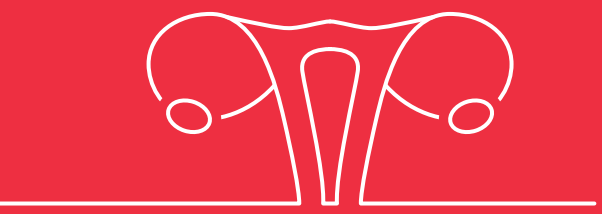

ANNE-LOTTE VAN DER KOOI

Clinical and genetic determinants of the IMPACT OF CHILDHOOD CANCER ON REPRODUCTIVE HEALTH

CLINICAL AND GENETIC DETERMINANTS OF THE IMPACT OF CHILDHOOD CANCER ON REPRODUCTIVE HEALTH

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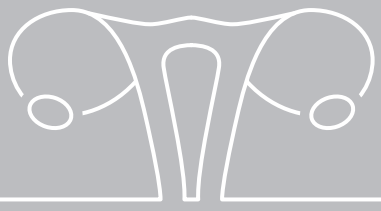

CHAPTER 1

General introduction

1. THE IMPACT OF CANCER

1.1 Childhood, adolescent and young adult cancer

The impact of a cancer diagnosis can hardly be overestimated. A cancer diagnosis bears tremendous consequences for the short and long-term prospective of patients and their loved ones. Annually, around 1.2 million children and young adults under 40 years of age are diagnosed with cancer around the world[1]. In 2018, around 600 children under the age of 18 received this diagnosis in The Netherlands. In the early 1970s, the reported 5-year cumulative survival for all childhood cancers diagnosed in Great Britain was slightly below 40%[2], while currently, survival rates in developed countries advance an average of 80%[3], doubling the chances of 5-year survival within 5 decades. The continuation of improvements in the treatment of paediatric cancer has led to a growing population of long-term survivors of cancer (Fig. 1). Unfortunately, as a result of the therapeutic treatment regimens used to achieve cure, many childhood cancer survivors (CCS) are at risk for developing complications later on in life[4,5]. These late effects may affect multiple organ systems, and can be both life-threatening and affect quality of life[6]. As cure rates improve, awareness of these late effects and the necessity to think beyond survival, has increased.

1.2 Late effects

Large cohort studies such as the Childhood Cancer Survivors Study (CCSS) and the European PanCare projects aim to identify these late effects and quantify its consequences. The impact on later health of survivors is high: quality of life is consistently lower in cancer survivors as compared to women without a history of cancer[7,8]. Approximately 75% of childhood cancer survivors have developed at least one health problem as a result of their cancer treatment[5], and childhood cancer survivors are 8.2 times more likely to have a severe chronic condition such as premature gonadal failure in comparison to their peers[6,9]. Increased awareness of the impact of these late effects on numerous organ systems has further stimulated the evaluation of treatment protocols for cancer: while survival remains the first priority, risks of late effects are weighted into the equation. As a result, mantle field radiation in Hodgkin lymphoma has been reduced or eliminated and replaced by more local therapy or chemotherapy, with a lower incidence of breast cancer later in life[10]. Cranial radiotherapy in acute lymphoblastic leukaemia is increasingly omitted as a prophylactic standard of care[11], without compromising overall survival yet reducing endocrine late effects resulting from an impaired central driver of the hypothalamic-pituitary axis. Consequently, the reduction of radiotherapy and chemotherapy exposures and the increased awareness for prevention and early detection of late effects have resulted in not only extension of the lifespan of CCS, but also extension of the *healthy* lifespan of CCS[12,13].

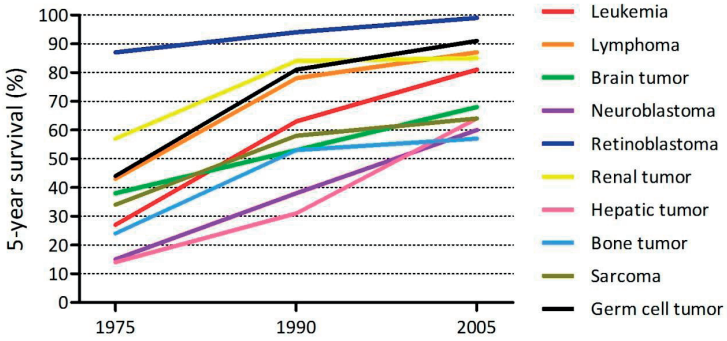

Figure 1. Survival rates for Dutch childhood cancer survivors diagnosed before the age of 18 years. Dutch Childhood Oncology Group (DCOG) national registration 2017.

2. GONADAL FUNCTION

2.1 Ovarian physiology

The female reproductive lifespan is limited as its function gradually decreases with age, mainly due to a depletion of the ovarian follicle pool. At the beginning of life, around twenty weeks post-conception, the female ovarian pool is at its peak with around 6-7 million primordial follicles[14,15]. These primordial follicles are around 0.03-0.05 mm in diameter and lie dormant in the ovary, covered in only one flat cell layer of granulosa cells. Each of these primordial follicles contain one immature primary oocyte, or "egg". After this peak during fetal development, the number of primordial follicles decrease steadily, and less than 1 million primordial follicles remain at birth. At the time of menarche, the ovarian pool consists of approximately 400,000 – 500,000 primordial follicles[16]. Even before menarche occurs, primordial follicles are activated to grow while the granulosa cells proliferate and form a cuboidal structure around the oocyte[17]. The follicles are now called primary and then secondary follicles, but are still independent of gonadotropins. This phase is called the initial recruitment and marks the initiation of growth of the follicles[16] (Fig. 2).

Signals involved in this pathway have long been undetermined, but since the beginning of this millennium one of these signals has been identified as anti-Müllerian hormone (AMH)[18]. In females, AMH is produced in the ovary by granulosa cells of small growing follicles and is considered a surrogate marker for ovarian function and ovarian reserve[19,20]. AMH regulates the pathway of folliculogenesis in at least two ways: by inhibiting the recruitment of more follicles from the primordial pool, protecting the ovary from excessive follicular recruitment and by inhibition of FSH sensitivity, regulating the maturation of follicles during the initial recruitment. The follicle now continues to proliferate, and the zona pellucida, lamina basalis, theca cells and non-functioning follicle-stimulating hormone-receptors begin to form. After more than 4 months since the start of initial recruitment, a cavity (or: antrum) is

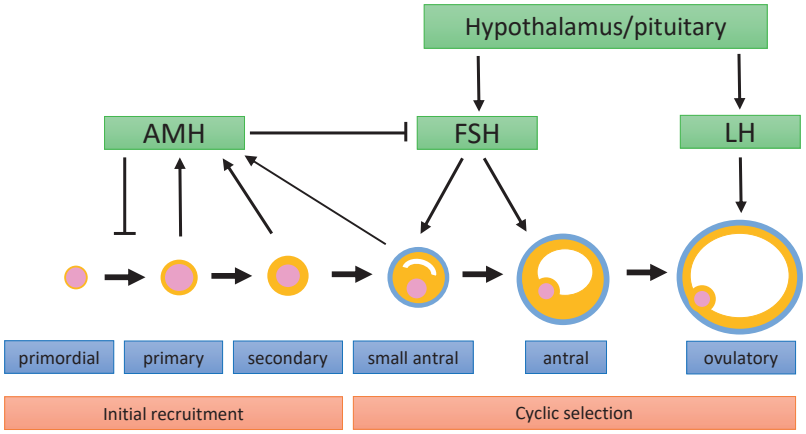

Figure 2. Simplified depiction of initial recruitment and cycle recruitment during folliculogenesis. AMH, anti-Müllerian hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone.

formed within the follicle[17]. The next phase is termed cyclic recruitment. This phase, which does not occur before menarche, is under endocrine control, and is, in contrast with the initial recruitment, gonadotropin dependent[16,17].

The follicle with an antrum is named the antral follicle, and the follicle-stimulating hormone-receptors have now become receptive for signals from the pituitary in the form of follicle stimulating hormone (FSH). AMH inhibits the sensitivity of the follicle to FSH. This inhibition regulates follicular recruitment via the PI3K/PTEN/Akt follicle activation pathway until AMH expression disappears gradually in larger antral follicles[21]. Around 10 antral follicles are typically recruited, and usually one follicle will emerge as the dominant follicle of the group: it will grow faster and produce higher levels of estrogens and inhibins than its competitors. These estrogens and inhibins send a negative feedback signal to the pituitary to suppress FSH secretion. The suppressed secretion results in lower FSH levels, which decreases the chances of the competing antral follicles to receive adequate FSH stimulation to survive. When the antral follicles do not receive this stimulation, they go into atresia, and only the sole leading follicle remains. The increasing estrogen levels, produced by the dominant follicle, exceed a threshold and now trigger the hypothalamus to signal the pituitary to secrete high levels of the luteinizing hormone (LH)[22]. As the follicle is luteinised, the oocyte and some cumulus cells are excreted, hoping to be picked up by the fallopian tubes, be fertilized and implanted in the uterus. The ruptured follicle which has now lost its oocyte is called the corpus luteum, and its granulosa and theca cells are transformed to now produce progesterone, inhibin A and estrogen. The uterine lining changes under influence of progesterone, to prepare for a potential implantation of an embryo[23]. Progesterone also inhibits LH secretion, and as the corpus luteum is dependent on LH stimulation it will degrade if not a look-a-like of LH, human chorionic gonadotropin (hCG), is produced by

the placenta to sustain it instead. If no pregnancy occurs, the drop in LH will lead to the degradation of the corpus luteum and a fall in progesterone and estrogen. Due to this drop in progesterone and estrogen the uterine lining cannot be sustained and will be expulsed: the onset of the menses[17]. The negative feedback that estrogen has exerted on FSH secretion also diminishes, and rising FSH levels cause a new cohort of antral follicles to continue its development.

The ovarian follicle pool slowly becomes depleted. There are less small growing follicles present in the ovary to secrete AMH, with a rise in FSH levels as a result. Increasing FSH levels lead to higher and earlier recruitment of follicles and the menstrual cycle becomes irregular, until only about 1,000 follicles remain and menopause occurs[17, 24].

2.2 Other important functions of steroids

Ovarian physiology is not only important in reproduction, but is a key determinant of health as a whole. In addition to the uterine lining, estrogens target breast, brain, bone, liver and heart, among others. Disruption of follicle recruitment can lead to sustained low levels of estrogen, and in the long term osteoporosis, lower HDL levels (increasing the risk for heart disease) and cognitive impairment. Testosterone is one of the biologically available androgens in the human body, and half of it is derived from the ovaries while the other half is produced by the adrenal glands. Women who undergo bilateral oophorectomy report decreased sex libido[25,26] as a result of low testosterone levels, while women with increased testosterone levels can have symptoms such as hirsutism, ace and alopecia[27].

2.3 Assessment of ovarian function

Ovarian function can be measured and defined in many ways[28-30]. In adult women, the evaluation of FSH/LH with estrogen, together with an ultrasound assessing the antral follicle count, is usually considered to be the gold standard. However, this evaluation needs to be assessed in the early follicular phase of the menstrual cycle in order to be reliable, as the assessor needs to be certain the observations are not done during an ovulation. In addition, FSH only starts to permanently increase when fecundity is already at risk, and a high FSH is therefore a relatively late sign of decreasing ovarian function, just as the self-reported onset of amenorrhea or menopause is only the very final stage of this decrease (Fig. 3). The antral follicle count does diminish gradually with age, but its assessment has the disadvantage of the need of an ultrasound – requiring an experienced sonographer, time, timing and introducing observant bias.

AMH has the advantage to be a more objective measurement, and can serve as a reliable surrogate marker for ovarian function while the primordial follicle pool is not yet depleted[19,20,31]. Prior to the clinical manifestation of amenorrhea and increased levels of FSH, impaired ovarian function can be detected by the measurement of decreased serum AMH levels[32]. AMH in females is produced solely in the ovary by granulosa cells of small

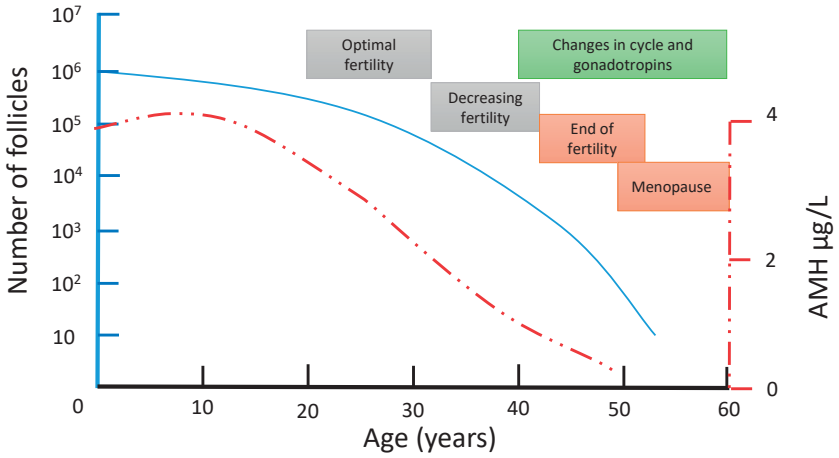

Figure 3. Decline in primordial follicle pool (line) and gradual decline of AMH levels (dotted line). Reproductive events that can be used for ovarian function assessment are indicated on the timeline where they usually occur. AMH = anti-Müllerian hormone.

growing follicles and is considered a surrogate marker for ovarian function and ovarian reserve[19,20]. Like the primordial follicle pool, AMH levels decrease from adolescence on, until menopause occurs. Even women who do not report premature menopause (or Primary Ovarian Insufficiency, POI, defined as menopause before the age of 40 years) can still have a poor ovarian function, potentially resulting in reduced fertility or a shorter reproductive window (e.g., early menopause or menopause between 40-45 years). This impairment of ovarian function can be identified by the evaluation of AMH levels.

2.4 Male gonadal physiology

While sperm quality and quantity does seem to decline by age, there is no natural end to the male reproductive life[33-35]. In the assessment of gonadal function in men, semen analysis is considered the gold standard[36]. An active hypothalamic-pituitary-gonadal axis is required for the production of sex steroid hormones and the production of healthy mature male gametes. Just as in females, LH and FSH play important roles in this pathway. In males, LH stimulates the Leydig cell in the testes to produce testosterone. Testosterone is binded to androgen-binding protein, which is produced in Sertoli cells under influence of FSH. The resulting high levels of androgens such as testosterone enable spermatogenesis in the seminiferous tubules and sperm maturation in the epididymis. Spermatogenesis starts with the mitotic division of spermatogonial stem cells, where one clone replenishes the stem cells and the other clone differentiates into spermatocytes, later on being transformed into spermatozoa or sperm cells[37]. As a result of this mitotic division of spermatogonial stem cells, this process can continue uninterrupted until death without a natural senescence as we see in females.

Apart from androgen-binding protein, Sertoli cells of the testes also produce inhibin B under influence of FSH. Both inhibin B and testosterone exert a negative feedback on the production of LH and FSH at the hypothalamus and pituitary level.

2.5 Assessment of male gonadal function

Semen analysis is considered the gold standard in the assessment of gonadal function in men[36], but with an inactive hypothalamic-pituitary-gonadal axis, no spermatogenesis or subsequent semen is produced that can be analyzed. However, the presence of inhibin B levels in pre-pubertal males indicates that basal inhibin B secretion takes place in the prepubertal testis despite very low levels of FSH and testosterone[38]. In adult males, inhibin B is a marker of spermatogenesis as it is positively correlated with sperm count and concentration in adulthood[39-41]. Given the substantial patient burden or impossibility of obtaining semen samples from young boys (by masturbation or electro-ejaculation), inhibin B is considered a feasible and adequate surrogate marker for gonadotoxicity in young boys[36,42-44], and reference values are available for both prepubertal as well as for pubertal boys[42,45].

3. TOXIC MECHANISMS OF CHILDHOOD CANCER THERAPY

Chemotherapy and radiotherapy are often important components of antitumor therapy, both targeting dividing cells and consequently the growing follicles of the ovaries. However, non-growing primordial follicles too can be damaged by both radiotherapy and cytotoxic chemotherapy.

