3–5)	3 (2–5)	3 (2–5)	3 (3–5)
	P	4 (2–5)	4.5 (3–5)	3 (2–5)	3 (2–5)	3 (2–5)
Testicular volume	**Left (ml)**	13.5 (6.0–20.0)	14.3 (8.0–20.0)	9.5 (6.0–15.0)	NA	9.5 (6.0–15.0)
	Right (ml)	12.8 (6.0–20.0)	13.5 (8.0–20.0)	9.5 (6.0–15.0)	NA	9.5 (6.0–15.0)
Semen analysis	**Volume (x10^6 ml)**	1.1 (0.0–4.5)	1.1 (0.0–4.5)	0.4 (0.02–3.0)	0.4 (0.4–0.4)	0.4 (0.02–3.0)
	Concentration (x10^6/ ml)	14.0 (0.0–323.0)	15.0 (0.0–323.0)	2.0 (0.1–14.5)	2.0 (0.1–5.5)	2.0 (0.1–14.5)
	Motility (%)	29.0 (0.0–69.0)	29.0 (0.0–69.0)	1.0 (0.0–4.0)	3.0 (2.0–4.0)	0
	pH	7.6 (6.4–8.0)	7.7 (7.1–8.0)	7.3 (6.4–8.0)	7.9	7.0 (6.4–8.0)

NA=not available; n=number. Numbers are depicted as median (range).

Table II | Case series and reports of EE in boys with cancer preceding treatment.

	Author and year	Study design	Age at EE	Diagnosis	Tanner stage	Testis V	Test (UI/l)	Semen analysis	Adequate yield
1.	Schmiegelow *et al* 1998 [5]	Case report	14	Relapse of pre-B ALL	G3P3	20-25 ml	NA	A: V 0.7 ml; M 1% R: C 1.6x10^6/ml; M 5%	+
2.	Müller *et al* 2000 [6]	Cohort study	13	NHL	>3	NA	NA	V 0.8ml; C 75x10^6/ml; M 38%	+
3.			14	ALL relapse	>3	NA	NA	V 3.2 ml; C 4.0x10^6/ml; M 10%	+
4.	Hovav *et al* 2001 [4]	Cohort study	15	Ewing sarcoma	NA	NA	NA	R: C 15x10^6/ml; M 6%	+
5.			15	Osteosarcoma	NA	NA	NA	R: C 24x10^6/ml; M 53%	+
6.			17	Osteogenic sarcoma	NA	NA	NA	A: C 0.65x10^6/ml; M 0% R: C 9x10^6/ml; M 0%	-
7.			18	TGCT	NA	NA	NA	A: C 35x10^6/ml; M 33%	+
8.	Hagenas *et al* 2010 [7]	Cohort study	12.7	HL	G5P4	8 ml	0.0	V 0.1 ml; C 0x10^6/ml; M 0%	-
9.			12.9	HL	P3	15 ml	8.0	V 1.3 ml; C 5.5x10^6/ml; M 15%	+
10.			13.8	Ewing sarcoma	G4	NA	4.1	V 0.1 ml; C 0x10^6/ml; M 0%	-
11.			13.9	Lymphoma	G3P4	8 ml	NA	V 0.1 ml; C 0x10^6/ml; M 0%	-
12.			14	Lymphoma	P5	20 ml	12.5	V 0.8 ml; C 99x10^6/ml; M 27%	+
13.			14.3	RMS	G4	15 ml	5.2	V 0.5 ml; C 0.3x10^6/ml; M 14%	+
14.			14.4	Osteosarcoma	P4	10 ml	13.1	V 6.1 ml; C 0.1x10^6/ml; M 0%	-
15.			14.5	Osteosarcoma	G3P3	10 ml	NA	V 0.03 ml; C 0x10^6/ml; M 0%	-
16.			15	HL	NA	NA	1.4	V 0.4 ml; C 0.24x10^6/ml; M 0%	-
17.			15.3	RMS	P5	15 ml	1.6	V 2.6 ml; C 0.8x10^6/ml; M 0%	-
18.			17.3	Testicular cancer	NA	8 ml	42.4	V 1.8 ml; C 0.1x10^6/ml; M 10%	+

Table II | *Continued*

	Author and year	Study design	Age at EE	Diagnosis	Tanner stage	Testis V	Test (UI/l)	Semen analysis	Adequate yield
19.	This Study	Retrospective cohort study	12.7	HL	G3P3	NA	3.1	V 0.4 ml; C 0x10^6/ml; M 0%	-
20.			13.0	HL	NA	6 ml	0.6	C 0x10^6/ml; M 0%	-
21.			14.6	HL	P2-3	10 ml	1.7	C 14.5x10^6/ml; M 0%	-
22.			15.9	HL	G3P3	9 ml	1.4	V 0.3 ml; C 0.1x10^6/ml; M 0%	-
23.			16.0	B-NHL	G5P5	NA	20.1	V 0.4 ml; C 2.0x10^6/ml; M 4%	+
24.			12.0	T-ALL	NA	NA	17.9	C 0x10^6/ml; M 0%	-
25.			13.7	B-NHL	NA	15 ml	3.4	V 1.9 ml; C 2.0x10^6/ml; M 0%	-
26.			12.5	Osteosarcoma	G3-4 P3-4	NA	7.4	V 0.4 ml; C 0.1x10^6/ ml	+
27.			15.0	ALL	G5P5	NA	6.0	V 0.02 ml; C 0x10^6/ml; M 0%	-
28.			13.8	HL	G2P2	NA	8.3	V 0.4 ml; C 5.5x10^6/ml; M 2%	+
29.			12.0	HL	NA	NA	0.4	V 3.0 ml; C 0x10^6/ml; M 0%	-
Total			14.0						**13/29**

Pt=patient number; EE=electroejaculation; A=antegrade ejaculation; R=retrograde ejaculation; V=volume; Test=testosterone; C=concentration; M=motility %; reprod hormones=reproductive hormones; ALL=acute lymphoblastic leukaemia; NHL=non-Hodgkin lymphoma; TGCT=testicular germ cell tumour; HL=Hodgkin lymphoma; RMS=rhabdomyosarcoma; NA=not available.

Median age at diagnosis of the boys that underwent electroejaculation was 14.0 years (12.0−18.0), median Tanner stage was 3 (3−5), and median testicular volume of cases with available information (n=13) was 10.0 ml (6.0−20.0). The 29 patients were diagnosed with leukaemia/non-Hodgkin lymphoma (5/9 successful), Hodgkin lymphoma (2/9 successful), sarcoma/PNET (4/9 successful) and testicular tumours (2/2 successful). In total, 13/29 (48%) of these selected patients produced semen samples fulfilling the criteria required for cryopreservation.

Hormone levels at time of cryopreservation were measured in 20/29 patients. Median FSH level was 2.1 UI/L (0.01−6.7), median LH level was 1.45 UI/L (0.01-4.9), median inhibin B level was 249 ng/L (56−320) and median testosterone level 4.7 nmol/L (0.01−42.4). Median semen volume was $0.4x10^6$ ml (0.02−6.1), median sperm concentration was $0.3x10^6$/ml (0-99.0) and median sperm motility was 0% (0-53). Electroejaculation was successful in 2/8 cases (25%) to whom it was offered primarily, and in 11/21 cases (52%) to those offered secondary (Table III).

No significant differences in age, Tanner stage and testicular volume between cases with and without adequate sperm yield were found (Table III). Also, hormone levels were similar in the two groups except for testosterone, which was significantly higher in patients with an adequate sperm yield by electroejaculation, i.e. respectively median 8.3 nmol/L (5.2−42.4) versus 1.7 nmol/L (0.01−17.9), p=0.006 (Figure I). FSH, LH and inhibin B levels were not correlated with the semen quality (Table III).

Table III | Characteristics of semen cryopreservation of patients who underwent electroejaculation (n=29).

	n	Successful yield (n=13) (range)	n	No successful yield (n=16) (range)	p
Age (years)	13	14.0 (12.5−18.0)	16	14.2 (12.0–17.0)	0.48
Tanner stage G	7	3 (2–5)	8	3.5 (3–5)	0.40
Tanner stage P	8	3 (2–5)	8	3.5 (2–5)	0.65
Testicular volume (ml)	4	15.0 (8.0–20.0)	9	10.0 (6.0–15.0)	0.20
FSH (UI/L)	7	2.1 (0.01–5.2)	12	1.9 (0.5–6.7)	0.83
LH (UI/L)	7	1.6 (0.01–4.9))	13	1.2 (0.5–4.1)	0.50
Inhibin B (ng/L)	7	277.0 (56.0–299.0)	13	194.0 (72.0–320.0)	0.75
Testosterone (nmol/L)	7	8.3 (5.2-42.4)	13	1.7 (0.01-17.9)	0.006

n=number; FSH=follicle-stimulating hormone; LH=luteinizing hormone. Numbers are depicted as median (range).
Mann-Whitney U test showed a significant difference for testosterone between the two groups. Semen cryopreservation was defined to be successful if motile spermatozoa were found and subsequently banked.