3.1 Chemotherapy

One of the first effective chemotherapeutic drug was mechlorathemine, a modification of mustard gas which had been used as a chemical warfare agent. During World War I, a lymphotoxic effect was observed after accidental exposure to the agent, and this observation gave way to the first successful treatment of lymphoma patients with chemotherapy. Designed after this agent, derivatives such as cyclophosphamide and ifosfamide continue to be key players in current cancer treatment strategies. These agents can damage Deoxyribo Nucleic Acid (DNA) by forming intrastand or interstrand crosslinks[46-48]. This linkage of DNA strands makes it impossible for the body to unfold the strands, a critical step in cellular metabolism and DNA replication and transcription. Without this mechanism intact, sooner or later programmed cell death known as apoptosis will occur inevitably[48] (Fig. 4).

These agents, known as alkylating agents because of their ability to bind DNA via their alkyl group, can do their damaging work at any moment of the cell cycle and can therefore also damage non-growing primordial follicles[49,50]. Accurate repair pathways of DNA crosslinks can save some cells and are vital for healthy cells, but can cause resistance to the

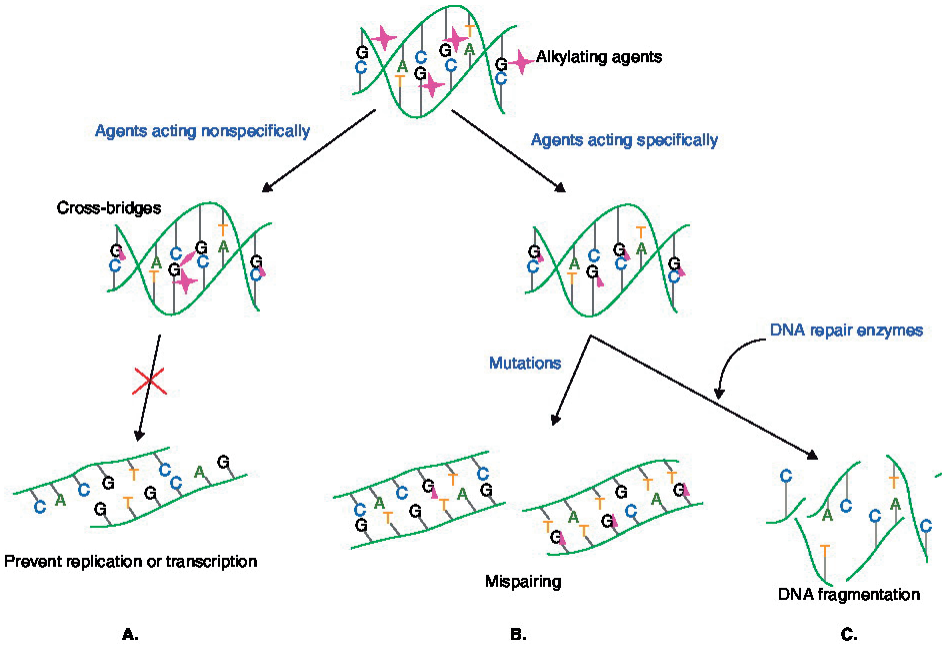

Figure 4. Mechanism of action of alkylating agents. A. Formation of cross-bridges, bonds between atoms in the DNA results in inhibition of replication or transcription. B. Alkylated G bases may erroneously pair with Ts. If this altered pairing is not correct it may lead to a permanent mutation. C. DNA fragmentation might occur as a result of attempt to replace alkylate bases by DNA repair enzymes. Reprinted with permission from Ralhan R., Kaur J. Alkylating agents and cancer therapy. Expert Opinion on Therapeutic Patents 2007.

agents on the other hand[49,50]. Alkylating agents have also been associated with a reduced uterine volume[51], but not consistently[52].

Another mechanism of follicle loss is known as 'burnout'. With the destruction of growing follicles, AMH levels decrease as a direct effect of administration of cyclophosphamide. This causes an upregulation in the PI3K/PTEN/Akt follicle activation pathway, triggering recruitment of a wave of primordial follicles causing the ovarian follicle pool to become exhausted[53,54].

Finally, another potential toxic mechanism of chemotherapy is through vascular damage of the ovary caused by chemotoxic agents. As small follicles do not have their own independent capillary network, microvascular damage of the cortex might impair ovarian function[55-57].

3.2 Radiotherapy

The dose of radiotherapy at which damage to an extent of POI occurs is around 20 Gy when administered at birth, 18.4 Gy at 10 years, 16.5 at 20 years and 14.3 Gy at 30 years of age at administration[58]. Less than 2 Gy appears to be needed to destruct 50% of the ovarian follicle

pool[59]. Not only the ovaries are damaged by radiotherapy. Women treated with total body irradiation during childhood (14.4 Gy) have a relatively small uterus, poor blood flow and a poor endometrial lining[51,52,60,61]. In addition, it has been suggested that the elasticity of the uterine musculature is damaged by radiotherapy[62,63]. Although some reports have shown an improvement of uterine volume and blood flow after administration of sex steroids[60,61], a larger study reported the radiotherapy-induced damage to be irreversible[64].

4. GENETICS

4.1 Genetic variation

In our discussion of chemotherapy (paragraph 3.1) we already briefly touched upon DNA. In most cells of the human body (all cells excluding red blood cells, and cornified cells in the skin, hair and nails) harbors a large string of DNA in its cell nucleus. DNA is folded so efficiently, every cell hosts approximately 2 meters of DNA, and if all DNA of one human would be stretched out it would be about four times the distance from the earth to the sun, and back. DNA is made up of four nucleotides, abbreviated C (cytosine), G (guanine), A (adenine) and T (thymine), in a very extensive sequence[65,66]. The nucleotides bind in pairs (C with G and A with T) coiling around each other and resulting in the configuration of a double helix. The order of these nucleotides carry the genetic codebook with all our genetic information. The combination of the resulting approximately 3.2 billion pairs is called our human genome[67]. The DNA sequence can be copied or transcribed into RNA, a process controlled by other DNA sequences such as promotors. The information on the RNA copy is then translated into the correct sequence of amino acids, which are the basis of all our proteins – the building blocks of our body[68].

The human genome is identical in all for about 99.9%. Nonetheless, every person is born with genetic differences, called variation, accounting for each individual uniqueness at the level of genes, traits and diseases. Different versions of a DNA sequence at a specific locus or position in the human genome, are called alleles. A variation in the single nucleotide or alleles that occurs at a specific position on the genome is known as a single nucleotide polymorphism (SNP) if the occurrence of both alleles is present in at least 1% of the population. SNPs can lie in the non-coding regions of the genome or in the protein-coding regions. SNPs in these coding region can either have no effect on the resulting amino acid sequence (synonymous mutation) or can result in the coding for another amino acid (missense) or a final stop of the coding usually resulting in a non-functional protein product (nonsense)[66]. SNPs that are not in protein-coding regions may still affect gene expression and therefore susceptibility of certain traits or diseases[66,69].

Recent genome-wide association studies (GWAS) have identified over 100 genetic variants that are associated with age of onset of natural menopause. Genetic variants that

determine age at menopause seem to be mainly involved in DNA repair and genome maintenance. Interestingly, the identified menopausal genes involved in genome maintenance pathways, are mainly linked with DNA repair processes, which preserve proper genome function and protect from DNA damage induced cell death primarily during replication or by transcription-coupled repair. The link between ageing of the soma on the one hand and fertility and menopause on the other hand implies a common genetic background for these phenomena. Indeed, functional biology data as well as epidemiology data do suggest that the ageing soma determines when reproduction and subsequently menopause will occur. This new paradigm challenges the old dogma that women age as a consequence of menopause. Finally, reproductive performance seems to constitute a very good marker for a woman's general health later on life. This offers new possibilities for developing preventive strategies, which might further improve women's health[70].

4.2 Candidate-gene approach

Differences in ovarian damage in women who received the same treatment suggest that genetic variation may be an important determinant of ovarian damage. Genetic association studies test if a higher frequency of a SNP is observed in a series of individuals with a trait as compared to a series of individuals without the trait[66]. Disorders or traits caused by one mutation or variation are commonly known as single gene disorder and can be evaluated using Mendelian inheritance patterns[71]. However, most traits and diseases are the result of many small differences in the human genome, as well as environmental factors, and are therefore called multigenic or complex disorders.

The association between a SNP and a disease or trait such as ovarian function can be assessed by various types of genetic association studies. The first method is called a candidate-gene study. Based on prior knowledge of the mechanism of the trait or previous reported associations of the SNPs with the trait in other populations, SNPs are selected for association analysis.

4.3 Genome-wide association studies (GWAS)

Where the method of candidate-gene studies have a hypothesis for the association, the design of the genome-wide association study (GWAS) takes a hypothesis-free approach. In theory, each locus of the human genome is analysed for a correlation with the trait of interest. In practice, a large proportion of the genome of many hundreds of thousands SNPs are analysed without any prior assumption on mechanism or known association. Using knowledge of the non-random correlation of genetic variants (known as linkage disequilibrium) and reference genotype datasets such as 1000 Genomes Project[72], genotypes that are not directly measured can be imputed and still be analysed for an association with the trait. The subsequent abundance of statistical tests that have been performed within a GWAS have a direct implication for the level of statistical significance. Statistical testing is based

on rejecting the null hypothesis of 'no association' if the likelihood of the observed association under the null hypotheses is low. If multiple associations are tested, the likelihood of incorrectly rejecting a null hypothesis increases, with many 'false positive' associations as a result of chance. The Bonferroni correction is applied to correct for this increase. The usual statistical significance is arbitrarily set at 0.05 in most health sciences, and the Bonferroni correction is commonly 5×10^{-8}, obtained by dividing 0.05 by 1,000,000 assessed SNPs.

5. AIM AND OUTLINE OF THIS THESIS

The general aim of research described in this thesis is to evaluate reproductive health in men and women who have been treated for cancer. In this thesis, the focus is mainly on female survivors of childhood cancer. In part I, we start with trends in gonadal function markers using longitudinal data on AMH and inhibin B. In Chapter 2 we focus on gonadal function as reflected by serum inhibin B and testosterone levels, before the start of treatment in boys with newly diagnosed cancer. In Chapter 3 we describe the impact childhood cancer treatment has on gonadal function markers in both girls and boys. In Chapter 4 we evaluate longitudinal data from female adult childhood cancer survivors at a longer follow-up time, and evaluate if the long-term decline of ovarian function, as reflected by a decrease in AMH, accelerates over time as compared to the physiological decline in women of the same age.

The observed reduced ovarian function among CCS is only partially explained by treatment and baseline patient characteristics. In part II of this thesis we consider this inter-individual variability, and hypothesize that genetic variation possibly modifies this association. In Chapter 5 we review the available literature on genetic susceptibility of late toxicity after childhood cancer treatment related to components of gonadal impairment, as well as of metabolic syndrome, bone mineral density, and hearing impairment. In this chapter, we also discuss future directions for genetic association studies of late toxicities. In Chapter 6 we describe the design of the PanCareLIFE study to evaluate genetic association of chemotherapy-induced gonadal impairment in a large European cohort, with a large independent replication cohort. In Chapter 7 we evaluate whether SNPs that have been associated with age at natural menopause in the general population are of influence on alkylating agent related reduced ovarian function in female CCS from the Dutch nationwide DCOG LATER-VEVO study, the PanCareLIFE study and the St. Jude Lifetime Cohort.

In the final part of this thesis, part III, we move away from gonadal function markers and turn our attention to obstetric outcomes in cancer survivors. In Chapter 8 we investigate the risk of adverse pregnancy and perinatal outcomes in survivors of cancer diagnosed before the age of 40 years compared to the general population. In Chapter 9 we review the literature of pregnancy and perinatal risk in cancer survivors and present a meta-analysis

of these risks. We offer international harmonized recommendations for counseling and surveillance of obstetric risks for female survivors of childhood, adolescent, and young adult cancer in Chapter 10. Chapter 11 concludes with a general discussion of this thesis in a broader context, and offers directions for future research and topics of debate.

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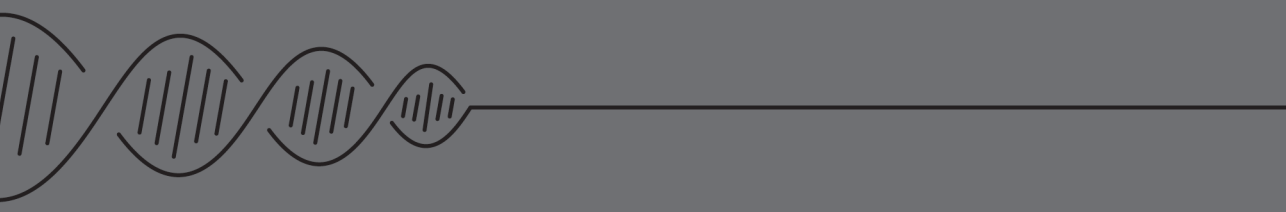
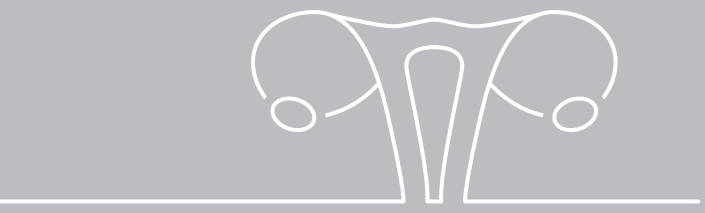


PART I

Clinical aspects of gonadal function



CHAPTER 2

Gonadal function in boys with newly diagnosed cancer before the start of treatment

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ABSTRACT

Study question: Are inhibin B and testosterone levels reduced in boys with newly diagnosed cancer prior to therapy?

Summary answer: Pretreatment serum levels of inhibin B and testosterone are significantly reduced in boys with newly diagnosed cancer, compared to reference values.

What is already known: Disease-related gonadal impairment has been demonstrated in girls and young women diagnosed with cancer, prior to therapy.

Study design, size, duration: We conducted a descriptive study in boys newly diagnosed with cancer between January 2006 and February 2014.

Participants/materials, setting, methods: Serum inhibin B and testosterone levels were determined in 224 boys, up to the age of 18 years, with newly diagnosed cancer prior to therapy. Hormone levels were compared with age-matched reference values. The cohort consisted of patients with acute lymphoblastic leukaemia (ALL), acute myeloid leukaemia (AML), Hodgkin lymphoma (HL), non-Hodgkin lymphoma (NHL), nephroblastoma, neuroblastoma and sarcoma.

Main results and the role of chance: This study demonstrates reduced serum levels of inhibin B in boys with newly diagnosed cancer, compared to reference values (standard deviation score (SDS) -0.9, $P < 0.001$). Median inhibin B level in patients was 103.5 ng/l (range 20-422). Of all patients, 78.6% showed inhibin B levels below the 50th percentile, and 58.5% had inhibin B levels below the 25th percentile. Serum testosterone levels were significantly lower than the reference range population (SDS -1.2, $P < 0.001$). Median testosterone level in pubertal patients was 7.3 nmol/l (range 0.1-23.6). No correlation with clinical signs of general illness and hormone levels were observed.

Limitations, reasons for caution: In this study, reproductive hormone levels were compared with age-matched reference values. Future studies may compare reproductive hormone levels with case controls.

Wider implications of the findings: Future longitudinal studies are necessary to determine whether pretreatment impaired gonadal function at the time of cancer diagnosis is an important determinant of ultimate recovery of spermatogenesis after treatment and later on in adulthood.

INTRODUCTION

Long-term survival of childhood cancer has steadily increased following optimized treatment regimens over the past decades[1]. As a result, the absolute number of survivors of childhood cancer is increasing[2]. Consequently, awareness of direct and long-term side effects after treatment of pediatric cancer is growing[3]. Gonadal dysfunction with the risk of impaired fertility is one of these long-term side effects in both childhood and adult cancer survivors[4,5]. Gonadal dysfunction depends on administered treatment modality as well as the total cumulative dosages[4]. Recently, we reported significantly reduced anti-Müllerian hormone (AMH) levels in girls with newly diagnosed cancer prior to treatment, indicating that not only gonadotoxic treatment but also the disease itself and the concomitant health status affects gonadal reserve in girls with cancer[6]. Currently, only scarce information is available about gonadal function in boys with newly diagnosed cancer before the start of treatment[7].

To assess gonadal function in men, semen analysis is considered to be the gold standard[4]. In addition, inhibin B and follicle-stimulating hormone (FSH) have been identified as reliable serum markers of spermatogenesis during adulthood, as significant associations between Inhibin B, as well as between FSH, and sperm concentrations have been reported[4,8-13].

Inhibin B is a dimeric hormone produced by Sertoli cells, which provides negative feedback on FSH secretion[8]. During the first months after birth, inhibin B levels are elevated as a result of transient activation of the hypothalamic-pituitary-testicular axis. After the postnatal peak, inhibin B levels gradually decline until the age of 2 years to a constant level during childhood. At the start of puberty, inhibin B levels increase until adult levels are reached. Both periods of high inhibin B production are associated with the presence as well as the proliferative activity of Sertoli cells[14-17]. It has been suggested that the number of Sertoli cells determines the spermatogenic potential later in life[14,15]. Therefore, serum inhibin B levels may provide a reflection of gonadal function even in young boys[14,16].

Sperm production requires testosterone production. This process of testosterone synthesis and secretion by Leydig cells in the testis, is stimulated by luteinizing hormone (LH)[18]. Testosterone levels show a similar increase as inhibin B following birth. Unlike inhibin B, testosterone levels rise to a peak at 1-3 months of age and then decline to barely detectable levels at 1 year of age till puberty, after which a second peak occurs during puberty. The postnatal as well as the pubertal peaks of testosterone levels follow the proliferation and maturation of Leydig cells[14].

Based on reports on disease-related gonadal impairment at time of diagnosis before cancer treatment in girls and young women, we hypothesized that similarly compromised gonadal dysfunction may occur in boys with newly diagnosed cancer. This would be similar to adult cancer patients where oligozoospermia is observed at time of diagnosis before any treatment has started[19-21]. The exact underlying mechanism is unknown yet. In boys

with cancer, such data on the effect of disease on gonadal hormone production, based on a substantial number of cases, is not available.

Knowledge of pretreatment gonadal function in boys with newly diagnosed cancer is of interest, as this may be the baseline for potential recovery of fertility after childhood cancer. Therefore, we evaluated gonadal function and disease-related determinants in boys with newly diagnosed cancer, using inhibin B and testosterone as markers of gonadal function.

MATERIALS AND METHODS

Study population

We included boys up to the age of 18 years with newly diagnosed cancer at our Paediatric Oncology Centre between January 2006 and February 2014. Patients with brain tumours were excluded due to potential hypothalamic-pituitary-axis dysfunction, and patients with germ cell tumours were excluded because of the localization of the tumour in the testes and/or direct influence on hormone production. We only included groups of tumour subtype with at least nine patients. Details on age, diagnosis, pubertal stage and clinical parameters were retrieved from patient record files. Pubertal status at diagnosis was assessed clinically and classified as prepubertal (Tanner stage 1), midpubertal (Tanner stage 2 – 3) and postpubertal (Tanner stage 4 – 5) as previously described[22]. Baseline inhibin B levels were measured in all subjects and testosterone, FSH and LH levels at time of presentation with cancer were measured in boys from Tanner stage 2 onwards, since these hormones are barely measurable before puberty[14,16]. Because of the small number of pubertal boys, testosterone levels were evaluated only in patients with acute lymphoblastic leukaemia (ALL), Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL). To identify the determinants of the pretreatment hormonal deviations at diagnosis, we used the surrogate markers, body temperature, C-reactive protein (CRP) and haemoglobin (Hb) levels at diagnosis, as proxy for general health status. CRP has been shown to be sufficiently reliable in cohort studies as an acute phase protein and provides together with Hb a reflection of being chronically unwell[23]. Increased CRP as acute phase protein and Hb as a reflection of being chronically unwell are indeed not very hard indicators for disease as they are not specific. However, as anaemia and enhanced inflammation can be signs of an unhealthy status, and as these markers were available in the majority of all patients, we decided to include them as surrogate markers of being unwell. Using these surrogate markers as reflection of chronic well-being has been recently applied in a similar study with female childhood cancer patients[6]. Informed consent was obtained from all included patients and parents to use left-over material for additional research including this study, according to the standards of the Institutional Review Board.

Laboratory measurements

Serum samples were stored at -20° C until analysis. Inhibin B was used as a surrogate marker for gonadal function[4,8]. Inhibin B levels were measured using an enzyme-linked immunosorbent assay (inhibin B Gen II ELISA kit, Beckman Coulter, Inc. Brea, CA). Within-assay coefficients of variation (CVs) were 4.4% at 11.6 ng/L and 1.9% at 146.3 ng/L, respectively. Between-assay CVs for inhibin B were 14.3% at 15 ng/L and 11.4% at 162 ng/L. In addition, serum FSH and LH were determined with the Immulite assay (Siemens DPC, Los Angeles, CA, USA). Within-assay and between-assay CV were <6% and <9% and <5% and 11% for FSH and LH respectively. Serum total testosterone levels were determined using standard coated-tube radioimmunoassays (Siemens DPC) and liquid chromatography tandem mass spectrometry (LC-MS-MS) methods. Within-assay and between-assay variation coefficients were 3% and 4.5% for radioimmunoassays. For LC-MS, within-assay CVs were 6.1% at 0.029 nmol/L, 3.5% at 0.0073 nmol/L and 7.1% at 1.127 nmol/L. Between-assay CVs were 9.2% at 0.038 nmol/L, 4.2% at 0.08 nmol/L and 6.8% at 1.04 nmol/L. Hormone levels were compared with age-matched reference values from previously published studies[16,24-26].

Statistics

After log-transformation, inhibin B levels turned out to be normally distributed. Inhibin B and testosterone levels were analyzed as continuous variables. Standard deviation scores (SDS) were used to be able to adjust for age, using reference values of inhibin B and testosterone, which is important as these reproductive hormones vary with age and development stage[16,25,26]. The one-sample Wilcoxon signed rank test was performed to compare inhibin B, testosterone, FSH and LH SDS in boys with newly diagnosed cancer with reference values. The Kruskal-Wallis test was used to compare inhibin B and testosterone levels of cases in various diagnostic subgroups. Both the Kruskal-Wallis test and the Mann-Whitney *U*-test were used to explore the association between inhibin B SDS, respectively, testosterone SDS and Tanner stage. The correlation between SDS of reproductive hormones and the indirect markers of general health condition (body temperature, CRP levels and Hb levels at diagnosis) was studied using the Spearman rank correlation test. These analyses have been performed in both the entire cohort, as divided by Tanner stages. *P*-values <0.05 were considered significant. Statistical analyses were performed using the IBM Statistical Package for Social Sciences version 20 (IBM Corp., Armonk, NY, USA).

RESULTS

Reproductive hormone levels were analyzed in 224 boys with newly diagnosed cancer. The cohort consisted of patients with ALL, acute myeloid leukaemia (AML), HL, NHL, nephro-

blastoma, neuroblastoma and sarcoma. The median age of the boys was 5.7 years (range 0.1 – 17.7) (Supplementary data, Table S1).

The median pretreatment inhibin B level in boys with newly diagnosed cancer was 103.5 ng/l (range 20 – 422) (SDS -0.9), which was overall significantly low as compared to reference values ($P < 0.001$) (Table 1). Inhibin B levels were below the 50th percentile in 78.6% of all boys with childhood cancer, and below the 25th percentile in 58.5% (Fig 1). Twenty-eight (12.5%) had inhibin B levels of -2 SDS or lower at time of cancer diagnosis. Inhibin B SDS levels were low in all tumour types, with the exception of nephroblastoma patients (Fig. 2). No significant differences in inhibin B SDS levels were observed between cancer types (Table 1).

Table 1. Univariate analysis of pretreatment absolute Inhibin B levels and Inhibin B standard deviation scores (SDS) by diagnosis in boys presenting with cancer ($n = 224$).

Diagnosis (n)	Inhibin B (ng/l), median [range]	Inhibin B SDS, median [range]	p-value [a,b]
ALL (92)	96 [34-376]	-0.9 [-2.7 to 1.0]	<0.001
AML (31)	92 [20-273]	-1.2 [-3.7 to 0.7]	<0.001
HL (24)	135 [30-318]	-1.2 [-2.9 to 0.7]	<0.001
NHL (28)	124.5 [26-299]	-0.6 [-3.4 to 0.7]	0.003
Nephroblastoma (9)	103 [57-182]	-0.3 [-1.6 to 1.1]	0.26
Neuroblastoma (20)	99 [27-422]	-0.8 [-2.9 to 1.4]	0.01
Sarcoma (20)	109 [29-299]	-0.6 [-3.0 to 2.0]	0.02
Total group (224)	103.5 [20-422]	-0.9 [-3.7 to 2.0]	<0.001

ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; HL, Hodgkin lymphoma; NHL, non-Hodgkin lymphoma. [a] *P*-values were calculated using the one-sample Wilcoxon signed rank test. [b] Inhibin B levels did not differ between diagnostic subgroups ($P = 0.29$: Kruskal-Wallis test).

Testosterone levels were studied in 38 pubertal boys diagnosed with ALL (n = 13), AML (n = 2), HL (n = 10), NHL (n = 10) and sarcoma (n = 3). The median pretreatment testosterone level in patients was 7.3 nmol/l (range 0.1 – 23.6), i.e., significantly lower compared to reference values (SDS -1.2, $P < 0.001$) (Supplementary data, Table SII). Only three subsets of tumour types (ALL, HL, NHL) showed appropirate numbers in order to analyse testosterone levels by diagnosis, separately (Supplementary data, Table SIII). In these three tumour types, we found relatively low testosterone SDS levels in all cases. In addition pretreatment testosterone SDS were reduced in all boys diagnosed with AML (range -0.92 to -1.21) and sarcoma (range -0.74 to -2.44), as well. Of the 38 pubertal boys with available testosterone levels, 4 (10.5%) boys showed testosterone levels of -2 SDS or lower before the start of treatment. Testosterone SDS levels showed no significant differences between the three malignancies (ALL, HL, NHL).

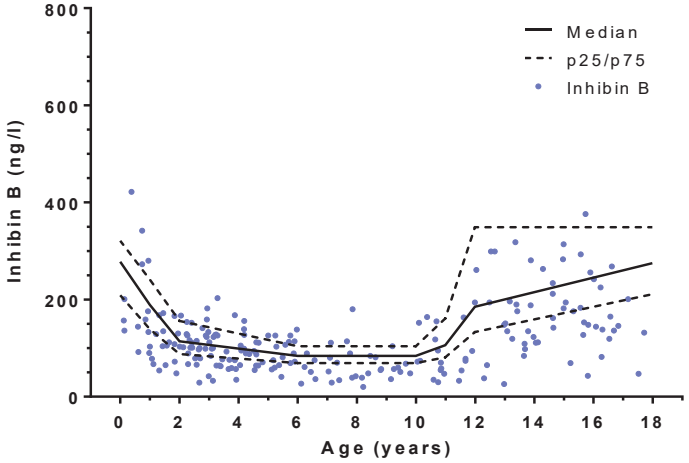

Figure 1. Pretreatment inhibin B levels in boys with newly-diagnosed cancer (n = 224) as compared to reference values (P < 0.001, one-sample Wilcoxon signed rank test). p75, p50 and p25 refer to 75th, 50th and 10th percentiles, respectively. Of all boys, 78.6% had inhibin B levels below the 50th percentile, and 58.5% had inhibin B levels below the 25th percentile.

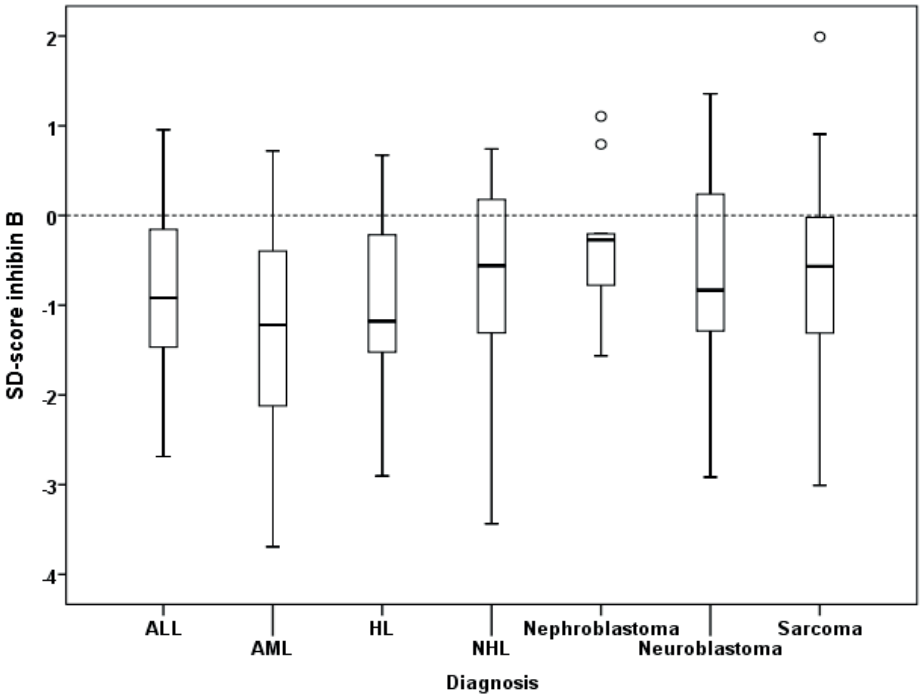

Figure 2. Pretreatment inhibin B standard deviation scores (SDS) by diagnosis in boys with cancer (n = 224). ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; HL, Hodgkin lymphoma; NHL, non-Hodgkin lymphoma.

Serum levels of FSH SDS at diagnosis did not differ significantly from reference values (SDS – 0.04, $P = 0.9$) (Supplementary data, Table SIV). LH SDS serum levels were significantly higher as compared to reference values (SDS 0.2, $P = 0.01$) (Supplementary data, Table SV).

Data on pubertal status were available in 200/224 boys. Of those, 162 were prepubertal (72.3%) and 38 boys (17%) were mid- and postpubertal (Supplementary data, Table SVI). At diagnosis, inhibin B and testosterone levels were significantly different ($P < 0.001$, $P = 0.03$) between the subcategories of pubertal status.

Median body temperature was 37.2 °C (range 35.0 – 39.9). At diagnosis median CRP level was 13.0 mg/l (range 1.0 – 296) and Hb level was 5.9 mmol/l (range 2.2 – 9.8) (Supplementary data, Table S1). None of the variables was significantly associated with inhibin B nor testosterone SDS levels (Supplementary data, Tble SVII). Also, when we stratified the analyses by pubertal status, none of the correlations were significant.

DISCUSSION

Over the past decades, optimization of paediatric cancer therapy has improved long-term survival of childhood cancer tremendously. Consequently, post-treatment quality of life, including fertility, is a critical issue for childhood cancer patients[4]. Pretreatment testicular function is of interest as this is the baseline for the gonadotoxicity and potential recovery to be monitored after childhood cancer treatment[19]. Also, in pubertal boys, it may reflect the feasibility and success rate of common but also novel preservation options, such as sperm cryopreservation or testicular sperm extraction (TESE). In adult males with cancer, the disease status was shown to hamper such options[4,27]. As in boys, this has not been systematically investigated. We studied baseline gonadal function in boys with newly diagnosed cancer before the start of treatment by hormonal evaluation.

This study shows for the first time that serum inhibin B and testosterone levels are reduced in young boys with newly diagnosed cancer prior to therapy. The cause of these reduced reproductive hormone levels is unclear. We anticipated that hormone alterations in boys with newly diagnosed cancer may be negatively affected by stress, downregulation by endocrine substances or cytokines produced by some tumours and metabolic conditions or malnutrition[28]. Such a phenomenon was shown to occur in girls with cancer that demonstrated reduced AMH levels before the start of treatment, and we could show that general health status accompanying the de novo cancer determined gonadal impairment[6]. Here, we show that in boys a correlation with disease state (as reflected by body temperature, CRP and Hb) does not seem to influence gonadal status, defined as reduced inhibin B and/or testosterone levels. The reason for this difference between boys and girls with this respect remains unclear. Previously, histological abnormalities such as Leydig cell atrophy and loss of Sertoli cells were observed in adult male HL patients at diagnosis[29]. Despite

these histological abnormalities, it might be that male gonads are less susceptible than female gonads for the impact of cancer by overall health status, but the exact mechanism is unclear. In adult male patients with HL, a high erythrocyte sedimentation rate (ESR) at diagnosis was associated with low semen quality[30]. It should also be considered that for male gonadal impairment testing, body temperature, CRP and Hb may not be the most relevant surrogate markers for general health status. We suggest that other possible markers, e.g., ESR, are potentially more relevant surrogate markers of general health in boys diagnosed with cancer before the onset of treatment[31,32].