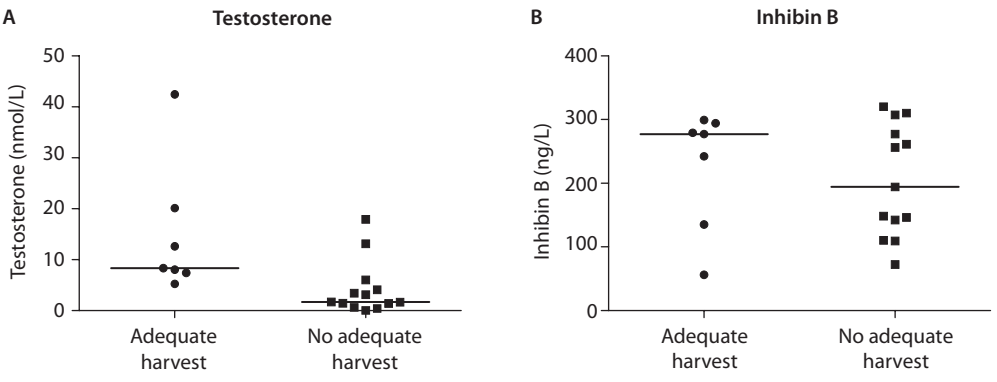


Figure 1 | Hormone levels of patients who underwent electroejaculation (n=29); A) testosterone levels, B) inhibin B levels. Lines indicate median values. Semen cryopreservation was defined to be successful if motile spermatozoa were found and subsequently banked.

Discussion

It has become good clinical practice to offer cryopreservation of semen in pubertal boys with cancer before starting gonadotoxic treatment to allow the possibility to father their genetically own child. Adolescent males with cancer have been reported to be good candidates for sperm banking [12-14]. Currently, according to literature, approximately 77% of boys aged 12−18 years with new or relapsed cancer is offered sperm banking before start of therapy [15], and 28-69% of these patients attempt to cryopreserve semen [15,16], of which approximately 65% is successful [15,17]. A success rate of 74% was found in our cohort.

Electroejaculation is an alternative technique to cryopreserve semen, but so far, information on the success of electroejaculation procedures in children is scarce. Summarizing all available information on reported cases, including the present study, 45% of the procedures resulted in a yield that was sufficient for banking. Although this represents a selected series, this illustrates that electroejaculation is a meaningful sperm harvest alternative for young boys who are not able to produce a semen sample by masturbation. It has to be emphasized, though, that only 1 out of 29 cases produced a sufficient semen sample according to the WHO criteria. Nevertheless, 13/29 patients had semen samples stored, as motile spermatozoa were present and, currently, potential pregnancies can be achieved in ICSI programs with only few functional spermatozoa [18,19]. It is not known whether such programs will ultimately be of help in childhood cancer survivors, since to our knowledge no data on pregnancy outcome of cryopreserved semen of childhood cancer patients are available. In survivors of adult males with cancer approximately 18−46% of ART cycles resulted in pregnancy [20-22] and 75% of the pregnancies resulted in a live birth [20,22]. A registry documenting sperm banking in childhood cancer patients, as well as follow-up data on the usage of cryopreserved sperm and pregnancy outcome of these patients would be of utmost value.

In the selection of children to offer electroejaculation, the efficacy, costs, as well as the medical and psychological burden of electroejaculation should carefully be weighted. For that reason, identifying predictive factors for a successful yield would be of great value. It is obvious that, based on current knowledge in this small series, evidence based guidelines cannot be provided. However, our study suggests that testosterone, but not inhibin B seems to be the most valuable predictor for a successful sperm yield retrieved by electroejaculation. In addition, Tanner stage and age may also be used as parameters for considering electroejaculation. We show that even in cases with early puberty (G2P2), electroejaculation may be feasible, and especially in these patients, testosterone may guide the decision. Additionally, the patients' emotional and sexual development should also play an important role in the decision to offer cryopreservation by electroejaculation.

Unfortunately, pre-pubertal boys with cancer are unable to produce an adequate ejaculate by masturbation or electroejaculation due to immature spermatogenesis. Testicular sperm extraction (TESE), which is the removal of testicular tissue by an incision in the testis in an attempt to retrieve spermatozoa, is used in pubertal boys [23,24], but is still no option for pre-pubertal boys. Retrieving spermatogonial stem cells by testis biopsies in order to preserve fertility in pre-pubertal boys needs to be further developed [24,25]. Issues such as tumour contamination, testis haemorrhage after biopsy, as well as the delay of starting treatment waiting for such procedures should be included in the further development of clinical practice guidelines by paediatric oncologists and fertility preservation experts.

We conclude that all pubertal boys with cancer should be offered semen cryopreservation before gonadotoxic therapy has started from Tanner stage G2P2 onwards. If masturbation fails, electroejaculation can be considered as a useful option for semen cryopreservation, and leads to adequate material for cryopreservation in about half of the cases.

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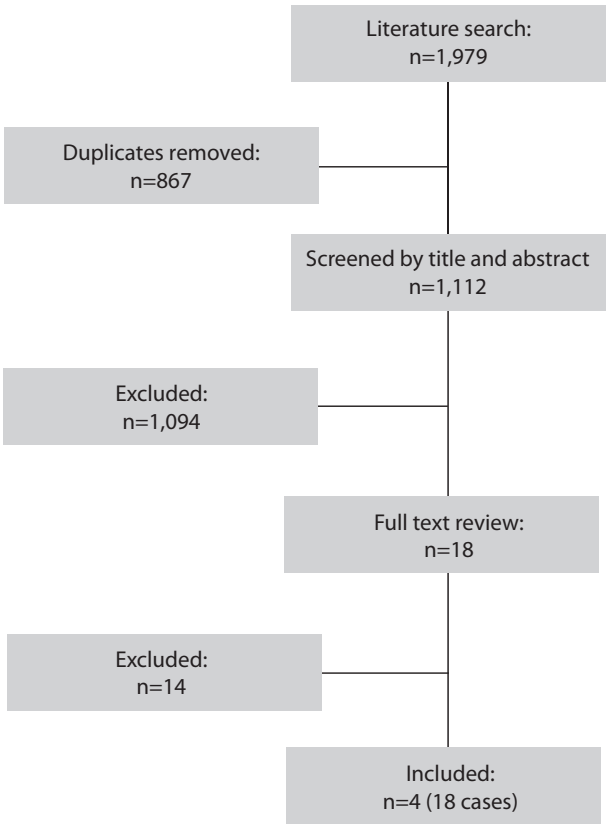

Supplementary Figure I | Flowchart of literature search.

CHAPTER 12

General discussion and perspectives

The increasing survival of childhood cancer over the last decades stresses the need for awareness for late treatment effects. Gonadal dysfunction is one of the important late effects. The impact of cancer treatment on gonadal function has extensively been studied [1-8]. However, the extent of loss of gonadal function differs between equally treated childhood cancer survivors. Information on determinants of ovarian function in childhood cancer survivors other than previous cancer treatment is largely lacking. Therefore, in this thesis we aimed to identify determinants of gonadal function and its potential recovery after childhood cancer, and, where possible, to evaluate alternative fertility options for survivors of childhood cancer.

12.1 | Gonadal function in female survivors of childhood cancer

Determinants of female gonadal function following childhood cancer treatment

Baseline gonadal function before treatment. Pre-treatment gonadal function does determine ovarian reserve after cancer treatment in survivors of adult cancer. In these adults, ovarian reserve was found to be impaired even before cancer treatment [9]. As reported in adults, anti-Müllerian hormone (AMH) levels were reduced in girls with newly diagnosed cancer, even before cancer treatment has started [10]. The cause of these reduced AMH levels is unclear. However, impaired ovarian function was also found in adults with type 2 diabetes mellitus and systemic lupus erythematous, suggesting that disease-related health status may affect AMH levels [10-12].

Whether an improved general health status is associated with increasing AMH levels is currently unknown. So far, ovarian reserve was mainly assessed after childhood cancer treatment. It is likely that baseline gonadal function affects post-treatment ovarian reserve. AMH is a reliable marker for the number of primordial follicles in the general population, and pre-treatment AMH has been described as a predictor of ovarian function after breast cancer treatment [13-19]. However, since an impaired health status seems to affect granulosa cell function as well, AMH is a marker of the actual ovarian function, indicative for a reduced primordial follicle pool, or a combination of these two in girls with newly diagnosed cancer. We are currently performing a longitudinal study of AMH levels in girls before and after cancer treatment in order to gain more insight in AMH as potential determinant of ovarian function after childhood cancer. This also has the potential to study the relationship between improving health status and AMH recovery.

Tumour infiltration of the gonads. The tumour localisation and subsequent treatment may also affect long-term gonadal function. The most striking examples are primary ovarian tumours, such as germ cell tumours, but also ovarian infiltration, which may occur in patients with for example non-Hodgkin lymphoma (NHL) [20]. The latter condition is very rare. Although chemotherapy alone cures NHL, over half of the NHL patients reported in literature were treated with upfront ovariectomy, because these tumours were thought to be primary ovarian tumours (10). Ovariectomy obviously leads to a reduced ovarian reserve, and might lead to premature menopause [20,21]. We thus recommend to avoid ovariectomy as treatment of ovarian infiltrated NHL. In girls with ovarian tumours with negative tumour markers (α-fetoprotein (AFP) and human chorionic gonadotropin (β-HCG)), tumour biopsies should be done to avoid unnecessary ovariectomy.

Moreover, the presence of malignant cells in ovarian tissue is of importance for pre-treatment cryopreservation because of the potential recurrence of cancer after re-implantation. The risk of ovarian metastasis depends on cancer type, as presented in Table I [22]. Both paediatric oncologists and gynaecologists should be aware of these risks when cryopreservation is offered to newly diagnosed patients.

Table I | Risk of ovarian metastasis according to cancer type.

High risk	Moderate risk	Low risk
Leukaemia	Breast cancer	Breast cancer
	Stage IV	Stage I-II
	Infiltrating lobular subtype	Infiltrating ductal subtype
Neuroblastoma	Colon cancer	Squamous cell carcinoma of the cervix
Burkitt lymphoma	Adenocarcinoma of the cervix	Hodgkin lymphoma
	Non-Burkitt non-Hodgkin lymphoma	Osteogenic carcinoma
	Ewing sarcoma	Non-genital rhabdomyosarcoma
		Wilms tumour

Modified from Dolmans *et al*, Fertil Steril, 2013.

Obesity. In the general population, obesity is associated with subfertility [23-26]. Similarly, we found that obesity is associated with decreased ovarian function in survivors of childhood cancer [27]. Because obesity and gonadal dysfunction are both major late effects of anticancer treatment, survivors and their health care providers should be aware of the potential relationship between obesity and gonadal function. The mechanism behind this association is not fully understood. It has been hypothesized that bilateral ovariectomy and subsequent oestradiol deficiency lead to obesity and insulin resistance, similar to observations in animal models [28,29]. However, adiposity and insulin resistance may also affect granulosa cell function [11,30,31]. We recommend longitudinal intervention studies addressing the impact of weight loss on gonadal function in female survivors of childhood cancer.

Genetic factors. In the general population, age at menopause is associated with specific single nucleotide polymorphisms (SNPs) mainly involved in DNA repair [32-35]. We showed that one of these SNPs, rs1172822, was also associated with impaired ovarian reserve and lower predicted age at menopause in adult female survivors of childhood cancer, even independent of previous cancer treatment [36]. In contrast, other candidate SNPs that were relevant in the general population were not associated with ovarian reserve in childhood cancer survivors. However, it should be noted that the power to detect the association with these SNPs was moderate to poor because of low minor allele frequencies requiring larger numbers of cases. Therefore, including larger numbers of cases in future studies is advised. Currently, national and international efforts are being developed to solve

methodological issues by building adequately powered study designs, and still addressing the issue of variation in ethnic backgrounds [37,38], but also bring together expertise of geneticists, paediatric oncologists, fertility experts, epidemiologists and health scientists. Current advances in genetic technologies including genome-wide association studies and whole-genome sequencing, provide opportunities to identify rare variants associated with a premature diminished ovarian reserve in female childhood cancer survivors. It is anticipated that these efforts will aid to the integration of genetic variation in future risk prediction of gonadal dysfunction after childhood cancer.

Female fertility after childhood cancer

Pregnancy outcome. Impaired fertility is a major issue in female survivors of childhood cancer, and occurs especially after treatment with high doses of alkylating agents and abdominal irradiation [39,40]. Female childhood cancer survivors have a ten-fold risk of developing premature menopause compared to their siblings (8% versus 0.8%) [41]. Still, despite equal efforts to offer fertility counselling and treatment, survivors were less likely than their siblings to receive fertility treatment [39]. This might be caused by either a provider concern about treating cancer survivors for infertility and potential medical comorbidities, or patients' reluctance against taking drugs after previous extensive treatment. Either way, it stresses the importance to gain and spread knowledge about fertility counselling and -treatment after childhood cancer. Literature on this topic, including pregnancy outcome and pregnancy complications of artificially conceived pregnancies is scarce. Recently, the number of oocytes retrieved by in vitro fertilisation (IVF) was found to be decreased in survivors of adult cancer treated with chemotherapy [42]. To our knowledge, data on IVF treatment and pregnancy outcome in adult survivors of childhood cancer is not available yet.

Currently, oocyte donation was suggested as a successful treatment option for infertility in patients with primary ovarian insufficiency, either idiopathic, genetic or after gonadotoxic cancer treatment. Oocyte donation in survivors of childhood cancer has been described in case reports only. All four reported cases had embryo transfers, after which three became pregnant, resulting in two healthy children and one mid-trimester loss of pregnancy [43,44]. We showed that women treated with oocyte donation, including 12 cancer survivors, had an increased risk for pregnancy induced hypertension [45], which is in line with previous studies [46-52]. Therefore, close monitoring of oocyte donation pregnancies is recommended. Even more awareness in childhood cancer survivors who previously received cancer treatment, in particular survivors treated with cardiotoxic therapy, might be needed. However, studies including larger numbers of childhood cancer survivors are needed to evaluate whether these survivors indeed are at increased risk of cardiovascular complications during pregnancy compared to other women treated with oocyte donation.

Fertility preservation. In order to preserve fertility in patients who will be treated with gonadotoxic cancer therapy, both gonadal suppression and cryopreservation methods have been used. Gonadotropin-releasing hormone-analogues (GnRH-a), suppressing LH and FSH production, were suggested to protect the ovaries [53-55]. However, to date, it is questionable whether GnRH treatment will work, since primordial follicles are not gonadotropin sensitive. Recently, the local administration of Sphingosine-1-phosphate, an apoptosis inhibitor, was found to reduce ovarian

damage during cytotoxic treatment in rodents, non-human primates, but also recently in human ovarian tissue [56,57]. Although promising, the functionality of the rescued human ovarian follicles needs to be evaluated, as well as the impact of the apoptosis inhibitor. Before puberty, there is no rationale for GnRH-a treatment because of the absence of activation of the hypothalamic-pituitary-ovarian axis.

Oocyte vitrification in adults and embryo cryopreservation in adults with a partner are potential options for gonadal preservation. However, in most cases the time span available to perform such procedures (i.e. IVF or intracytoplasmic sperm injection (ICSI)) is limited. Moreover, the exact number of oocytes that should be harvested to assure a pregnancy ending in a live birth is unknown but will be generally spoken higher than in one cycle might be harvested. Finally, in case of sex steroid sensitive tumours the increase in sex steroids as a result of the initiated IVF treatment is a major point of concern. Adjuvant therapeutic modalities are emerging but are not yet indicated for this specific use and might also constitute an extra risk factor. To summarize, although treatment options have been improved, several uncertainties remain, and requires further evaluation.

In pre-pubertal girls and adolescents, ovarian tissue cryopreservation is an option for gonadal preservation [58]. As mentioned earlier, the risk of re-implanting malignant cells must be considered for all malignant diseases, particular in case of systemic malignant diseases, such as leukaemia (Table I). In patients with leukaemia, metastases were repeatedly detected in ovarian tissue obtained for cryopreservation purposes, while no metastases were found in ovarian tissue from lymphoma patients [59-63]. However, so far, oncological recurrence has been described in two survivors, of cervical cancer and breast cancer, who had their ovarian tissue auto-transplanted. Though, the recurrence may not be related to the transplantation [60,64].

Ovarian cryopreservation, although still experimental, may be offered to pre-pubertal girls with non-ovarian, non-haematological cancer [65-69]. In vitro maturation of ovarian follicles to harvest mature oocytes eliminates the risk of cancer cell re-introduction, but this technique is still technically challenging and not very successful so far [70]. However, the field of fertility preservation is rapidly evolving, and, though based on experimental protocols, ovarian cryopreservation may be considered. To date, the ASCO guideline recommends that ovarian cryopreservation and transplantation procedures should only be performed in centres with the necessary expertise [71]. To provide success rates of cryopreservation, all procedures should be documented and analysed [65].