Nevertheless, this study does show reduced pretreatment inhibin B levels in paediatric patients with HL, thereby confirming observations in adult studies[20,29,30,33], which revealed impaired gonadal function in male patients with HL before therapy, using testosterone[20,29,30,33]. Several other authors suggested that there may be an association between gonadal impairment and patients with HL; however, other types of disease are hardly studied[20,29]. In children, we show here that inhibin B levels are statistically signifcantly decreased in all tumour subtypes with the exception of nephroblastoma. Interestingly, in our group of pubertal boys, testosterone levels were low in ALL and NHL but not in HL patients.

In order to obtain more insight in the reason for impaired inhibin B and testosterone production, serum levels of FSH and LH were also measured in the subset pubertal boys at diagnosis. Although we expected increased pretreatment FSH levels, our results show that FSH levels did not differ significantly from reference values, thereby insinuating that the feedback mechanism (central axis) may be less sufficient. Hence, future studies are needed to further study this phenomenon. In contrast, we demonstrate elevated pretreatment LH levels, illustrating that reduced testosterone levels are indeed due to primary testicular failure, while Leydig cell function tends to compensate the relative testicular insufficiency. Previously, experimental studies in rats have suggested that inflammatory cytokines, such as interluekin-1, may play a role[34,35]. Also, in adult male patients, it has been suggested that Leydig cell suppression in case of acute stress may be considered as a protective mechanism for temporarily less vital functions, in order to preserve energy and metabolic substrates[36].

Apart from critical ilness, reduced inhibin B and testosterone levels could conceivably be due to stress. In adult males, it has been suggested that stress associated with the cancer process reduces reproductive function by the effect of stress hormones, which eventually suppresses the secretion of GnRH[37]. This may induce a disturbance of the hypothalamic-pituitary axis and therefore secondary testicular failure. This hypothesis might explain why FSH at diagnosis is not significantly higher compared to reference values. Though, this hypothesis is inconsistent with the demonstrated elevated levels of LH at diagnosis. Previously, experimental studies on the effect of acute stress in baboons and rats have suggested that glucocorticoids might have a suppressive effect on the steroidogenesis resulting in declined testosterone levels[38,39]. Therefore, acute stress may also affect gonadal function at testicular level. Measuring early morning stress hormone cortisol levels could provide

insight in the influence of stress on gonadal function in future studies. Unfortunately, fasting cortisol levels at diagnosis were not available in our patients.

A previous study showed normal inhibin B levels in 16 boys with childhood cancer, diagnosed with ALL, NHL, neuroblastoma, sarcoma and Wilms tumor, before the start of treatment[7]. We here show in a larger cohort of paediatric patients that pretreatment inhibin B levels are low as compared to normal controls. The discrepancy between these findings may be influenced by the previously limited sample size or a difference in tumor subgroups. As we present the first large series here, on pretreatment male gonadal function in children, we appreciate the fact that confirmation of our data in even larger cohorts of paediatric oncologic patients is important.

In this study, reproductive hormone levels were compared with age-matched reference values. The hormone levels of the reference serum used in our assays for calibration were within the range of the normal controls described in the literature, nevertheless measurement bias cannot be totally excluded. We suggest that future studies should compare reproductive hormone levels with case controls. Also we recommend to assess gonadal function in larger cohorts of pubertal patients for replication.

In summary, we show reduced inhibin B and testosterone levels in boys with newly diagnosed cancer already before starting treatment. The reason for the reduced levels of these reproductive hormones remains unclear. Future longitudinal studies are necessary to determine whether pretreatment impaired gonadal function at the time of cancer diagnosis is an important determinant of ultimate recovery of spermatogenesis after treatment and later on in adulthood.

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SUPPLEMENTAL DATA

Table SI. Characteristics of study population.

	n	Percentage (%)	median, [range]
Age (y)			5.7 [0.1-17.7]
Tumour subtype	224	100	
ALL	92	41.1	
AML	31	13.8	
HL	24	10.7	
NHL	28	12.5	
Nephroblastoma	9	4.0	
Neuroblastoma	20	8.9	
Sarcoma	20	8.9	
Pubertal stage	200	89.3	
Tanner 1	162	72.3	
Tanner 2-3	18	8.0	
Tanner 4-5	20	8.9	
Markers of general health			
Body temperature (°C)	130		37.2 [35.0-39.9]
CRP (mg/l)	123		13.0 [1.0-296]
Hb (mg/l)	215		5.9 [2.2-9.8]

ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; HL, Hodgkin lymphoma; NHL, non-Hodgkin lymphoma; Tanner 1, prepubertal; Tanner 2-3, midpubertal; Tanner 4-5, postpubertal; CRP, C-reactive protein; Hb, haemoglobin.

Table SII. Univariate analysis of pretreatment testosterone standard deviation scores (SDS) and absolute testosterone levels by diagnosis in boys with cancer (*n* = 33).

Diagnosis	Testosterone (nmol/l), median [range]	Testosterone SDS, median [range]	p-value[a,b]
ALL (13)	6.3 [0.1-16.4]	-1.6 [-2.0-0.2]	0.002
HL (10)	6.1 [0.3-23.6]	-1.2 [-2.0-1.0]	0.05
NHL (10)	10.8 [0.5-20.1]	-0.6 [-2.0-0.4]	0.01
Total group (33)	7.3 [0.1-23.6]	-1.2 [-2.0-1.0]	<0.001

ALL, acute lymphoblastic leukaemia; HL, Hodgkin lymphoma; NHL, non-Hodgkin lymphoma. [a]: *P*-values were calculated using the one-sample Wilcoxon signed rank test. [b]: Testosterone levels did not differ between diagnostic subgroups (*P* = 0.39: Kruskal-Wallis test).

Table SIII Univariate analysis of pretreatment testosterone standard deviation scores (SDS) and absolute testosterone levels according to pubertal stage in boys with cancer (*n* = 38).

Stage of puberty, (n)	Testosterone (nmol/l), median [range]	Reference value (nmol/l), median [range]	Testosterone SDS, median [range]	p-value [a]
P1G1 (162)	n.a.	n.a.	n.a.	n.a.
P2G2 (11)	1.7 [0.1-16.4]	5.3 [0.1-10.5]	-1.82 [-2.03-0.20]	0.004
P3G3 (7)	9.6 [1.4-23.6]	14.95 [0.4-29.5]	-0.74 [-1.86-1.03]	0.13
P4-5G4-5 (20)	8.0 [2.74-20.1]	17.5 [5.6-29.4]	-1.10 [-2.44-0.44]	<0.001
Total group (38)	7.34 [0.10-23.6]	n.a.	-1.21 [-2.44-1.03]	<0.001

P2G2, Tanner stage 2; P3G3, Tanner stage 3; P4-5G4-5, Tanner stage 4-5. n.a., not applicable. [a]: *P*-values were calculated using one-sample Wilcoxon signed rank test.

Table SIV. Univariate analysis of pretreatment absolute Inhibin B and testosterone levels by pubertal stage in boys with cancer.

Pubertal stage (n)	Inhibin B (ng/l)		Pubertal stage (n)	Testosterone (nmol/l)	
	median, [range]	p-value[a]		median, [range]	p-value[a]
Tanner 1 (162)	91 [20-422]	<0.001	**Tanner 1 (n.a.)**	n.a.	0.03
Tanner 2-3 (18)	152.5 [26-299]		**Tanner 2-3 (18)**	2.4 [0.1-23.6]	
Tanner 4-5 (20)	164.5 [47-314]		**Tanner 4-5 (20)**	8.0 [2.7-20.1]	

Tanner 1, prepubertal; Tanner 2-3, midpubertal; Tanner 4-5, postpubertal; [a]: *P*-value was calculated using the Kruskal-Wallis test. [b]: *P*-value was calculated using the Mann-Whitney *U*-test.

Table SV. Univariate analysis of pretreatment FSH standard deviation scores (SDS) and absolute FSH levels by diagnosis in pubertal boys with cancer (*n*=37).

Stage of puberty	FSH (nmol/l) median [range]	FSH SDS, median [range]	p-value[a]
P1G1 (162)	n.a.	n.a.	n.a.
P2G2 (10)	3.2 [0.9-5.2]	-0.1 [-0.5-0.5]	0.48
P3G3 (7)	3.5 [1.1-15.9]	0.2 [-0.4-3.4]	0.74
P4-5G4-5 (20)	3.6 [1.5-11.3]	-0.03 [-0.8-1.6]	0.90
Total group (37)	3.4 [0.9-15.9]	-0.04 [-0.8-3.4]	0.93

P2G2, Tanner stage 2; P3G3, Tanner stage 3; P4-5G4-5, Tanner stage 4-5 [a]: *P*-values were calculated using one-sample Wilcoxon signed rank test.

Table SVI. Univariate analysis of pretreatment LH standard deviation scores (SDS) and absolute LH levels by diagnosis in pubertal boys with cancer (*n*=38).

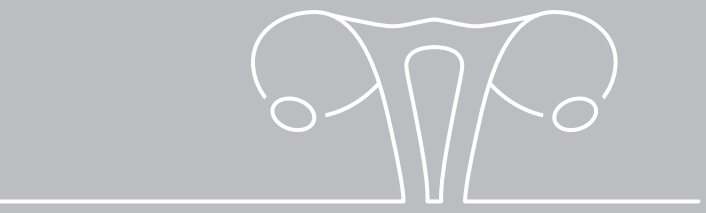
Stage of puberty	LH (nmol/l) median [range]	LH SDS, median [range]	p-value[a]
P1G1 (162)	n.a.	n.a.	n.a.
P2G2 (11)	1.1 [0.2-2.9]	-0.02 [-0.7-0.8]	0.48
P3G3 (7)	2.4 [1.1-9.7]	0.1 [-0.2-1.7]	0.24
P4-5G4-5 (20)	3.0 [1.1-7.7]	0.3 [-0.2-2.7]	0.001
Total group (38)	2.2 [0.2-9.7]	0.2 [-0.7-2.7]	0.01

P2G2, Tanner stage 2; P3G3, Tanner stage 3; P4-5G4-5, Tanner stage 4-5 [a]: *P*-values were calculated using one-sample Wilcoxon signed rank test.

Table SVII. Correlation between pretreatment Inhibin B and testosterone standard deviation scores (SDS) and markers of general health in boys with cancer (*n* = 224).

Pubertal Stage	Markers of general health	Inhibin B SDS, *R* (*p*-value)[a]	Pubertal Stage	Markers of general health	Testosterone SDS, *R* (*p*-value)[a]
Tanner 1 (162)	Body temperature (100)	-0.13 (0.22)	**Tanner 1 (162)**	Body temperature	**n.a.**
	CRP (91)	-0.08 (0.48)		CRP	
	Hb (157)	-0.04 (0.59)		Hb	
Tanner 2 (18)	Body temperature (7)	-0.57 (0.18)	**Tanner 2 (18)**	Body temperature (7)	-0.61 (0.15)
	CRP (10)	-0.38 (0.28)		CRP (10)	-0.02 (0.96)
	Hb (17)	0.49 (0.05)		Hb (17)	0.37 (0.15)
Tanner 3 (20)	Body temperature (13)	-0.16 (0.61)	**Tanner 3 (20)**	Body temperature (13)	0.30 (0.33)
	CRP (10)	0.25 (0.49)		CRP (10)	-0.12 (0.75)
	Hb (19)	0.07 (0.77)		Hb (19)	0.35 (0.14)
Total group (224)	Body temperature (130)	-0.10 (0.26)	**Total group (38)**	Body temperature (20)	-0.09 (0.70)
	CRP (123)	-0.10 (0.26)		CRP (20)	0.003 (0.99)
	Hb (215)	-0.06 (0.38)		Hb (36)	0.22 (0.21)

Tanner 1, prepubertal; Tanner 2, midpubertal; Tanner 3, postpubertal; CRP, C-reactive protein; Hb, haemoglobin, *R*, Correlation coefficient. [a]: Correlation coefficients and *p*-values were calculated using the Spearman rank correlation test.



CHAPTER 3

Changes in anti-Müllerian hormone and inhibin B in children treated for cancer

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ABSTRACT

Purpose: Diminished reproductive function can be a major late effect of childhood cancer treatment. This study evaluates the changes, and occurrence of possible recovery, in gonadal function markers in children treated for cancer.

Methods: Gonadal function markers were measured before (T0), directly after (T1) and one year after (T2) end of treatment of childhood cancer. Anti-Müllerian hormone (AMH) was measured in girls and inhibin B in boys and compared to reference populations. Repeated measures ANOVA and t-tests were employed for data analysis.

Results: Baseline gonadal function markers (T0) at diagnosis were available in 129 girls and 150 boys. Paired gonadal function markers were available in 49 girls and 54 boys for T0-T1, and in 27 girls and 32 boys for T1-T2. Gonadal function markers were significantly lower than the reference population at each time point ($p<0.001$). Postmenarcheal girls showed a decrease in AMH between T0 and T1 (SDS -0.72 to -1.32, $p=0.007$) and in the boys cohort a decrease in inhibin B (SDS -1.14 to -1.43, $p=0.045$) was observed. Impaired gonadal function levels ($<5^{th}$ percentile) at T1 were observed in 15 of 27 (56%) girls and in 15 of 32 (47%) boys. However, gonadal function had recovered at T2 in 7 girls and 6 boys.

Conclusions: Our data suggest that gonadal function is already compromised at diagnosis and is further decreased by childhood cancer treatment. Nevertheless, about half of the children with gonadal impairment recovered over time. Evaluation of gonadal function markers before one year after end of treatment may therefore be unreliable.

INTRODUCTION

Childhood cancer survival rates have improved significantly over the last decades, with 5-year survival rates averaging 80% in Western countries[1]. The increase in the absolute number of childhood cancer survivors (CCS) has caused a concomitant increase in the incidence of complications related to cancer treatment[2,3]. A well-known late effect of childhood cancer treatment is loss of gonadal function, which is especially observed after treatment with high doses of alkylating chemotherapeutics, stem cell transplantation and/or after radiation on the gonads[4,5]. In addition, we recently reported that already at diagnosis of childhood cancer, gonadal function markers were reduced in both boys and girls, indicating that gonadal function in children with cancer is affected not only by cancer treatment but also by the disease itself prior to treatment[6,7].

Children with cancer and their parents consider fertility an important future health issue and are often anxious to find out if their treatment has caused gonadal impairment[8,9]. To prevent unnecessary grief or unwarranted hope, knowledge on the trends of gonadal function and possibly the appropriate timing of analysis of gonadal status after childhood cancer would be valuable. However, longitudinal studies on the effects of cancer treatment on gonadal function in children are scarce.

In the assessment of gonadal function in men, semen analysis is considered the gold standard[5]. Inhibin B, mainly produced by Sertoli cells of the testis under influence of FSH, is a marker of spermatogenesis as it is positively correlated with sperm count and concentration in adulthood[10-12]. Given the substantial patient burden or impossibility of obtaining semen samples from young boys (by masturbation or electro-ejaculation), inhibin B is considered a feasible and adequate surrogate marker for gonadotoxicity in young boys[5,13-15].

To assess gonadal function in women, the gold standard combines endocrine measurements of FSH, LH, estradiol and progesterone with antral follicle counts and menstrual history[16]. In prepubertal girls, gonadotrophins and antral follicle count are unreliable as the hypothalamic-pituitary-gonadal axis might still be dormant. A useful marker that reflects gonadal function is anti-Müllerian hormone (AMH). AMH is produced by the granulosa cells of the preantral and small antral follicles in the ovaries and reflects the quantitative status of the ovarian reserve in adult women[17-20]. AMH levels rise during infancy until a peak in the mid-twenties, after which the levels slowly decline in parallel with the gradual decline in ovarian reserve, until the end of reproductive age[21,22]. Over the last decade both AMH and inhibin B have been used in clinical settings, and previous studies demonstrated its clinical usefulness to evaluate gonadal function in children with cancer[14,23,24].

The aim of the current study was to study the direct effect of disease and cancer treatment on gonadal function markers and to assess the frequency of gonadal function impairment in children before and after childhood cancer treatment. Furthermore, potential recovery of gonadal function markers and its determinants were explored.