12.2 | Gonadal reserve in male survivors of childhood cancer

In men, semen quality is an important marker of fertility. Inhibin B, a product of the Sertoli cells, seems a reliable first screening marker for sperm quality after childhood cancer treatment [3,4,72]. While Sertoli cells are susceptible to damage by cytotoxic treatment, Leydig cells seem relatively resistant to this treatment, which explains previous studies reporting normal testosterone levels while sperm quality and inhibin B levels were low after treatment with alkylating agents [3,73].

Determinants of male gonadal function following childhood cancer treatment

In male survivors of childhood cancer, alkylating agents and testis irradiation are associated with impaired gonadal function [3,4,7,74]. Impaired pre-treatment spermatogenesis [75,76], testicular tumour infiltration [77] as well as genetic variation [78] have been described, though sparsely, as determinants of gonadal dysfunction after childhood cancer. In the general population, obesity determines testicular production of testosterone in a negative manner. It has been hypothesized that low testosterone levels are the result of high aromatase activity, expressed in white adipose tissue, that converts testosterone into oestradiol [79,80]. Consequently normal levels of LH and FSH are expected, because of the negative feedback of oestradiol on the pituitary gland [81]. We found, similar to female childhood cancer survivors, an association between obesity and gonadal dysfunction in male survivors of childhood cancer, independent of previous gonadotoxic cancer treatment [82]. Future longitudinal intervention studies should address the impact of weight loss on recovery of gonadal function.

Male fertility after childhood cancer

Fertility. As reported in female childhood cancer survivors, impaired fertility occurs particularly after treatment with high doses of alkylating agents or abdominal irradiation [3]. The Childhood Cancer Survivor Study showed that young male survivors were less likely to father a child after treatment with cyclophosphamide or procarbazine [7]. IVF and ICSI may be good alternatives available for survivors with reduced fertility.

Fertility preservation. In post-pubertal boys with cancer, semen cryopreservation is offered before the start of gonadotoxic treatment [83-85]. If semen retrieval by masturbation is unsuccessful, electroejaculation techniques should be considered [86]. This can be performed in combination with other procedures for which general anaesthesia is required. It is important to know, however, whether these semen samples are sufficient for IVF or ICSI. Recently, a small study showed no differences in pregnancy rates of IVF or ICSI between couples with and without a cancer history [87]. In survivors of adult cancer, half of couples achieved pregnancy and life birth using the cryopreserved semen samples [88].

If semen cryopreservation is unsuccessful, testicular sperm extraction (TESE) might be a reliable option [89,90]. TESE is a procedure to retrieve sperm from the testicular tissue of survivors with ejaculatory azoospermia who may have reduced but preserved fertility [91,92]. Studies on the feasibility of TESE in cancer patients have only been reported after chemotherapy. In these post-treatment azoospermic patients, viable sperm retrieval rates of 37% to 60% have been reported [91,92].

Semen cryopreservation by masturbation, electroejaculation or TESE is not feasible in pre-pubertal boys. In these boys, retrieval of spermatogonial stem cells by testis biopsy might be an option, but this procedure needs to be further developed before clinical implementation [90,93-95]. However, future research needs to ensure the elimination of reintroducing malignant cells. Patients diagnosed with non-solid tumours would be at high risk, since testis-infiltrated tumour cells may be present in the biopsy material [95] in contrast to solid tumours that rarely metastasize to the testes [96]. The

risk of testis haemorrhage after biopsy as well as the potential cancer treatment delay should also be included in the decision whether or not to perform cryopreservation.

Potential recovery of gonadal function. Knowledge on potential recovery of gonadal dysfunction and its determinants in survivors of childhood cancer is important for counselling survivors about their potential fertile life span. Recovery of spermatogenesis has been reported even 5–15 years after initial cancer treatment in adult Hodgkin's lymphoma survivors treated with procarbazine. Testicular function is better in survivors treated with ABVD compared to survivors treated with MOPP, as demonstrated by the return to normal fertility in the vast majority of survivors treated with ABVD [97-103]. We found that inhibin B levels, a serum marker of spermatogenesis, still increase in very long-term survivors of childhood cancer, but only in those survivors without critically low serum inhibin B levels at first screening [104]. Survivors with critically low levels are treated with high dose alkylating agents, testis irradiation or total body irradiation. This suggests that cancer treatment remains an important determinant of recovery. The mechanism of recovery after such a long follow-up time is not fully understood. Type A dark spermatogonia, which show no proliferative activity under normal conditions, and type A pale spermatogonia, which divide at 16-day interval [105], are less responsive to cytostatics than the rapidly destroyed type B spermatogonia because these other cells have little or no mitotic activity [106]. However, it remains important that threshold cumulative cytostatic doses are not exceeded. Previous studies showed that recovery occurs as long as 3–9 years after therapy [107,108], and our results suggest that even after a longer time period recovery may occur [104].

12.3 | Gonadal dysfunction surveillance after childhood cancer

As stated above, several determinants may impact a survivor's gonadal function and subsequent fertility after childhood cancer treatment. To endorse early detection of and intervention for late effects resulting from childhood cancer treatment, clinical practice guidelines for long-term follow-up of childhood cancer survivors have been developed in Europe and North America [109-112]. However, the definition of patient risk groups, and surveillance modalities and frequencies differ between these guidelines. This inconsistency leads to uncertainty about which guideline to follow. An international endeavour was initiated to harmonize guidelines for childhood cancer survivors [113]. This already resulted in the harmonized recommendations for breast cancer surveillance for female childhood, adolescent and young adult cancer survivors treated with chest irradiation [114]. Following that effort we enforced surveillance recommendations for primary ovarian insufficiency for female childhood cancer survivors by the international Harmonization Working Group. After reviewing all existing literature and guided by expert opinions, the following surveillance recommendations at late effect clinics were formulated:

Table II | Harmonized recommendations for screening female survivors of childhood cancer.

STRONG recommendations
Counselling regarding the risk of POI and its complications for future fertility is recommended for post-pubertal females treated with gonadotoxic chemotherapy and/or ovarian radiation
Monitoring of pubertal development for pre-pubertal females treated with gonadotoxic chemotherapy and/or ovarian radiation is recommended
Laboratory evaluation (FSH and oestradiol) and referral to paediatric endocrinology/gynaecology of pre-pubertal females who fail to initiate or progress through puberty is recommended
Laboratory evaluation (FSH and oestradiol) and referral to gynaecology/(reproductive) endocrinology is recommended for post-pubertal females who present with menstrual cycle dysfunction suggestive of POI
Referral to gynaecology/reproductive endocrinology and laboratory evaluation is recommended for post-pubertal females treated with gonadotoxic chemotherapy and/or ovarian radiation without signs and symptoms of primary ovarian insufficiency who desire assessment about potential for future fertility
Treatment with sex steroid replacement therapy is recommended in pre-pubertal and post-pubertal females diagnosed with POI
MODERATE recommendation
AMH level measurement may be reasonable for female survivors 25 years or older who present with menstrual cycle dysfunction suggestive of POI or who desire assessment about potential for future fertility

Similarly, several clinical practice guidelines for long-term follow-up of male childhood cancer survivors have been developed in Europe and North America [109-112]. As they differ in their recommendations, a harmonized guideline for the surveillance of gonadal dysfunction in male childhood cancer survivors is currently in development.

12.4 | General perspectives

Collaboration and centralisation of care and research is necessary to further improve cure rates for children with cancer while on one hand reducing the side effects of therapy and on the other hand to optimize the treatment of for instance iatrogenic infertility. So far, childhood cancer care was provided in five paediatric oncology centres and two transplantation centres in the Netherlands. These centres will be brought together in one national paediatric oncology centre, which is located in the centre of the Netherlands. In order to centralise research, a large nationwide study is currently being performed in 7,000 long-term survivors in the Netherlands, the so-called Dutch Childhood Oncology Group (DCOG) – LATER Q2008. In this study, the prevalence and risk factors of long-term treatment-related complications will be assessed. As a start, the DCOG LATER-VEVO study, initiated by the VUMc, is ongoing. This study will provide valuable information on the reproductive potential long-term survivors of childhood cancer [115]. These efforts are supported by several national grants. Likewise, PanCareLife is an EU Framework 7 Programme in the Health Theme that studies the impact of drug treatment regimens on childhood cancer patient outcomes. This project will focus on fertility in survivors of childhood cancer, including the influence of genetic variation on fertility

outcome (*www.pancarelife.eu*). These large scale studies are expected to increase the knowledge on determinants of gonadal function after childhood cancer as partly described in this thesis.