MATERIALS AND METHODS

Patients

We included boys and girls newly diagnosed with cancer up and until the age of 18 years between January 2010 and January 2014, who had available remnant serum after routine work-up. Ovarian cryopreservation was not yet standard of care at that time. Patients with brain or germ cell tumors were excluded. Patients who received stem cell transplantation, experienced a relapse or who died were only measured at diagnosis. Details on age, gender, pubertal stage, diagnosis and treatment were retrieved from our local database. In boys, pubertal status was assessed clinically at diagnosis and classified as prepubertal (Tanner stage 1) or pubertal (Tanner stage 2–5)[25]. Girls were classified according to status before or after menarche. Treatment was categorized as low or high risk for gonadal function impairment (high-risk treatment defined as radiation on the abdomen and/or a cyclophosphamide equivalent dose (CED) score > 4000[26].

This study was approved by the Medical Ethics Committee Erasmus MC of Rotterdam, The Netherlands with waiver of the requirement for patients' written informed consent (MEC-2018-1399).

Hormone level assessment

Remnant material was collected if available before start of treatment (T0), directly after treatment (T1, median 1 month, range 0-6 months after end of treatment) and later after end of treatment (T2, median 10 months, range 6-23 months). Blood samples were stored at -80°C until analysis. AMH and inhibin B levels were measured at the end of the study at the Erasmus MC laboratory, Rotterdam, the Netherlands. This data was supplemented with clinically measured AMH and inhibin B levels, which was available in ~10% of the participants. In 2010, clinically measured AMH levels were obtained using an ultrasensitive ELISA (Immunotech-Coulter, Marseille, France). These AMH values were batch-by-batch adjusted to allow comparison with the currently used ELISA (commercially available as the Gen II Beckman Coulter, Beckman Coulter, Inc., Webster, TX, USA) which was employed at the end of the study enrolment. Intra- and inter-assay variation coefficients were <5 and < 10% respectively[27]. Age-matched reference values were based on a cohort with 250 healthy girls, whose samples were measured in the same laboratory using the same assays[22]. After the statistical analyses were performed, FSH, LH, estradiol and progesterone levels were measured in available remnant plasma to assess our hypothesis of the recovery of gonadal function.

Inhibin B levels were measured using an ELISA (Inhibin B Gen II ELISA kit; Beckman Coulter, Inc. Brea, CA, USA). Intra-assay variation coefficient was <5% over the whole measuring range and inter-assay variation coefficient was 5% (at the average concentration of 195 ng/L) to <15% (at the average concentration of 22 ng/L), respectively. Inhibin B levels were compared with age-matched reference values from previously published literature[13].

Statistical analysis

To account for age-dependency of the repeated gonadal function markers, standard deviation scores (SDS) were calculated using AMH and inhibin B reference values[13,22].

The one sample t-test was used to test a statistical difference between the mean SDS of the included participants at each time point as compared to the reference population. Repeated measure ANOVA was used to test differences in AMH SDS (for girls) and inhibin B SDS (for boys) at the three time points. Greenhouse–Geisser's epsilon adjustment was used in all cases when Mauchly's test indicated that the sphericity assumption had been violated. When significance was demonstrated in the repeated measure ANOVA, the paired t-test was employed for any significant main effect.

To examine the associations between patient and treatment characteristics and the gonadal function markers univariable linear regression was used. Variable groupings of the potential risk factors were selected based on clinical relevance and to assure adequate numbers of persons within groups for statistical power. Variables that were associated with the gonadal function maker with a p-value <0.20 in the univariable analysis were included in the multivariable linear regression model with the gonadal function markers in SDS as outcome. Analysis were repeated with the crude AMH (µg/L) and inhibin B (ng/L) levels. Results of both analyses were similar and, therefore, only the analysis of the SDS is reported.

A small change in SDS, especially in the higher regions, is unlikely to be of clinical relevance and could obscure the results. We therefore classified the gonadal function markers as a likely sign of gonadal function impairment when the markers were on or below the 5th percentile (or below a SDS of -1.645, the corresponding z-score) of the reference values. Univariable logistic regression analyses were used to identify potential determinants for gonadal function impairment. Within the group with impaired gonadal function markers directly after treatment (T1), the Mann-Whitney U test was used to compare the groups that did and did not show recovery at T2. Statistical analyses were performed with the Statistical Package for Social Sciences version 24.0 (SPSS, Chicago, IL, USA).

RESULTS

Baseline gonadal function markers were available in 129 girls and 150 boys with newly diagnosed childhood cancer. In total 49 girls and 59 boys had gonadal function markers available on at least two time points. The group with available longitudinal data was similar with respect to their baseline gonadal characteristics as the group without longitudinal data (Table 1). At diagnosis, the mean age of the girls was 7.6 year (range 0.9 – 17.4) and 75.5% of the girls were premenarcheal. Mean age at diagnosis of the boys was 7.3 year (range 0.6 – 16.2) and 79.7% of the boys were prepubertal (Table 1). Ovarian tissue cryopreservation was not offered in our hospital at this time yet to these newly diagnosed girls.

Table 1. Baseline characteristics of included children with childhood cancer with the total group of children with childhood cancer

	Total newly diagnosed children	Girls with AMH at diagnosis	Girls with paired AMH	Boys with Inhibin B at diagnosis	Boys with paired inhibin B
Total	437	129	49	150	59
Age at diagnosis (years)	8.1 (0.0-18.2)	8.7 (0.6 – 17.9)	7.6 (0.9 – 17.4)	7.6 (0.1-18.2)	7.3 (0.6-16.2)
Before treatment gonadal marker SDS (inhibin B or AMH)		-0.8 (-1.8 – 2.1)	-0.9 (-1.8 – 0.2)*	-1.1 (-3.7 – 2.0)	-1.1 (-3.7 – 0.8)*
Gonadal <p5		11 (8.5)	1 (2.0)	39 (26.0)	16 (27.1)
Gonadal <p10		25 (19.4)	6 (12.2)	60 (40.0)	21 (35.6)
Prepubertal/ menarcheal			37 (75.5)		47 (79.7)
Postpubertal/ menarcheal			8 (16.3)		6 (10.2)
Diagnosis					
- ALL & T-NHL	139 (31.8)		26 (53.1)		24 (40.7)
- AML	35 (8.0)		3 (6.1)		10 (16.9)
- B-NHL	15 (3.4)		2 (4.1)		6 (10.2)
- Hodgkin lymphoma	41 (9.4)		8 (16.3)		5 (8.5)
- Renal tumor	26 (5.9)		3 (6.1)		4 (6.8)
- Neuroblastoma	39 (8.9)		2 (4.1)		1 (1.7)
- LCH	21 (4.8)		1 (2.0)		2 (3.4)
- Ewing	15 (3.4)		2 (4.1)		2 (3.4)
- Osteosarcoma	4 (0.9)		0 (0.0)		0 (0.0)
- Sarcoma	31 (7.1)		1 (2.0)		5 (8.5)
- Other	34 (7.8)		1 (2.0)		0 (0.0)
Radiation					
- Cranial			1 (45.0)		2 (55.0 – 55.8)
- Chest			1 (19.8)		2 (19.8 – 45.0)
- Abdominal			3 (14.4-54.0)		3 (10.8 – 19.8)
- Other			1 (50.4)		1 (45.0)
CED in mg/m²					
- 0			12 (24.5)		23 (39.0)
- 0-<4,000			28 (57.1)		27 (45.8)
- ≥4,000			9 (18.4)		9 (15.3)

Data are expressed as mean (range) or frequencies (%). Radiation is depicted as n (range total dose in Gray). *difference in SDS between cohorts with one gonadal marker and with available follow-up markers: p-value 0.61 (boys) and 0.25 (girls). SDS, standard deviation score; AMH, anti-Müllerian hormone; ALL, acute lymphoblastic leukaemia; T-NHL, T-cell non-Hodgkin lymphoma; AML, acute myeloid leukaemia; B-NHL, B-cell non-Hodgkin lymphoma; LCH, Langerhans Cell Histiocytosis; CED, cyclophosphamide equivalent dose mg/m^2.

FSH, LH, estradiol and progesterone serum concentrations were not correlated with AMH or inhibin B levels. None of the children had elevated FSH levels above 10.0 U/L (data not shown).

Girls

Before start of treatment (T0), AMH levels were below the 50th percentile in 43 girls (87.8%) and 1 (2.0%) was below the 5th percentile. After a median of one month after treatment (range 0-6 months, T1), AMH levels were below the 50th percentile in 44 girls (89.8%), and below the 5th percentile in 21 (42.9%) girls (Figure 1).

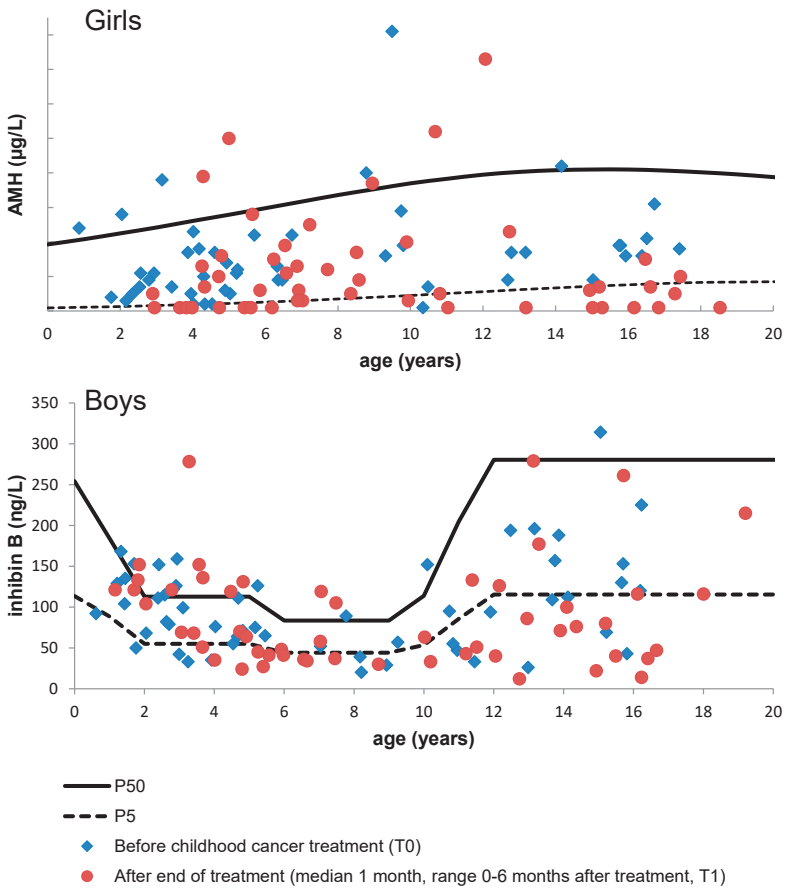

Figure 1. Girls' AMH and boys' inhibin B levels before and after cancer treatment
Anti-Müllerian hormone (AMH, upper figure) (in 49 girls) and inhibin B (lower figure, in 54 boys) levels before childhood cancer treatment (T0) and one month after childhood cancer treatment (T1; median 1month, range 0-6 months). P_{50} and P_5 refer to 50th and 5th percentiles of reference range populations.

Mauchly's test indicated that the sphericity assumption had not been violated (p=0.077), with a Greenhouse–Geisser's estimate of sphericity (Ɛ=0.844). The repeated measures ANOVA showed a significant difference in AMH over time (p=0.027). Paired analysis showed that the decrease in AMH levels in postmenarcheal girls between before treatment (T0: mean SDS -0.72) and one month after treatment (T1: mean SDS -1.32) was significant (p=0.007, Table 2). However, in premenarcheal girls this decrease was not significant (from mean SDS -1.00 to SDS -1.04, p=0.766). The decrease was significant (p=0.002) in the high-risk treatment group (from mean SDS -0.82 to SDS -1.44) (Table 2).

After treatment, between T1 and T2, AMH levels significantly increased from a mean SDS of -1.31 to a mean SDS of -0.87 (p=0.018). This increase was specifically observed in the premenarcheal subgroup and in those not in the high-risk treatment group, but there was no significant change in AMH levels in postmenarcheal girls and girls in the high-risk treatment group (Table 2). Table 3 shows the univariable and multivariable linear regression models for AMH levels at T1 an T2. After multivariate linear regression, no determinants remained significantly associated with AMH levels at T1 or T2.

Of the 27 girls with paired data at T1 (1 month after end of treatment) and T2 (10 months after end of treatment), 15 girls (56%) had AMH levels below the 5th percentile at T1 (Figure 2). Only 8 of these 15 girls still had AMH levels below the 5th percentile 10 months after end of treatment (T2) (positive predictive value 0.53). In the other 7 girls AMH levels increased to levels above the 5th percentile at T2. Of the 15 girls with gonadal impairment one month after treatment, 9 were premenarcheal and 7 of them showed recovery 10 months after end of treatment, while all 5 postmenarchal girls remained below the 5th percentile. Of the 15 girls with gonadal impairment 6 had been treated with high-risk treatment and 5 of these 6 girls remained below the 5th percentile (Supplementary Table 1).

Boys

Inhibin B levels were below the 50th percentile in 45 (83.3%) boys with newly diagnosed cancer (at T0) and below the 5th percentile in 16 (29.6%) boys. One month after treatment (T1) inhibin B levels were below the 50th percentile in 46 (85.2%) boys and below the 5th percentile in 27 (50.0%) boys (Figure 1).

Mauchly's test indicated that the sphericity assumption had been violated (p=0.015), with a Greenhouse–Geisser's estimate of sphericity (Ɛ=0.777). The repeated measures ANOVA did not show a significant difference in inhibin B over the three time points (p=0.168). Analysis of all paired samples available for T0 and T1, showed that the decrease in inhibin B levels between T0 (mean SDS -1.14) to T1 (mean SDS -1.43) was significant (p=0.045) (Table 2). After multivariable linear regression, only initial inhibin B levels remained significant determinants of inhibin B levels at T1 (Table 4). No statistical significant change in linear inhibin B levels was observed during follow-up after end of treatment (Table 2). Multivariable analysis of inhibin B at T2 showed only a significant association with the inhibin B levels (in SDS) at T1 (Table 4).

Table 2. Paired analysis of gonadal function markers in children with cancer

Girls	N	AMH at T0	AMH at T1	Change in AMH between T0 and T1 (95%-CI)	N	AMH at T1	AMH at T2	Change in AMH between T1 and T2 (95%-CI)
Total	49	-0.95 (0.07)†	-1.09 (0.09)†	-0.14 (-0.37 – 0.08)	27	-1.31 (0.06)†	-0.87 (0.19)†	0.44 (0.08 – 0.81)^
Pubertal stage at diagnosis								
- Premenarcheal	37	-1.00 (0.08)	-1.04 (0.12)	-0.04 (-0.32 – 0.24)	21	-1.29 (0.08)	-0.74 (0.24)	0.55 (0.08 – 1.01)^
- Postmenarcheal	8	-0.72 (0.13)	-1.32 (0.08)	-0.60 (-0.98 – -0.23)^	5	-1.35 (0.11)	-1.31 (0.10)	0.04 (-0.01 – 0.09)
CED score								
- 0	12	-0.81 (0.16)	-1.47 (0.10)	-0.66 (-1.01 - -0.31)^	8	-1.50 (0.09)	-0.63 (0.60)	0.87 (-0.46 – 2.20)
- > 0 – 4000	28	-1.02 (0.08)	-0.81 (0.14)	0.22 (-0.09 – 0.52)	13	-1.14 (0.09)	-0.84 (0.12)	0.30 (0.10 – 0.50)^
- > 4000	9	-0.89 (0.17)	-1.47 (0.09)	-0.58 (-0.98 – -0.19)^	6	-1.43 (0.12)	-1.23 (0.22)	0.20 (-0.20 – 0.59)
High-risk radiation field								
- No	44	-1.00 (0.07)	-1.06 (0.11)	-0.06 (-0.30 – 0.18)	25	-1.27 (0.06)	-0.82 (0.20)	0.45 (0.06 – 0.84)^
- Yes	5	-0.51 (0.18)	-1.41 (0.18)	-0.90 (-1.60 – -0.18)^	2	-1.79 (0.03)	-1.40 (0.26)	0.39*
High-risk treatment								
- No	38	-0.98 (0.08)	-0.99 (0.12)	-0.01 (-0.28 – 0.26)	21	-1.25 (0.07)	-0.72 (0.24)	0.53 (0.06 – 0.99)^
- Yes	11	-0.82 (0.14)	-1.44 (0.10)	-0.62 (-0.95 – -0.29)^	6	-1.53 (0.12)	-1.38 (0.12)	0.16 (-1.15 – 0.47)
Boys	**N**	**Inhibin B at T0**	**Inhibin B at T1**	**Change in inhibin between T0 and T1 (95%-CI)**	**N**	**Inhibin B at T1**	**Inhibin B at T2**	**Change in inhibin B between T1 and T2 (95%-CI)**
Total	54	-1.14 (0.14)†	-1.43 (0.16)†	-0.29 (-0.57 - -0.01)^	32	-1.44 (0.25)†	-1.19 (0.18)†	0.25 (-0.09 – 0.60)
Pubertal stage at diagnosis								
- Prepubertal	40	-1.07 (0.18)	-1.26 (0.20)	-0.19 (-0.53 – 0.15)	25	-1.22 (0.30)	-0.94 (-0.20)	0.28 (-0.14 – 0.70)
- (Post)pubertal	9	-1.31 (0.30)	-1.55 (0.26)	-0.24 (-0.88 - 0.41)	4	-1.86 (0.32)	-2.15 (0.31)	-0.29 (-1.52 – 0.94)
CED score								
- 0	19	-1.10 (0.25)	-1.29 (0.28)	-0.19 (-0.72 - 0.34)	17	-1.42 (0.35)	-0.96 (0.22)	0.47 (-0.08 – 1.02)
- > 0 – 4000	26	-1.15 (0.22)	-1.36 (0.26)	-0.22 (-0.63 – 0.19)	9	-1.15 (0.57)	-1.26 (0.43)	-0.12 (-0.86 – 0.63)

Table 2. Paired analysis of gonadal function markers in children with cancer (continued)

Girls	N	AMH at T0	AMH at T1	Change in AMH between T0 and T1 (95%-CI)	N	AMH at T1	AMH at T2	Change in AMH between T1 and T2 (95%-CI)
- > 4000	9	-1.20 (0.33)	-1.90 (0.26)	-0.71 (-1.44 – 0.03)	6	-1.94 (0.38)	-1.74 (0.40)	0.20 (-0.14 – 0.54)
High-risk radiation field								
- No	44	-1.13 (0.17)	-1.34 (0.19)	-0.21 (-0.53 – 0.11)	25	-1.40 (0.30)	-1.10 (0.21)	0.30 (-0.14 – 0.74)
- Yes	10	-1.19 (0.30)	-1.83 (0.25)	-0.63 (-1.30 – 0.03)	7	-1.60 (0.46)	-1.51 (0.41)	0.10 (-0.28 – 0.47)
High-risk treatment								
- No	44	-1.13 (0.17)	-1.34 (0.19)	-0.21 (-0.53 – 0.11)	25	-1.40 (0.30)	-1.10 (0.21)	0.30 (-0.14 – 0.74)
- Yes	10	-1.19 (0.30)	-1.83 (0.25)	-0.63 (-1.30 – 0.03)	7	-1.60 (0.46)	-1.51 (0.41)	0.10 (-0.28 – 0.47)

Gonadal function markers (AMH and inhibin B) are reported in standard deviation scores from reference populations. Data are presented as mean (standard error) or mean (95% confidence interval of the difference). †p-value <0.001 (one sample t-test). ^p-value <0.05 (paired samples t-test). *analysis was not performed because of small number of patients. SDS, Standard Deviation Score; AMH, anti-Müllerian hormone; T0, before start of treatment; T1, directly after end of treatment (median 1 month, range 0-6 months;. T2, median interval after end of treatment 10 months (range 6-23 months); CED score, Cyclophosphamide Equivalent Dose Score[40]; High-risk radiation field, (for iatrogenic hypogonadotropic or hypergonadotropic hypogonadism) is defined as radiation on the abdomen or cranium; High-risk treatment, is defined as radiation on the abdomen and/or a CED score > 4000.

Of the 32 boys with longitudinal data after treatment, 15 boys (47%) had inhibin B levels below the 5th percentile at one month after treatment (T1) (Figure 2). Nine of these 15 boys (positive predictive value 0.60) remained below the 5th percentile ten months after treatment (T2). Of the 15 boys with gonadal impairment one month after end of treatment 5 boys had been treated with high-risk treatment, and 4 of these 5 boys remained below the 5th percentile, while in the lower-risk treatment group 5 out of 10 boys recovered (Supplementary Table 1).

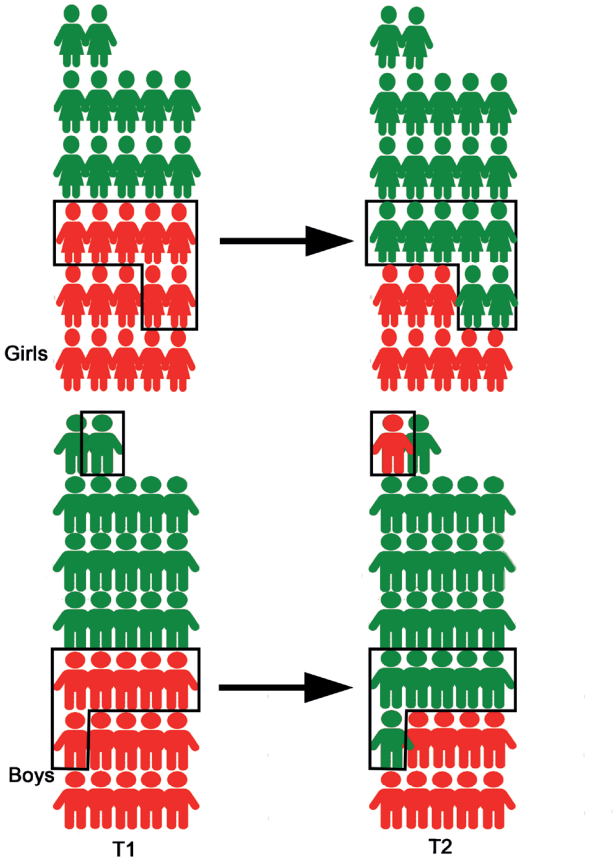

Figure 2. Recovery of gonadal function markers after cancer treatment
Infographic depicting the frequency of impaired gonadal function levels after childhood cancer treatment. Children with impaired levels (defined as ≤5th percentile of reference values (22, 41) are depicted in red, and children without impaired markers are depicted in green. Children were evaluated directly after end of treatment (T1, median 1 month, range 0-6 months) and later after end of treatment (T2, median 10 months, range 6-23 months). Above: paired levels of anti-Müllerian hormone after treatment were available in 27 girls. Of the 15 girls with impaired markers at T1, 7 did not have impaired levels anymore at T2. Below: paired levels of inhibin B after treatment were available in 32 boys. Of the 15 boys with impaired markers at T1, 6 did not have impaired levels anymore at T2, while one of the boys without impairment at T1 did have an impaired inhibin B level at T2.

Table 3. Association of patient and treatment factors with girls' AMH (in SD) at T1 and at T2

	Univariable		Multivariable	
AMH (SDS) (n=49) at T1	**B (95% CI)**	**p-value**	**B (95% CI)**	**p-value**
AMH (in SDS) at T0	0.13 (-0.29 – 0.54)	0.54		
Age at T0	-0.01 (-0.05 – 0.03)	0.66		
Pubertal stage at T0				
- Premenarcheal	1 (ref)			
- Postmenarcheal	-0.28 (-0.83 – 0.27)	0.31		
Interval between T0 and T1	0.39 (0.15 – 0.62)	<0.01	0.20 (-0.10 – 0.49)	0.18
CED score				
- 0	1 (ref)		1 (ref)	
- > 0 - 4000	0.66 (0.24 – 1.08)	<0.01	0.47 (-0.03 – 0.98)	0.06
- > 4000	0.001 (-0.54 – 0.54)	0.99	0.03 (-0.70 – 0.75)	0.95
High-risk radiation field				
- No	1 (ref)			
- Yes	-0.35 (-0.96 – 0.30)	0.28		
High-risk treatment				
- No	1 (ref)		1 (ref)	
- Yes	-0.45 (-0.91 – 0.01)	0.05	-0.03 (-0.69 – 0.63)	0.93
	Univariable		**Multivariable**	
AMH (SDS) (n=27) at T2	**B (95% CI)**	**p-value**	**B (95% CI)**	**p-value**
AMH (in SDS) at T0	0.40 (-0.37 – 1.17)	0.29		
Age at T0	-0.05 (-0.14 – 0.03)	0.18	-0.05 (-0.15 – 0.04)	0.26
Pubertal stage at T0				
- Premenarcheal	1 (ref)			
- Postmenarcheal	-0.56 (-1.61 – 0.46)	0.26		
Interval T0-T1	0.27 (-0.24 – 0.78)	0.28		
AMH (in SDS) at T1	1.17 (0.02 – 2.31)	0.05	1.14 (-0.12 – 2.41)	0.08
Age at T1	-0.05 (-0.14 – 0.03)	0.22		
Interval T1-T2	-0.57 (-2.70 – 1.56)	0.58		
CED score				
- 0	1 (ref)			
- > 0 - 4000	-0.21 (-1.15 – 0.73)	0.65		
- > 4000	-0.60 (-1.73 – 0.53)	0.28		
High risk radiation field				
- No	1 (ref)			
- Yes	-0.57 (-2.09 – 0.94)	0.44		
High-risk treatment				
- No	1 (ref)		1 (ref)	
- Yes	-0.66 (-1.58 – 0.27)	0.16	-0.01 (-1.17 – 1.15)	0.99

Variables with p-values < 0.20 from the univariable linear regression analysis were included in the multivariable linear regression model. AMH, anti-Müllerian hormone; SDS, Standard Deviation Score; T0, before start of treatment; T1, directly after end of treatment (median 1 month, range 0-6 months); T2, median interval after end of treatment 10 months (range 6-23 months); 95% CI, 95% confidence interval; CED score, Cyclophosphamide Equivalent Dose Score[40]; High-risk radiation field, is defined as radiation on the abdomen or cranium; High-risk treatment, is defined as radiation on the abdomen and/or a CED score > 4000.

Table 4. Association of patient and treatment factors with boys' inhibin B (in SDS) at T1 and at T2

	Univariable		Multivariable	
Inhibin B (SDS) (n=54) at T1	**B (95% CI)**	**p-value**	**B (95% CI)**	**p-value**
Inhibin B (in SDS) at T0	0.67 (0.42 – 0.93)	<0.01	0.62 (0.36 – 0.88)	<0.01
Age at T0	-0.07 (-0.14 – -0.01)	0.02	-0.04 (-0.10 – 0.01)	0.10
Pubertal stage at T0				
- Prepubertal	1 (ref)			
- (Post)pubertal	-0.28 (-1.24 – 0.67)	0.55		
Interval between T0 and T1	-0.25 (-0.65 – 0.16)	0.23		
CED score				
- 0	1 (ref)			
- > 0 – 4000	0.07 (-0.67 – 0.81)	0.85		
- > 4000	-0.53 (-1.56 – 0.49)	0.30		
High-risk radiation field				
- No	1 (ref)			
- Yes	-0.25 (-1.12 – 0.63)	0.57		
High-risk treatment				
- No	1 (ref)			
- Yes	-0.25 (-1.12 – 0.63)	0.57		
	Univariable		**Multivariable**	
Inhibin B (SDS) (n=32) at T2	**B (95% CI)**	**p-value**	**B (95% CI)**	**p-value**
Inhibin B (in SDS) at T0	0.58 (0.18-0.97)	<0.01	0.10 (-0.23 – 0.44)	0.53
Age at T0	-0.10 (-0.18 - -0.02)	0.02	-0.02 (-0.44 – 0.41)	0.94
Pubertal stage at T0				
- Prepubertal	1 (ref)		1 (ref)	
- (Post)pubertal	-1.21 (-2.29 - -0.14)	0.03	-0.77 (-1.76 – 0.22)	0.12
Interval T0-T1	-0.19 (-0.77 – 0.40)	0.52		
Inhibin B (in SDS) at T1	0.55 (0.36-0.73)	<0.01	0.68 (0.33 – 1.03)	<0.01
Age at T1	-0.10 (-1.17 - -0.03)	<0.01	0.03 (-0.40 – 0.47)	0.88
Interval T1-T2	-0.57 (-1.88 – 0.74)	0.38		
CED score				
- 0	1 (ref)		1 (ref)	
- > 0 – 4000	-0.31 (-1.18 – 0.57)	0.48	-0.58 (-1.33 – 0.17)	0.12
- > 4000	-0.79 (-1.80 – 0.23)	0.12	-0.15 (-0.91 – 0.61)	0.69
High-risk radiation field				
- No	1 (ref)			
- Yes	-0.41 (-1.33 – 0.52)	0.38		
High-risk treatment				
- No	1 (ref)			
- Yes	-0.41 (-1.33 – 0.52)	0.38		

Variables with p-values < 0.20 from the univariable linear regression analysis were included in the multivariable linear regression model. SDS, Standard Deviation Score; T0, before start of treatment; T1, directly after end of treatment (median 1 month, range 0-6 months); T2, median interval after end of treatment 10 months (range 6-23 months); 95% CI, 95% confidence interval; CED score, Cyclophosphamide Equivalent Dose Score[40]; High-risk radiation field, is defined as radiation on the abdomen or cranium; High-risk treatment, is defined as radiation on the abdomen and/or a CED score > 4000.

DISCUSSION

The results of this longitudinal study of childhood cancer, suggest that levels of gonadal function markers at the time of diagnosis are significantly lower compared to those in healthy age matched controls. Moreover, treatment causes an additional decrease in gonadal function markers. However, gonadal function markers recovered in about half of the patients.

The finding of decreased gonadal function markers at the time of diagnosis, even before treatment has started, confirms findings in smaller cohorts[6,7,28-31]. This indicates that gonadal function in children with cancer is affected not only by gonadotoxic treatment but also by the disease itself, as a possible effect of chronic disease. Similar findings were reported by Brougham et al in 22 pre- and postpubertal girls with slightly lower AMH levels prior to treatment[23]. The subsequent decrease in AMH levels during treatment is an expected finding that was also observed in other studies[32,33]. In other longitudinal studies addressing AMH before and after childhood cancer treatment, high-risk groups did not show recovery whereas the lower risk groups did recover[23,32,33]. This is in line with the results of our study, where more than half of the female survivors with impaired AMH levels directly after the treatment recovered after 1 year. In fact, the positive predictive value of an AMH level below the 5th percentile for persistent impairment up to ten months after treatment was very poor. Although this study was not powered to determine which groups have the highest odds for this recovery, our study confirmed the earlier findings of recovery in lower risk groups[23,32,33]. Moreover, after treatment, girls in a high-risk treatment group were more likely to stay below the 5th percentile of age-adjusted AMH levels when compared to girls in a low-risk treatment group.

Our observations also suggest that gonadotoxicity occurs more often in postmenarcheal girls and that premenarcheal girls are more likely to recover from a very low AMH level than postmenarcheal girls. This corresponds with the hypothesis that gonadotropin-releasing hormone (GnRH) agonists are effective in preventing ovarian function loss in young adult women treated for breast cancer with chemotherapy. Although debate on this topic is still ongoing, large randomized controlled trials indicate a protective effect of GnRH analogues[34-37]. Alternatively, the observation that premenarcheal girls seem to be less susceptible for long lasting gonadotoxicity could also be due to the fact that ovaries from premenarcheal girls have a larger pool of follicles, making them more resistant to chemotherapy.

The increase or recovery of AMH levels after cancer treatment exemplifies the dynamic nature of the female gonad. The ovarian pool is determined before birth and cannot be replenished. Therefore, the observed increase in AMH levels must indicate restoration of folliculogenesis and the concomitant growth of follicles that produce AMH. In terms of physiological senescence AMH can be referred to as a surrogate marker for quantitative

ovarian reserve, - although not of its qualitative status[20]. We feel that in the context of childhood cancer however, the term 'ovarian reserve' may be misleading and should be replaced by the more accurate 'ovarian function'. This term allows for a better description of the dynamic nature of the gonads as well as of AMH after childhood cancer.