By centralising care, specialised survivorship care can be commenced by a variety of expert providers with different backgrounds including subspecialists as fertility experts, cardiologists and endocrinologists, nurse practitioners and oncologists, committed to understand late effects and up to date on recommendations regarding screening and education. This specialised model of care gives instant credibility to survivorship care to the patients and their families [116], and provides proper fertility counselling to families with a newly diagnosed child.

12.5 | Conclusions

This thesis presents determinants, apart from cancer treatment, contributing to gonadal dysfunction in childhood cancer survivors. A thorough knowledge on these clinical and genetic factors should lead to improved understanding of variation in gonadal function after childhood cancer. In the near future, our findings may be used to design a model for predicting gonadal dysfunction after childhood cancer. This enables health care professionals to provide personally tailored counselling to patients and their parents, before and after childhood cancer treatment.

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CHAPTER 13

Summary
Samenvatting

13.1 | Summary

Each year, 600–700 children are diagnosed with cancer in the Netherlands. Over the last decades, the overall long-term survival of childhood cancer has increased up to ~75%. Due to the expansion of the childhood cancer survivor population, chronic health sequelae resulting from cancer and its treatment have become more prevalent and require our attention. Approximately 75% of the childhood cancer survivors have developed at least one long-term complication of cancer treatment. One-third of these effects has even been classified as severe or life threatening. **Chapter 1** provides the background of childhood cancer and its survival, some examples of late effects of cancer treatment including gonadal dysfunction, physiological gonadal function and reserve throughout life. Moreover, it described the background of gonadal dysfunction after childhood cancer. Previous studies in female and male survivors obviously showed that the occurrence of late effects of cancer treatment in survivors depends on former cancer diagnosis, but more importantly on former treatment. In **chapter 2**, we describe the available literature on endocrine late sequelae after childhood Hodgkin's lymphoma as an example of a childhood cancer type that reached high survival rates and is treated with chemotherapy, radiotherapy or a combination of both. In general, the severity of the endocrine toxicity after childhood Hodgkin lymphoma mainly depends on the type of treatment in both male and female survivors, but other determinants, such as genetic variation and lifestyle, probably explain variation in gonadal damage after equal cancer treatment.

As described above, variation in long-term gonadal function of childhood cancer survivors who received equal treatment regimens suggests that factors other than cancer treatment impact gonadal function after cancer treatment. In order to improve knowledge and understanding of variation in gonadal function after childhood cancer treatment, we studied potential factors other than cancer treatment that may influence gonadal function after childhood cancer. In **chapter 3**, pre-treatment ovarian function was studied in girls with newly diagnosed cancer and compared with that in healthy girls. We found that anti-Müllerian hormone (AMH) levels were already reduced before treatment has started. These levels were associated with general health status markers, i.e. temperature, haemoglobin levels and C-reactive protein levels at diagnosis, suggesting that the disease itself or subsequent health status may impact gonadal function.

Ovarian infiltration in girls with non-Hodgkin lymphoma might be important for their ovarian function after childhood cancer. In **chapter 4**, we summarised characteristics and outcome of this rare presentation, and performed a narrative review of all cases reported in literature so far. Albeit treatment should preferably consist of chemotherapy, our literature study showed that in many previously reported cases, (bilateral) ovariectomy was performed as first treatment option, obviously resulting in a decreased ovarian function after treatment. We emphasize that, in case of ovarian tumours with negative tumour markers (alpha-fetoprotein (AFP) and β-human chorionic gonadotropin (β-HCG)), the diagnosis of NHL, and subsequent biopsy, should be considered in order to avoid unnecessary surgery.

Obesity and gonadal dysfunction are both major late effects of childhood cancer treatment. In **chapter 5**, we studied the occurrence of decreased ovarian reserve in obese female survivors of childhood cancer. As previously described in the general population, we observed that obesity and

insulin resistance were associated with impaired ovarian reserve, as reflected by AMH and reduced follicle counts, independent of previous administered gonadotoxic treatment regimens.

Besides clinical determinants, genetic variants may also influence gonadal function after childhood cancer. In the general population, several single nucleotide polymorphisms (SNPs) were associated with age at menopause. In **chapter 6**, we questioned whether the genetic impact on gonadal function in childhood cancer survivors may be exceeded by gonadotoxic treatment regimens. Interestingly, we found an association between the CT genotype of rs117822 (*BRSK1*) and impaired ovarian reserve in female childhood cancer survivors, independent of previous gonadotoxic cancer treatment.

Since childhood cancer survivor subsets have an increased risk of developing primary ovarian insufficiency (POI), clinical practice guidelines are necessary to warrant that survivors receive optimum care, and thereby improve optimal treatment of late effects of cancer treatment. Accordingly, several nationwide guidelines have been developed, but recommendations vary among the existing guidelines, resulting in uncertainty for both survivors and providers which guideline to follow. Recognizing the importance of a global consensus, an international effort was organized to harmonize existing late effects screening recommendations for survivors of childhood cancer. One of the efforts is the development of recommendations for POI surveillance in childhood cancer survivors, which are described in **chapter 7**. Next to consensus on recommendations, an important result is the demonstration of a research agenda, based upon the identification of key gaps in knowledge.

Oocyte donation has been used as successful last resort treatment option for infertility. One of the most prevalent indications is POI. **Chapter 8** describes treatment- and pregnancy outcome of oocyte donation in patients with POI. We found that younger donor age and acceptors with previous pregnancies were associated with increased pregnancy rates. Oocyte pregnancies are associated with increased risk of pregnancy-induced hypertension compared to IVF pregnancies, stressing the importance of monitoring oocyte donation pregnancies.

As in the female general population, obesity is also associated with decreased fertility in men. **Chapter 9** describes a retrospective study in male survivors of childhood cancer on the association between obesity and gonadal dysfunction. We found an association between obesity, as represented by high total fat percentage, and impaired gonadal function, independent of previous cancer treatment.

Chapter 10 describes a longitudinal study on potential recovery of gonadal function in male survivors of childhood cancer, using paired inhibin B levels. Several potential determinants of recovery were studied within this chapter. We showed that in general, inhibin B levels increase over time in most survivors, suggesting that recovery of gonadal function may occur even long time after discontinuation of treatment, apart from cases in which critically low serum inhibin B levels are already reached. The latter includes survivors treated with high risk gonadotoxic treatment regimens such as alkylating agents and abdominal irradiation.

In **chapter 11**, the feasibility of electroejaculation in boys with newly diagnosed cancer is described. Because one of the important long-term effects of childhood cancer treatment is infertility, especially after treatment with alkylating agents and testis radiation, fertility

preservation is important to give these boys the chance to father their own offspring. Although semen cryopreservation by masturbation is easy and non-invasive, in particular young patients are frequently unable to produce sperm by masturbation. In these cases, electroejaculation might be an option to obtain motile spermatozoa. Combining literature review and our electroejaculation cases, we concluded that if masturbation fails, electroejaculation can be considered as a useful option for semen cryopreservation, and leads to semen cryopreservation in about half of the cases.

Chapter 12 provides a general discussion on the combined results of the studies in a broader perspective. We recommend future research and elaborate on replications of our findings. Thereafter, the identified determinants should be integrated into prediction models for gonadal function after childhood cancer treatment.

13.2 | Samenvatting

Jaarlijks worden in Nederland 600-700 kinderen gediagnosticeerd met kanker. De overleving van kinderkanker is gedurende de afgelopen decennia gestegen tot ~75%. Door de groeiende populatie van overlevenden zijn de chronische late effecten van kinderkanker en de behandeling ervan zichtbaar geworden. Hierdoor is aandacht voor en onderzoek naar deze late effecten belangrijk geworden. Ongeveer 75% van de overlevenden van kinderkanker ontwikkelt minimaal één laat effect van de vroegere kankerbehandeling. Een derde van deze effecten is ernstig of zelfs levensbedreigend. In **hoofdstuk 1** wordt een overzicht gegeven van de incidentie van kinderkanker, de overleving van kinderkanker en de late effecten van de behandeling, waaronder gonadale dysfunctie. Aangezien de focus van dit proefschrift ligt op gonadale dysfunctie na kinderkanker, wordt een overzicht gegeven van de fysiologie van gonadale functie en reserve gedurende het leven, en gonadale dysfunctie als laat effect van de behandeling van kinderkanker. Eerdere studies bij zowel vrouwelijke als mannelijke overlevenden van kinderkanker lieten duidelijk zien dat het voorkomen van late effecten afhankelijk is van de diagnose, maar nog meer van de behandeling. In **hoofdstuk 2** geven we een overzicht van de literatuur over endocriene late effecten van de behandeling van het Hodgkin lymfoom als voorbeeld van een kinderkanker type dat een goede overleving kent en behandeld wordt met chemotherapie, radiotherapie of een combinatie van beiden. In het algemeen hangt de ernst van de endocriene late effecten na Hodgkin lymfoom behandeling af van het type behandeling. Echter, andere determinanten, zoals genetische variatie en leefstijlfactoren, verklaren mogelijk de variatie in gonadale schade bij patiënten die een gelijke behandeling gehad hebben.