Our finding that inhibin B levels in boys were lower than the range of the reference population at all time points contrasts with the results of a previously reported longitudinal study[14]. In this study, chemotherapy had only limited effect on inhibin B levels in prepubertal boys[14].

Previously, we showed normalization of mildly decreased inhibin B levels in very long-term adult survivors is possible, while a normalization in inhibin B levels did not occur in survivors who had levels below a critical level of 60 ng/L[38]. Although we here report a different cohort, it could be hypothesized that survivors with gonadal impairment that have some recovery potential, demonstrate their recovery only shortly after end of treatment. If recovery does not occur at that time, it is unlikely that they will do so later in life.

Inhibin B is secreted from the Sertoli cells which seem more susceptible to alkylating agents than Leydig cells. This explains why earlier studies reported normal levels of testosterone, produced in the possibly more cytotoxic resistant Leydig cells, in cancer survivors who did show compromised inhibin B levels[5,39]. The recovery of inhibin B levels could be a result of reactivation of Sertoli cell production of inhibin B, as a result of normalized gonadotrophins, or of restored function of seminiferous epithelium.

Although the number of patients analyzed in this study is larger than those in previous studies on this topic and presents longitudinal data for both boys and girls, the number is still relatively small for statistical analysis in subsets in this heterogeneous cohort. Future replication in a larger independent prospective cohort of childhood cancer patients with follow-up beyond 2 years after end of treatment compared to a healthy control group is necessary.

CONCLUSION

Our results indicate that in children diagnosed with cancer, levels of AMH in girls and inhibin B in boys are compromised at the time of diagnosis and are further decreased directly after treatment. However, AMH as well as inhibin B levels recover after one year in a relatively large proportion of children, especially in the lower-risk group and in premenarcheal girls. This has implications for how and when these children should be counseled regarding their future fertility. Evaluation of gonadal function markers within the first year after treatment may not be advisable.

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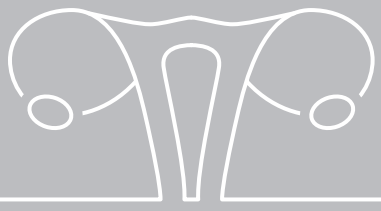
Suppl. Table 1. Recovery after AMH or inhibin B levels below the 5th percentile one month after treatment

	Girls N=15			Boys N=15		
	≤P_5 at T2	>P_5 at T2	p-value	≤P_5 at T2	>P_5 at T2	p-value
AMH or inhibin B (in SDS) at T0	-0.80 (-0.98 - -0.71)	-0.94 (-1.28 - -0.65)	0.54	-1.50 (-2.26 - -0.87)	-1.23 (1.87 - -0.61)	0.52
AMH or inhibin B (in SDS) at T1	-1.66 (-1.76 - -1.64)	-1.64 (-1.79 - -1.64)	0.46	-2.57 (-2.98 - -2.32)	-2.09 (-2.21 - -1.81)	0.11
Age at T0	13.47 (5.36 – 16.36)	4.17 (3.15 – 5.20)	0.09	11.44 (4.34 – 14.67)	5.17 (3.05 – 12.83)	0.36
Age at T1	14.98 (6.28 – 17.12)	5.60 (3.82 – 7.02)	0.05	12.73 (6.02 – 15.58)	5.56 (4.46 – 14.00)	0.22
Interval T0 and T1 (in yrs)	0.82 (0.69 – 1.47)	0.68 (0.41 – 1.99)	0.54	0.76 (0.59 – 2.00)	0.56 (0.36 – 2.013)	0.61
Interval T1 and T2 (in yrs)	0.93 (0.87 – 0.99)	0.97 (0.73 – 1.00)	0.16	0.86 (0.57 – 1.12)	0.93 (0.61 – 1.09)	0.69
Pubertal Stage						
- Prepubertal	2	7		5	4	
- Postpubertal	5	0		2	1	
Age category						
- 0-8 years	2	6		3	3	
- 8-14 years	2	1		4	2	
- >14 years	4	0		2	0	
CED score						
- 0	1	4		2	4	
- 0-4000	2	2		3	1	
- >4000	5	1		4	1	
Radiotherapy						
- None	7	5		5	7	
- Cranial	0	0		1	1	
- Chest	0	0		0	1	
- Abdomen	1	1		-	-	
- Other	0	1		-	-	
High risk radiation field						
- No	7	6		5	5	
- Yes	1	1		4	1	
High-risk treatment						
- No	3	6		5	5	
- Yes	5	1		4	1	

Data are expressed as median (interquartile range) for continuous variables, with p-values based on the Mann-Whitney U test. For categorical variables absolute numbers are depicted, on these variables analysis was not performed because of small number of patients. T0, before start of treatment. T1, directly after end of treatment (median 1 month, range 0-6 months). T2, median interval after end of treatment 10 months (range 6-23 months) SDS, Standard Deviation Score CED score, Cyclophosphamide Equivalent Dose Score. High-risk radiation field, is defined as radiation on the abdomen or cranium. High-risk treatment, is defined as radiation on the abdomen and/or a CED score > 4000. **P_5** refers to 5th percentile of reference range populations (41, 42).



CHAPTER 4

Longitudinal follow-up in female childhood cancer survivors: no signs of accelerated ovarian function loss

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ABSTRACT

Study question: Is the long-term decline of ovarian function, as reflected by a decrease in serum anti-Müllerian hormone (AMH) concentration, accelerated over time in female childhood cancer survivors (CCS) as compared to healthy women of the same age?

Summary answer: The median decline of AMH levels in long-term female CCS is not accelerated and similar to that observed in healthy controls.

What is known already: Gonadal function is compromised in female CCS treated with chemotherapy and/or radiation therapy. Ovarian function is most compromised in survivors treated with total body irradiation, abdominal or pelvic irradiation, stem cell transplantation or high doses of alkylating agents.

Study design, size, duration: Longitudinal single-centre cohort study in 192 CCS in Rotterdam, The Netherlands, between 2001-2014.

Participants/materials, setting, methods: Serum AMH levels of 192 adult female CCS were assessed, at least five years after cessation of treatment and at a follow-up visit with a median of 3.2 years (range: 2.1 -6.0) later and were compared to the age-based P_{50} of AMH in healthy controls.

Main results and the role of chance: Median AMH levels were below the P_{50} at both visit 1 (-0.59 µg/L) and at visit 2 (-0.22 µg/L).In women with a sustained ovarian function (AMH > 1.0 µg/L), the decline in AMH is similar to that in the normal population (difference in decline per year: -0.07µg/L (range: -2.86 - 4.92), p-value =0.75). None of the treatment modalities was correlated with a significant acceleration of decline of AMH per year.

Limitations, reasons for caution: We selected CCS that visited our late effects outpatient clinic and who had two AMH levels available. It is conceivable that women without any apparent late effects of treatment as well as women with extreme late effects, which might be the ones with the largest impact on ovarian function, could be more likely to be lost to follow-up. However, general characteristics did not differ between the included and excluded patients.

Wider implications of the findings: While prospective longitudinal research is required to strengthen our findings, they may help physicians to counsel female CCS about their expected reproductive lifespan.

INTRODUCTION

The number of childhood cancer survivors (CCS) is growing. These increased success rates confront health care providers with new challenges regarding long-term adverse health-related outcomes[1-3].

Potentially affected organ systems in survivors include the reproductive organs[4-6]. Gonadotoxicity in adult CCS varies depending on treatment modality and the administered dose[7-9]. Patients treated with total body irradiation, with or without stem cell transplantation, local irradiation on the gonadal area or high dosage of alkylating agents seem to carry a higher risk for gonadal impairment in the long-term[10-13]. Clinical manifestations of gonadal impairment include irregular menses, infertility or peri-menopausal complaints. Prior to clinical presentation, impaired gonadal function is preceded by low serum anti-Müllerian hormone (AMH) levels[14]. AMH is produced solely in the ovary by granulosa cells of small growing follicles and is considered to be a reliable marker of ovarian function[15,16]. Despite increasing knowledge from cross-sectional studies of direct treatment-related gonadotoxicity in CCS, little is known about the longitudinal changes in ovarian function over time. For instance, knowledge is lacking as to whether CCS show a more rapid loss of ovarian function compared to the normal population in the long-term. Considering the increase in age at which women have their first child especially in western societies, it is relevant to investigate whether CCS should be counselled about a potentially reduced reproductive lifespan and the individual implications of such shortening.

In adult male CCS the course of Inhibin B levels has been studied. Surprisingly, after initial impairment, recovery of male gonadal function was suggested long after treatment in subsets of male CCS with modest gonadal impairment (Inhibin B levels ≥ 105 ng/L)[17-19]. In female CCS only one 10-year follow-up study is available, showing a seemingly normal ovarian function after an interval of 10 years, but this analysis was performed in a small cohort of 35 women selected on the basis of having regular cycles which might constitute a group of CCS with a relative good prognosis[20].

As gonadotoxicity is considerable in CCS, it is conceivable that AMH may show a more rapid decline in female CCS than normally expected. In the current study, we investigated whether the decline in AMH levels in CCS differs from that observed in the healthy normal population.

MATERIALS AND METHODS

Subjects

This retrospective single centre study was performed in adult female CCS from the Erasmus MC- Sophia Children Hospital in Rotterdam who visited the adult late-effects outpatient

clinic. Survivors were diagnosed with a primary tumour between 1960 and 2005 and were in complete remission. Only CCS who visited our late-effects outpatient clinic twice or more between 2001 and 2014 were included. CCS were at least 16 years of age at first measurement of AMH and were at least 5 years after cessation of cancer treatment. A second blood sample was taken at least 2 years after the first sample. If more samples were available we used the two samples with the longest time interval between them. Patients over the age of 50 years and patients who had undergone a bilateral salpingo-oophorectomy were excluded.

Information on patients' characteristics, type of disease and treatment was retrieved from medical records. The alkylating agent dose (AAD) score was calculated to include the effect of high-risk chemotherapy as previously reported[9,17,21,22]. Patients not exposed to alkylating agents were assigned an AAD-score of zero.

Hormone assays

Peripheral blood samples were obtained while CCS visited the LATER outpatient clinic for patient care. Serum samples were taken randomly during the menstrual cycle. All serum measurements were performed in one laboratory at the Erasmus MC, Rotterdam, the Netherlands. In cohorts before 2011, AMH was measured with an ultrasensitive ELISA (Immunotech-Coulter, Marseilles, France). These AMH values were adjusted to allow comparison with the currently used ELISA (commercially available as the Gen II Beckman Coulter, Beckman Coulter, Inc., Webster, TX). Intra- and inter-assay variation coefficients were <5 and < 10% respectively[15]. The reference data were measured in a similar manner[23].

Statistics

To compare the longitudinal AMH levels of CCS with AMH levels of a healthy reference population of the same age, we used the cross-sectional data available from our earlier report[23]. The original data of this large Dutch reference population was not normally distributed for each age group. We could therefore not calculate Z-scores of AMH for our CCS. Moreover, due to the absence of sequential AMH data in a large group of healthy women, we could not compare slopes between CCS and the normal population. Instead, we calculated the difference between the observed AMH level and its specific age-based P_{50} in µg/L (observed AMH – age-based P_{50} AMH) for each measurement, and analysed the difference between visits 1 and 2.

In our full cohort, we analysed the difference between visits 1 and 2 with the related-samples Wilcoxon signed rank test. The Kruskal-Wallis test was used to compare the difference between visit 1 and 2 by age group and by type of irradiation, AAD score, pre- or post-menarche at diagnosis and stem cell transplantation. We identified five age groups in our previously reported nomogram[23] based on different slopes in AMH decline. The high AMH levels of women with polycystic ovary syndrome (PCOS) can possible skew our results.

We therefore additionally analysed the difference between visits 1 and 2 excluding patients with an initial AMH level > 5 µg/L and patients with an initial AMH level > 10 µg/L since information about follicle count and hyperandrogenism was not available.

Next, we stratified our cohort in two groups depending on baseline AMH levels, based on values considered clinically relevant for fertility. AMH levels below 1.0 µg/L were considered to be low, all other levels are considered not to be low and will be referred to as 'normal'. The group with an initial low AMH and the group with an initial 'normal' AMH were analysed separately, evaluating, e.g. the probability to stay 'normal' if your baseline AMH level was 'normal'. This was done for several patient characteristics and different treatment modalities, using a chi-square goodness-of-fit or Mann-Whitney U test for categorical and continuous variables, respectively. *P*-values <0.05 were considered statistically significant. Statistical analyses were performed with the Statistical Package for Social Sciences version 21.0 (SPSS, Chicago, IL, USA), part of the graphics was created with the free software environment R version 3.2.2 and GraphPad Prism version 7 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

Ethical approval

Informed consent was obtained from all included patients according to the standards of the Medical Institutional Review Board of Erasmus MC. This study was approved by the latter Review Board.

RESULTS

Initial AMH levels were available from 358 out of 460 female CCS visiting our outpatient clinic. The 192 adult female CCS in whom a second AMH measurement was performed were included in our study. Clinical characteristics and treatment details of the total cohort of female CCS of our centre and the survivors included in this study are shown Table I. The included sample is representative for the total cohort of female CCS of our centre although fewer survivors included in this study had received treatment without alkylating agents (AAD score = 0). A comparable percentage was treated with a higher cumulative AAD scores 3 and 4 which is known to be highly correlated with gonadotoxicity. Importantly, AMH levels at the first visit of the included women were similar (P = 0.69) to the available AMH levels of the full cohort.

The median time since cessation of treatment was 15.8 years (range: 5.0 – 43.2) at the first visit, the second visit was after a median interval of 3.2 years (range: 2.1 – 6.0). The median observed AMH level in the included CCS was lower than the age-based P_{50} level, both at the first visit and second visit: -0.59 µg/L (-4.07 to 17.05 µg/L) and -0.22 µg/L (range -3.75 to 20.50 µg/L), respectively (Fig. 1).

Table 1. Comparison of survivors with a second anti-Mullerian hormone (AMH) measurement and included in the study with the total group of female adult childhood cancer survivors (CCS).

	Total group of adult female CCS	Survivors included in this study	P-value[a]
Age at diagnosis (yrs)	5.4 (0.1-16.8)	6.1 (0-16.8)	0.04
Age at first visit (yrs)	21.4 (5.9-57.4)	23.6 (17.1-46.2)	<0.01
Age at second visit (yrs)	NA	26.9(20.0-49.2)	
Interval between stop treatment and first AMH level (yrs)	15.1 (4.0-43.2)	15.8 (5.0-43.2)	0.07
Interval between first and second visit (yrs)	NA	3.2 (2.1-6.0)	
BMI at first visit (kg/m^2)	22.9 (15.3-40.0)	23.0 (16.2-39.6)	0.83
AMH level at first visit (µg/L)	2.50 (0.00-25.90)	2.50 (0.00-21.01)	0.69
AMH level at second visit (µg/L)	NA	2.43 (0.01-24.03)	
Difference in AMH with P_{50}, first visit (µg/L)	NA	-0.59 (-4.07-17.05)	
Difference in AMH with P_{50}, second visit (µg/L)	NA	-0.22 (-3.75-20.50)	
Diagnosis n (%)			<0.01
ALL & T-NHL	128 (30)	69 (36)	
Acute myeloid leukemia	9 (2)	8 (4)	
B-cell non Hodgkin lymphoma	25 (6)	7 (4)	
Hodgkin lymphoma	29 (7)	15 (8)	
Sarcoma	57 (13)	15 (8)	
Renal tumour	47 (11)	25 (13)	
Neuroblastoma	32 (8)	24 (13)	
Germ cell tumour	12 (3)	0 (0)	
Brain tumour	52 (12)	10 (5)	
Other	34 (8)	19 (10)	
Radiotherapy			0.48
Abdominal radiotherapy	27 (7)	19 (10)	
Total body irradiation	13 (3)	7 (4)	
Chemotherapy (AAD score)			0.01
0	237 (56)	94 (49)	
1	47 (11)	26 (14)	
2	47 (11)	30 (16)	
3	70 (17)	34 (18)	
≥ 4	24 (6)	8 (4)	

Data are expressed as median (range) or frequencies NA, not applicable; AMH, anti-Müllerian hormone; BMI, Body Mass Index; ALL, Acute Lymphoblastic Leukemia, T-NHL, T-cell non Hodgkin lymphoma; AAD score, alkylating agent dose score. (%). [a]Comparison between groups by Mann-Whitney U-test for continuous outcome and Chi square test for categorical outcome.