Zoals hierboven beschreven, suggereert de variatie in gonadale dysfunctie na gelijke behandeling van kanker dat andere factoren, naast de behandeling zelf, invloed hebben op gonadale functie na kinderkanker. Om meer kennis over deze variatie te verkrijgen, hebben wij andere potentiële determinanten van gonadale functie bestudeerd. **Hoofdstuk 3** beschrijft de studie naar ovariële functie voorafgaand aan de behandeling van kinderkanker in vergelijking met deze functie bij gezonde meisjes. Anti-Müllers hormoon (AMH) bleek al voor de start van de kankerbehandeling verlaagd. Daarnaast bleek het AMH geassocieerd te zijn met surrogaat markers voor de algemene gezondheidsstatus, zoals temperatuur, hemoglobine en CRP waarden bij diagnose. Dit laatste suggereert dat de ziekte zelf of de algemene gezondheidsstatus als gevolg van de ziekte van invloed zijn op de gonadale functie bij meisjes die gediagnosticeerd worden met kanker, al voor de therapie gestart is.

Ovariële infiltratie bij meisjes met non-Hodgkin lymfoom (NHL) lijkt van belang voor de ovariële functie na therapie. In **hoofdstuk 4** hebben we de karakteristieken en uitkomsten van ovariële infiltratie bij NHL, een zeer zeldzame presentatie, beschreven. Tevens vatten we in dit hoofdstuk de literatuur samen. Ondanks dat NHL in het algemeen behandeld kan worden met chemotherapie laat onze literatuurstudie zien dat (bilaterale) ovariëctomie in veel gevallen de eerste behandelkeuze is. Deze ingreep resulteert vanzelfsprekend in een verminderde ovariële reserve na therapie. Wij benadrukken dat, in geval van ovariële tumoren met negatieve tumormarkers (AFP en β-HCG), NHL

en de daarbij behorende diagnostische biopsie overwogen moeten worden om onnodige chirurgie te voorkomen, en op deze wijze de ovariële reserve te beschermen.

Overgewicht en gonadale dysfunctie zijn beiden belangrijke late effecten van de behandeling van kinderkanker. In **hoofdstuk 5** wordt het vóórkomen van een verminderde ovariële reserve bij obese vrouwelijke overlevenden van kinderkanker beschreven. Zoals al eerder beschreven in de algemene bevolking vonden we dat obesitas en insulineresistentie geassocieerd zijn met een verminderde ovariële reserve, gebaseerd op AMH waarden en het totale aantal follikels in de ovaria. Tevens bleek dat deze invloed van obesitas op de gonadale functie onafhankelijk was van de eerdere kankerbehandeling.

Naast klinische determinanten zou genetische variatie de gonadale functie na kinderkanker kunnen beïnvloeden. In de algemene bevolking zijn verschillende 'single nucleotide polymorphisms' (SNPs) geassocieerd met de leeftijd waarop een vrouw in de menopauze komt. **Hoofdstuk 6** beschrijft de invloed van genetische variatie op de gonadale functie bij overlevenden van kinderkanker, en of deze invloed overschreden wordt door het gonadotoxische effect van de eerdere kankerbehandeling. We vonden een associatie tussen het CT genotype van rs117822 (*BRSK1* gen) en een verminderde ovariële reserve in vrouwelijke overlevenden van kinderkanker, onafhankelijk van voorgaande kankerbehandeling. Dit suggereert dat genetische variatie, naast de gonadotoxische behandeling van kinderkanker, een rol speelt bij de ernst van de gonadale dysfunctie na kinderkanker.

Aangezien een deel van de overlevenden van kinderkanker een verhoogde kans heeft op het ontwikkelen van primaire ovariële insufficiëntie (POI), zijn klinische richtlijnen van belang om ervoor te zorgen dat overlevenden optimale zorg en daarbij optimale behandeling van eventuele late effecten van kankerbehandeling krijgen. Als gevolg hiervan zijn verschillende internationale richtlijnen ontwikkeld, echter blijkt dat deze richtlijnen met bijbehorende aanbevelingen niet overeen komen, waardoor onzekerheid kan ontstaan welke richtlijn gevolgd dient te worden. Om internationaal een éénduidig beleid te kunnen vormen, is een internationale werkgroep opgericht. Eén van de projecten omvat het opstellen van een richtlijn voor het screenen van overlevenden van kinderkanker op POI, zoals beschreven in **hoofdstuk 7.**

Een deel van de overlevenden van kinderkanker blijkt onvruchtbaar als gevolg van de gonadotoxische behandeling. Eiceldonatie is een belangrijke en succesvolle behandeling van infertiliteit. Eén van de meest voorkomende indicaties is POI. **Hoofdstuk 8** beschrijft de behandel- en zwangerschapsuitkomsten van eiceldonatie bij vrouwen met POI. We vonden dat een jongere leeftijd van de donor en een eerdere zwangerschap van de acceptor geassocieerd waren met een hoger zwangerschapscijfer. Daarnaast zijn eiceldonatiezwangerschappen, in vergelijking met IVF zwangerschappen, geassocieerd met een verhoogde kans op zwangerschap-geassocieerde hypertensie, waardoor het belang van goede monitoring van deze eiceldonatiezwangerschappen benadrukt wordt.

Obesitas is, zoals bij vrouwen, ook bij mannen geassocieerd met een verminderde vruchtbaarheid in de algemene bevolking. **Hoofdstuk 9** beschrijft onze retrospectieve studie naar de associatie tussen obesitas en gonadale functie van mannelijke overlevenden van kinderkanker. We vonden dat obesitas (een hoog totaal vet percentage) geassocieerd was met een verminderde gonadale functie, onafhankelijk van eerdere kankerbehandeling.

Hoofdstuk 10 beschrijft de longitudinale studie naar potentieel herstel van gonadale dysfunctie bij mannelijke overlevenden van kinderkanker, gebruikmakend van inhibine B waarden. Verschillende potentiële determinanten van herstel werden onderzocht in dit hoofdstuk. We vonden dat, in het algemeen, inhibine B waarden gedurende follow-up in de meeste overlevenden stegen, met uitzondering van overlevenden met al kritisch lage inhibine B waarden. Deze laatste groep omvatte overlevenden behandeld met hoog risico gonadotoxische behandeling, zoals alkylerende middelen en abdominale radiotherapie.

In **hoofdstuk 11** wordt de toepasbaarheid van elektro-ejaculatie bij jongens met kinderkanker beschreven. Aangezien infertiliteit één van de belangrijke late effecten is van de behandeling van kinderkanker, vooral na behandeling met alkylerende middelen en/ of bestraling van de testes, is cryopreservatie van semen belangrijk om jongens na de behandeling een kans te geven op het krijgen van een genetisch eigen kind. Alhoewel cryopreservatie van semen via masturbatie gemakkelijk en niet-invasief is, blijkt het, vooral bij jonge patiënten, soms niet succesvol. In deze gevallen zou elektro-ejaculatie een mogelijkheid kunnen zijn om semen te verkrijgen voor cryopreservatie. Na het combineren van de bestaande literatuur en alle casus uit ons eigen cohort, vonden we dat, als de productie van semen via masturbatie niet lukt of niet mogelijk is, elektro-ejaculatie een goed alternatief is voor mid-pubertaire en post-pubertaire patiënten om semen voor cryopreservatie te verkrijgen.

Hoofdstuk 12 bevat een algemene discussie over de resultaten beschreven in dit proefschrift, waarbij deze resultaten in een breder perspectief geplaatst worden. We doen suggesties voor het opzetten van vervolgstudies, welke uiteindelijk kunnen leiden tot een predictiemodel waarin alle geïdentificeerde determinanten van gonadale dysfunctie samengevoegd kunnen worden. Dit model kan gebruikt worden om gonadale functie na de behandeling van kinderkanker beter te kunnen voorspellen en hierdoor patiënten gedetailleerder te kunnen counselen.

ADDENDUM

Authors and affiliations
List of abbreviations
Bibliography
PhD portfolio
About the author
Dankwoord

Authors and affiliations

M.C. Adank	Department of Paediatric Oncology/ Haematology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands
R.D. van Beek	Department of Paediatric Oncology/ Haematology, and Department of Paediatric Endocrinology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands
A. Beishuizen	Department of Paediatric Oncology/ Haematology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands
M.H. van den Berg	Division of Paediatric Oncology/ Haematology, Department of Paediatrics, VU University Medical Center, Amsterdam, the Netherlands
K. Blijdorp	Department of Paediatric Oncology/ Haematology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands Division of Endocrinology, Department of Internal Medicine, Erasmus MC University Medical Centre, Rotterdam, the Netherlands
N.J. van Casteren	Division of Andrology, Department of Urology, Erasmus MC University Medical Centre, Rotterdam, the Netherlands
E. van Dulmen-den Broeder	Division of Paediatric Oncology/ Haematology, Department of Paediatrics, VU University Medical Center, Amsterdam, the Netherlands
M.M. van den Heuvel-Eibrink	Department of Paediatric Oncology/ Haematology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands
I.M.M. van der Geest	Department of Paediatric Oncology/ Haematology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands
R. Haupt	Department of Epidemiology and Biostatistics Unit, Istituto G. Gaslini, Genova, Italy
W.C.J. Hop	Department of Biostatistics, Erasmus MC University Medical Centre, Rotterdam, the Netherlands
M.M. Hudson	Department of Oncology and Epidemiology and Cancer Control, St. Jude Children's Research Hospital, Memphis, United States
C.W.P.M. Hukkelhoven	The Netherlands Perinatal Registry, Utrecht, the Netherlands
N. di Iorgi	Department of Paediatrics, Istituto G. Gaslini, Genova, Italy
F.H. de Jong	Department of Internal Medicine, Erasmus MC University Medical Centre, Rotterdam, the Netherlands
L.C. Kremer	Department of Paediatric Oncology, Emma Children's Hospital/ Academic Medical Center, Amsterdam, the Netherlands

J.S.E. Laven	Division of Reproductive Medicine, Department of Obstetrics and Gynaecology, Erasmus MC University Medical Centre, Rotterdam, the Netherlands
A.J. van der Lely	Division of Endocrinology, Department of Internal Medicine, Erasmus MC University Medical Centre, Rotterdam, the Netherlands
J.L. Levine	Division of Paediatric Oncology/ Blood and Marrow Transplantation, Columbia University Medical Center, Amsterdam, the Netherlands
S.M.P.F. de Muinck Keizer-Schrama	Department of Paediatric Endocrinology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands
R.L. Mulder	Department of Paediatric Oncology, Emma Children's Hospital/ Academic Medical Center, Amsterdam, the Netherlands
S.J.C.M.M. Neggers	Division of Endocrinology, Department of Internal Medicine, Erasmus MC University Medical Centre, Rotterdam, the Netherlands
C. Owusuaa	Department of Paediatric Oncology/ Haematology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands
R. Pieters	Department of Paediatric Oncology/ Haematology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands
S.M.F. Pluijm	Department of Paediatric Oncology/ Haematology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands
J.M. Rietveld	Department of Obstetrics/ Gynaecology, Erasmus MC-University Medical Centre, Rotterdam, the Netherlands
I. Schipper	Division of Reproductive Medicine, Department of Obstetrics and Gynaecology, Erasmus MC University Medical Centre, Rotterdam, the Netherlands
M. Smit	Division of Andrology, Department of Urology, Erasmus MC University Medical Centre, Rotterdam, the Netherlands
L. Stolk	Department of Internal Medicine, Erasmus MC University Medical Centre, Rotterdam, the Netherlands
A.G. Uitterlinden	Department of Internal Medicine, Erasmus MC University Medical Centre, Rotterdam, the Netherlands
J.A. Visser	Department of Internal Medicine, Erasmus MC University Medical Centre, Rotterdam, the Netherlands
A.C.H. de Vries	Department of Paediatric Oncology/ Haematology Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands
W.H. Wallace	Department of Paediatric Oncology, Royal Hospital for Sick Children, Edinburgh, United Kingdom

List of abbreviations

AAD score	alkylating agent dose score
ABP	androgen binding protein
ABVD	adriamycin, bleomycin, vinblastine, dacarbazine
AFC	antral follicle count
ALCL	anaplastic large cell lymphoma
AFP	alpha-fetoprotein
AHA	American Heart Association
ALL	acute lymphoblastic leukaemia
AMH	anti-Müllerian hormone
ARHGEF7	rho guanine nucleotide exchange factor 7 gene
ART	assisted reproductive technology
ASCO	American Society of Clinical Oncology
AML	acute myeloid leukaemia
Azo	azoospermia
b-HCG	beta-human chorionic gonadotropin
B-NHL	B-cell non-Hodgkin lymphoma
BiOv	bilateral ovariectomy
BL	Burkitt lymphoma
BM	bone marrow
BMD	bone mineral density
BMI	body mass index
B-NHL	B-cell non-Hodgkin lymphoma
BRSK1	BR serine/ threonine kinase 1 gene
CCR	complete clinical remission
CCS	childhood cancer survivors
ChlVPP	chlorambucil, vinblastine, procarbazine, prednisolone
Chr	chromosome
CNS	central nervous system
COG	North American Children's Oncology Group
COMP	COPP, methotrexate replaces procarbazine
COP	cyclophosphamide, vincristine, procarbazine
COPP	cyclophosphamide, vincristine, procarbazine, prednisolone
CRP	C-reactive protein
CS	caesarean section
CT	chemotherapy
CV	coefficients of variation
DCOG	Dutch Childhood Oncology Group
DLBCL	diffuse large B-cell lymphoma
DXA	dual-energy x-ray absorptiometry

E2	oestradiol
EBVD	epirubicin, bleomycin, vinblastine, dacarbazine
EE	electroejaculation
EFS	event free survival
EM	early menopause
ET	embryo transfer
FC	total follicle count
FSH	follicle-stimulating hormone
FT4	free thyroxine
GnRH(-a)	gonadotropin-releasing hormone(-analogues)
GRADE	Grading of Recommendations Assessment Development and Evaluation
GWA(S)	genome-wide association (study)
Hb	haemoglobin
HOMA	homeostasis model assessment
HL	Hodgkin's lymphoma
HLH	Haemophagocytic lymphohystiocytosis
HRQoL	Health Related Quality of Life
HRT	hormone replacement therapy
HWE	Hardy-Weinberg equilibrium
ICS	intracytoplasmic sperm injection
IGF2R	insulin-like growth factor 2 receptor gene
Inh B	inhibin B
IRB	Institutional Review Board
IVF	in vitro fertilisation
LH	luteinizing hormone
MAF	minor allele frequency
MCM8	minichromosome maintenance complex component 8 gene
MDP	doxorubicin, procarbazine, prednisone, vincristine, cyclophosphamide
MOPP	mechlorethamine, vincristine, procarbazine, prednisone
NGF	non-growing follicle
NHL	non-Hodgkin lymphoma
OCP	oral contraceptive pill
OD	oocyte donation
OEPA	vincristine, etoposide, prednisolone, doxyrubicin
Oligo	oligospermia
PAVe	procarbazine, alkeran, vinblastine
PCOM	polycystic ovarian morphology
PCOS	polycystic ovary syndrome
PCSK1	proprotein convertase subtilisin/kexin type 1 gene
PIH	pregnancy-induced hypertension
PNET	Primitive Neuroectodermal Tumour

POI	primary ovarian insufficiency
POF	premature ovarian failure
PR	pregnancy rate
PRN	The Netherlands Perinatal Registry
prog	progesterone
RT	radiotherapy
SCT	stem cell transplantation
SDS	standard deviation score
SHBG	sex hormone-binding globulin
SIGN	Scottish Intercollegiate Guidelines Network
SIOP	International Society of Paediatric Oncology
SMN	second malignant neoplasm
SNP	single nucleotide polymorphism
SNS	sympathetic nervous system
SRD5A1	steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase 1 gene)
T-LBL	T-cell lymphoblastic lymphoma
T-NHL	T-cell non-Hodgkin lymphoma
T2DM	type 2 diabetes mellitus
TBI	total body irradiation
TCD	total cumulative dose
TESE	testicular sperm extraction
TLI	total lymphoid irradiation
TNF	tumour necrosis factor gene
TSH	Thyroid-stimulating hormone
TTF	total fertilisation failure
UKCCLG	United Kingdom Children's Cancer and Leukaemia Group
US	United States
VMB	vinblastine, bleomycin, methotrexate
WHR	waist-hip ratio

List of manuscripts related to his thesis

Chapter 2
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DCOG LATER-VEVO Study Group (as member):

Van den Berg MH, van Dulmen-den Broeder E, Overbeek A, Kremer LC, van den Heuvel-Eibrink MM, Tissing WJE, Loonen JJ, **van Dorp W**, Versluys AB, Bresters D, Lambalk CB, Kaspers GJL, van Leeuwen FE. Selecting appropriate comparison groups for late effects studies among survivors of childhood cancer: experiences from a Dutch cohort study on female fertility. *Submitted.*

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PhD portfolio

Summary of PhD training and teaching activities

Name PhD student:	Wendy van Dorp
Erasmus MC Department:	Obstetrics and Gynaecology, Division of Reproductive Medicine
	Paediatric Oncology and Haematology
Research schools:	NIHES
	MolMed
PhD period:	Feb 2010 – Apr 2014
Promotors:	prof.dr. J.S.E. Laven and prof.dr. R. Pieters
Supervisor:	dr. M.M. van den Heuvel-Eibrink

	Year	Workload (Hours/ ECTS)
1. PhD TRAINING		
General courses:		
– Methodologie van Patiëntgebonden Onderzoek en Voorbereiding van Subsidieaanvragen	2010	0.3 ECTS
– Biomedical English Writing and Communication	2011	4.0 ECTS
Research skills:		
– Introduction to Data-analysis (Summer Course)	2010	1.0 ECTS
– Endnote Course	2010	0.1 ECTS
– The SNP Course VII	2010	2.0 ECTS
– The Photoshop and Illustrator Workshop for PhD-students	2011	0.3 ECTS
– Biostatistical Methods I: Basic Principles	2012	5.7 ECTS
(Inter)National Conferences – participation and presentations:		
– 11th International Conference on Long-Term Complications of Treatment of Children and Adolescents for Cancer, Williamsburg, Virginia (poster presentation)	2010	1.0 ECTS
– Annual Research meeting of the Department of Obstetrics and Gynaecology (oral presentation)	2011	1.0 ECTS
– 37th Annual Meeting of the European Group for Blood and Marrow Transplantation, Paris, France (poster presentation)	2011	1.0 ECTS
– European Symposium on Late Complications after Childhood Cancer (poster presentation)	2011	1.0 ECTS

– 67th Annual Meeting of the American Society for Reproductive Medicine (poster presentation)	2011	1.0 ECTS
– 59th Annual Meeting of the Society for Gynecologic Investigation, San Diego, USA (poster presentation)	2012	1.0 ECTS
– Wladimiroff Symposium, Erasmus MC (oral presentation)	2012	1.0 ECTS
– Gynaecongres, Arnhem (oral presentation)	2012	1.0 ECTS
– 28th Annual Meeting of the European Society of Human Reproduction And Embryology (oral presentation)	2012	1.0 ECTS
– 12th International Conference on Long-Term Complications of Treatment of Children and Adolescents for Cancer, Williamsburg, USA (poster presentation)	2012	1.0 ECTS
– 44th Congress of the International Society of Paediatric Oncology, London, England (poster presentation, poster award)	2012	1.0 ECTS
– 11th PanCare Meeting, Genova, Italy (coordinating meeting of the International Female Gonadal Dysfunction Harmonisation Work Group)	2013	1.0 ECTS
– 13th International Conference on Long-Term Complications of Treatment of Children and Adolescents for Cancer, Memphis, USA (oral and poster presentations)	2013	1.0 ECTS
– 95th Annual Meeting of The Endocrine Society, San Francisco, USA (poster presentations)	2013	1.0 ECTS
– 12th PanCare meeting, Amsterdam (coordinating meeting of the International Female Gonadal Dysfunction Harmonisation Work Group)	2013	1.0 ECTS
– 1st Research Retraite, SKION-Prinses Máxima Centrum, 2013 (oral presentation)	2013	1.0 ECTS
– Najaarsvergadering Vereniging voor Fertiliteitsstudie, 2013 (oral presentation)	2013	1.0 ECTS
– KiKa Promovendi dag 2014 (oral presentation)	2014	1.0 ECTS
– 60th Annual Meeting of the Society for Gynecologic Investigation, Florence, Italy (oral presentation, SGI President's award)	2014	1.0 ECTS
– Weekly research meetings Dept. of Obstetrics and Gynaecology, division of Reproductive Medicine	2010–2014	2.0 ECTS
– Weekly research meetings Dept. Paediatric Oncology and Haematology	2010–2014	2.0 ECTS
– Weekly QCAT research meeting during and after Cancer Treatment	2010–2014	2.0 ECTS
– Weekly multidisciplinary gynaecology- endocrinology meeting	2010–2014	2.0 ECTS

Seminars and workshops:

– Erasmus MC PhD day	2010	0.2 ECTS
– Masterclass Kinderendocrinologie, Utrecht	2011	0.4 ECTS
– SKION promovendi dag 2012	2012	0.4 ECTS
– AAV Wetenschapsmiddag 2013	2013	0.4 ECTS
– Erasmus MC PhD day	2013	0.2 ECTS
– Themaweek late effecten (presentation at Dept. of Paediatric Oncology/ Haematology)	2013	0.4 ECTS

2. TEACHING ACTIVITIES

Lecturing:

– Curriculum Bachelor Medicine, Faculty Medicine and Health Sciences, Erasmus University Medical Center Rotterdam	2010–2012	5.0 ECTS
– Curriculum Master Medicine, Faculty Medicine and Health Sciences, Erasmus University Medical Center Rotterdam	2013	1.0 ECTS

Supervising Master's theses:

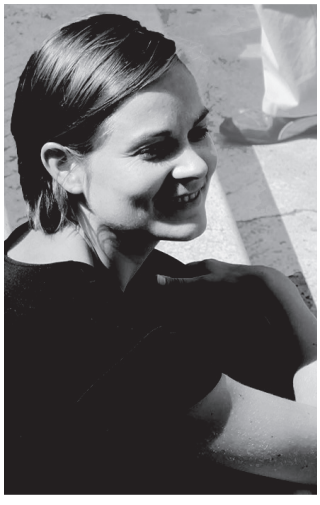
Supervising elective research programs (21 weeks) of medical students:

– Mw. Chantal den Bakker	2012	1.5 ECTS
– Mw. Catherine Owusuaa	2013	1.5 ECTS
– Mw. Annemarijne Adank	2013	1.5 ECTS

Other skills:

– Collaborator of the DCOG-VEVO study group	2010–2014	3.0 ECTS
– Coordinator of the International Female Gonadal Dysfunction Harmonisation Working Group	2012–2013	2.0 ECTS
– Peer reviewing of articles for scientific journals	2012–2014	2.0 ECTS

About the author



Wendy van Dorp was born on 19th June 1984 in Vlaardingen. She grew up in Rotterdam. In 2002, she finished her pre-university education (VWO) at the Sint-Laurenscollege. She studied Medicine in Rotterdam, and obtained her medical degree at the Erasmus University in Rotterdam in 2009. During her 'co-schappen', she worked for 9 weeks in the Aarhus Universitetshospital in Denmark. Wendy worked as a junior resident at the department of Obstetrics and Gynaecology at the Maasstad Ziekenhuis in Rotterdam. In February 2010 she started as a PhD student at the department of Obstetrics and Gynaecology, subdivision Reproductive Medicine, under the supervision of prof.dr. J.S.E. Laven, and the department of Paediatric Oncology and Haematology under the supervision of prof.dr. R. Pieters and dr. M.M. van den Heuvel-Eibrink (Erasmus MC).

Wendy van Dorp is geboren op 19 juni 1984 in Vlaardingen. Ze is opgegroeid in Rotterdam. In 2002 behaalde zij haar VWO diploma aan het Sint-Laurenscollege. Ze heeft geneeskunde gestudeerd aan de Erasmus Universiteit in Rotterdam, waar zij haar studie in december 2009 heeft afgerond. Tijdens haar keuze co-schappen heeft zij 9 weken lang meegelopen op de afdelingen Kindergeneeskunde en Obstetrie/Gynaecologie in het Aarhus Universitetshospital in Denemarken. Vervolgens is zij als arts-assistent niet in opleiding (ANIOS) gaan werken in het Maasstad Ziekenhuis in Rotterdam. Op 1 februari 2010 is zij gestart als arts-onderzoeker bij de subafdeling Voortplantingsgeneeskunde van het Erasmus Medisch Centrum Rotterdam en de afdeling Kinderoncologie en Hematologie. Haar promotie onderzoek staat onder supervisie van prof.dr. J.S.E Laven, prof.dr. R. Pieters en dr. M.M. van den Heuvel-Eibrink.

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Wendy