Figure 2 represents a Boxplot of the calculated absolute differences between the observed AMH level and the expected age-based AMH level according to the 50th percentile

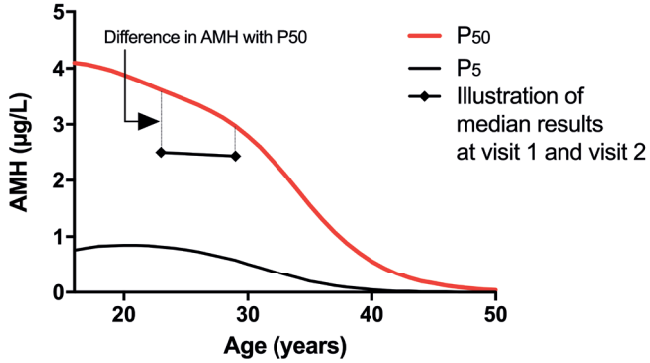

Figure 1. P_{50} and P_5 from healthy females (Lie Fong et al, 2012). Median results from our cohort (median age and median anti-Müllerian hormone (AMH) at first and second visit) are depicted. P_{50} and P_5 refer to 50^{th} and 5^{th} percentiles, respectively.

(P_{50}) of the healthy controls at visit 1 and at visit 2, for each age category. This difference did not vary significantly across the age categories (P-value 0.16) between visits 1 and 2 and our analysis was not stratified based on age.

Analysis of the AMH levels compared to the P_{50} of the normal healthy controls is presented in Table II. After a median follow-up interval of 3.2 years (range: 2.1-6.0 years), the median AMH levels were still below the P_{50} but the distance to the P_{50} was not increased. This indicates that the AMH levels of CCS remain well below the AMH levels of normal healthy controls, also at very long-term follow-up. However, there is no additional acceleration in the loss of AMH in CCS as compared to normal healthy controls (Fig. I). Whether the decrease in difference from the P_{50} is a clinically relevant observation requires more investigation.

Analysis of the cohort excluding women with a possible PCOS (based on AMH levels > 5 or > 10 µg/L) did not change these results (Supplementary Table I). In addition we analysed the effect of the treatment modalities on the distances to the P_{50}. These analyses showed similar results, i.e. no statistically significant association of any treatment modality with change in AMH (Table II).

There were 139 CCS (72%) who had an initial AMH level above 1.0 µg/L. In these women with a supposedly sustained 'normal' ovarian function after treatment, the decline in AMH is not different from what is known in normal healthy controls (difference in decline per year: -0.07 µg/L (range: -2.86 to 4.92), P = 0.75). In this group with retained ovarian function, 122 (87.8%, group D) remained above the threshold of 1.0 µg/L at follow-up (group D). Survivors with an AAD-score of 3 were more likely to show reduced ovarian function i.e. an AMH below 1.0 µg/L at follow-up (28.6% instead of 12.2%, P-value of difference 0.02), although this group is too small to draw definitive clinically relevant conclusions. No other risk factors in treatment modality could be determined (Supplementary Table II)

Table II. Analysis of difference between the observed anti-Müllerian hormone (AMH) value at visits 1 and 2 and the P_{50} of healthy peers stratified by treatment and menarche prior or after diagnosis, in female childhood cancer survivors.

	N	Difference with p50 at T1 (AMH µg/L) median (range)	Difference with p50 at T2 (AMH µg/L) median (range)[a]	P-value[b]
Total	192	-0.59 (-4.07 – 17.05)	-0.22 (-3.75 – 20.50)	0.04
Radiotherapy				0.68
No irradiation	123	-0.27 (-3.73 – 17.05)	-0.18 (-3.66 – 20.50)	
Abdomen/pelvis	7	-1.7 (-3.96 – 0.56)	-1.26 (-3.69 – 2.26)	
Half of abdomen	12	-0.54 (-2.39 – 13.81)	-0.43 (-3.36 – 16.54)	
Thorax	8	-0.88 (-4.07 – 2.82)	-0.30 (-2.10 – 10.63)	
Cranial and nerve system	27	-0.14 (-3.09 – 11.60)	-0.25 (-2.70 – 12.33)	
Total body irradiation	7	-3.35 (-4.02 - -2.31)	-3.16 (-3.75 - -2.30)	
Others	8	-0.88 (-3.23 – 2.67)	-1.17 (-2.60 – 7.01)	
Chemotherapy (AAD-score)				0.21
0	94	-0.08 (-3.88 – 13.81)	0.49 (-3.57 – 20.50)	
1	26	-1.20 (-3.91 – 17.05)	-0.72 (-3.16 – 18.53)	
2	30	-0.82 (-3.96 – 7.46)	-1.11 (-3.69 – 7.90)	
3	34	-0.95 (-4.07 – 14.46)	-0.83 (-3.75 -17.25)	
4	8	-1.18 (-3.73 – 6.98)	-0.14 (-3.66 – 4.77)	
Menarche				0.25
Pre-treatment	130	-0.69 (-4.07 – 17.05)	-0.26 (-3.75 – 20.50)	
Post-treatment	29	-0.88 (-3.88 – 4.22)	-0.21 (-3.66 – 10.63)	
Stem cell transplantation				n.a.
Yes	4	-3.44 (-4.02 – 2.31)	-3.37 (-3.75 - -2.30)	
No	132	-0.50 (-3.88 – 17.05)	-0.18 (-3.66 – 18.53)	

[a]After a median interval of 3.2 yr (range 2.1 – 6.0); T1, visit 1; minimally 5 years after stop treatment; T2, visit 2; minimally 2 years after T1; AAD score, Alkylating Agent Dose score; group 4, 4 or greater than 4. [b]Kruskal-Wallis test, testing the change in AMH over time; n.a., not applicable due to too small groups to test.

In our cohort, 53 CCS (28%) had an initial low AMH level (Table III, group A + B). Of these women, 60.4% still had a low AMH level at the second visit (group A), while 39.6%, mainly young CCS, increased towards an AMH level above 1.0 µg/L (group B).

Figure 3 shows the sequential AMH measurements of CCS in each group according to their AMH level at T1 and T2 in comparison to the AMH nomogram previously reported by[23]. This indicates that while the variation in AMH over time within the groups is substantial, most AMH levels remain within the normal range (P_5-P_{95}) during follow-up with the exception of the low-to-low group. The high-to-low group also fell below the fifth percentile at T2 but most began at relatively low serum levels.

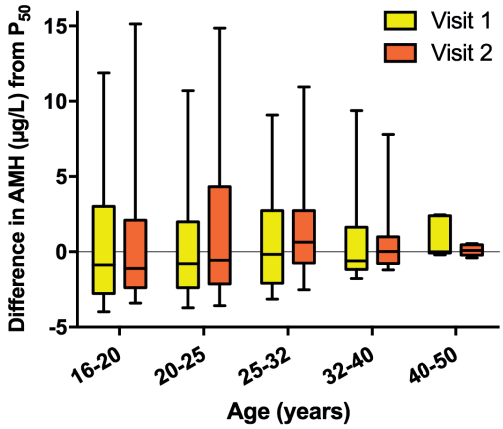

Figure 2. Difference in anti-Müllerian hormone (AMH) (in µg/L) from the observed AMH value of Childhood Cancer Survivors with the P50 of healthy women of same age at visit and 2 per age category. Horizontal small bars represent the 5-95th percentile range, and the boxes indicate the 25-75th percentile range. The horizontal line in each box corresponds to the median.

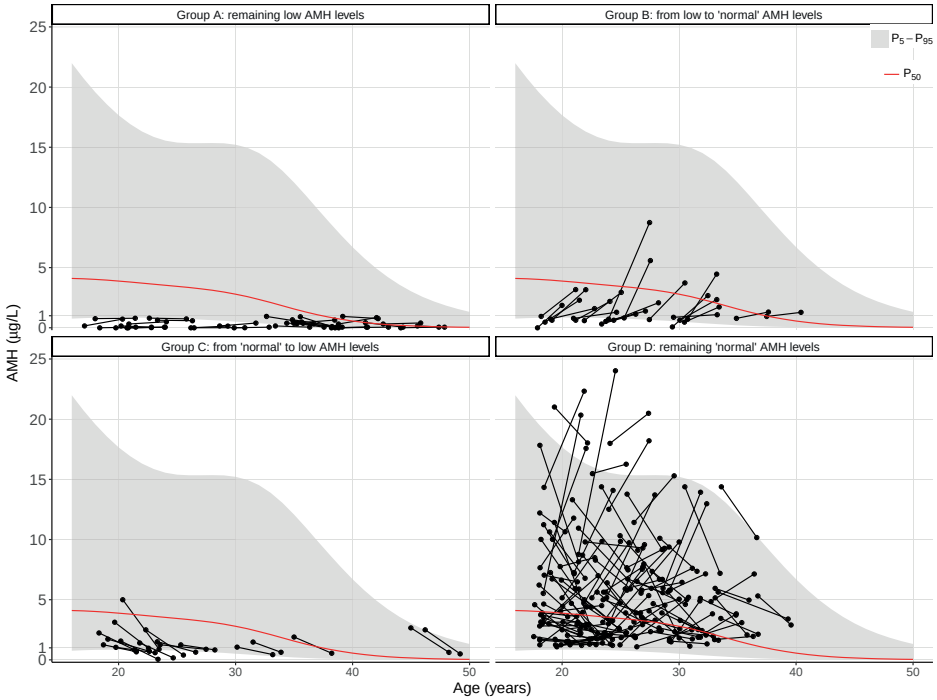

Figure 3. Individual longitudinal anti-Müllerian hormone (AMH) levels of childhood cancer survivors in each group according to their AMH level at visit 1 (minimally 5 years after stop treatment) and visit 2 (after an median interval of 3.2 years) in comparison to the AMH nomogram previously reported by (Lie Fong et al., 2012). Low AMH = <1.0 µg/L; Normal AMH = >1.0 µg/L; P_{50} and P_5-P_{95} refer to 50th and 5th until 95th percentiles, respectively, of healthy females.

Table III. Low or normal anti-Müllerian hormone (AMH) at second visit, groups stratified based on low or normal AMH at first visit.

		AMH first visit <1.0 µg/L N = 53 (28%)			AMH first visit >1.0 µg/L N = 139 (72%)	
Change per year of AMH compared to P_{50} (slope)		0.22 (-0.05– 2.40) [a]			-0.07 (-2.86-4.92)[c]	
	N	**Group A: AMH at T2 < 1.0 µg/L**	**Group B: AMH at T2 > 1.0 µg/L**	**N**	**Group C: AMH at T2 < 1.0 µg/L**	**Group D: AMH at T2 > 1.0 µg/L**
Age at T1	53	33.9 (17.1-45.6)	24.0 (17.9-37.5)[b]	139	22.3 (18.3-46.2)	23.0 (17.6-36.7)
BMI at T1	48	23.3 (16.2-39.6)	23.1 (16.6-34.7)	114	23.6 (18.8-39.4)	22.9 (17.1-37.2)
Interval T1-T2	53	3.2 (2.3-4.3)	3.0 (2.1-3.8)	139	3.0 (2.4-3.9)	3.2 (2.1-6.0)
AMH at T1	53	0.24 (0.00-0.94)	0.63 (0.00-0.94)[a]	139	1.57 (1.04-5.00)	4.93 (1.09-21.01)[a]
Overall incidence	**53**	**32 (60.4)**	**21 (39.6)**	**139**	**17 (12.2)**	**122 (87.8)**

Data are presented as median (range) or N (%). T1 = visit 1, minimally 5 years after stop treatment; T2 = visit 2, minimally 2 years after T1; BMI, Body Mass Index; Mann–Whitney U-test for change per year of AMH compared to P_{50} (slope); [a]p-value <0.001; [b]p-value = 0.02; [c]p-value = 0.75

DISCUSSION

The current study indicates that CCS seem to experience a single assault on their ovarian function caused by their disease and/or treatment. While this initial impairment is still evident after long-term follow-up, the decay of ovarian function seems not to be accelerated after the initial impairment has occurred. Given the gonadal impairment, CCS follow a similar rate of decline over time as compared to normal healthy controls.

While this study was not designed to detect early onset of menopause, our data do not give reason to assume CCS have an earlier onset of menopause other than would already be expected based on their, on average low, age-specific AMH levels. Long-term follow-up studies assessing ovarian function over time in female CCS are scarce. Shorter follow-up has been done quite extensively[9,12,24]. To our best knowledge only one previous study has examined ovarian function in CCS after 10 years of follow-up[20]. This study reported the comparison of ovarian function only of the 30 women with regular menstrual cycles, which might represent a relatively healthy cohort of CCS. Our study confirmed these findings, in a larger cohort, indicating that no further deterioration of ovarian function is to be expected after the initial impairment[20].

Chemotherapy has been identified as a key risk factor for ovarian impairment, and the extent of gonadotoxicity is related to cumulative dose[7-9,11,25]. However, neither chemotherapy, nor any treatment modality, was found to be associated with a change in AMH levels at long-term follow-up in our cohort.

AMH represents the activity of small antral follicles and is the best measure of ovarian function for different clinical conditions currently available[26,27]. AMH levels are influenced by several factors that have an impact on ovarian follicles. For instance, women with PCOS are known to reveal higher AMH levels due to a surplus of antral follicles while women with endometriosis and/or medical history of surgery of the ovaria generally have lower AMH levels. The variation of AMH levels throughout the menstrual cycle is generally believed to be limited[15,28]. The influence of oral contraceptives on AMH levels is still debated, with studies showing decreased AMH levels[14,28,29] in contrast to others indicating no effect at all of hormonal contraceptives[26,27,30-32]. In this study, we did not adjust for oral contraceptive usage due to missing data.

It has been established that even in CCS with low circulating AMH levels pregnancies can occur[20,33]. This suggests that a low AMH value in young CCS may still be accompanied by a relatively good oocyte quality in contrast to older healthy women with a similar low AMH value and poor oocyte quality as a result of cumulative acquired damage during most of their reproductive period. Therefore, despite a low AMH, oocyte quality seems not to be compromised as in older women. Indeed, data, albeit scarce, on pregnancy rates in these CCS do indicate virtually normal chances for successful conception[34]. In addition, our study suggests a possible clinical relevant recovery of ovarian function in almost 40% of the CCS initially showing signs of gonadal impairment. Such a recovery is seen mainly in young women under the age of 32 years. Even though this finding could be partly attributed to varying storage or assay conditions[35] and regression towards the mean, this observed phenomenon underlines the importance of counselling patients with low AMH levels about their fertility and the risk of (unintended) pregnancy. This study suggests that in CCS, after initial impairment of ovarian function, the decline in ovarian function is not accelerated compared to normal healthy fertile women. We hypothesize that the stabilization of ovarian function as measured by serum AMH levels is due to impairment of only part of the ovarian reserve. The remaining smaller yet undamaged ovarian pool may develop and decrease similar to that observed in normal healthy women.

There are certain limitations that must be taken into account when interpreting these data. We selected CCS that visited our late effect outpatient clinic and had two AMH levels available. It is conceivable that women without any apparent late effects of treatment could be more prone to become lost to follow-up as well as woman with extreme late effects (such as secondary neoplasms) and accompanying morbidity or even mortality. The latter group might constitute the one with the largest impact on ovarian function. However, general characteristics did not differ between the excluded patients with only one AMH level and the included patients with multiple available AMH levels. Due to the absence of normal sequential data on AMH during a woman's reproductive lifespan, we used the cross-sectional data available from our earlier reports[23]. We could therefore not compare the slopes between CCS and the normal population, but we were able to assess

the change in distance to the P_{50} at each time point. Based on our sample size of n=192 and assuming a normal distribution with a mean difference of 0.55, a standard deviation of 3.30 and an alpha of 0.05, the beta (type II error) would be 0.7 (power 30%). This indicates that the current study lacked power to completely rule out a false negative conclusion, i.e. that there is an acceleration of ovarian function loss while we did not find one. We feel our data nevertheless conveys an important message: in our cohort, we observed no additional decline in in AMH in CCS compared to healthy women. We recommend confirming our data in larger prospective cohort studies with a healthy reference group. This would also enable to include various treatment modalities as independent variables.

The presented study was conducted to investigate the longitudinal decline of ovarian function in female CCS at very long-term follow-up. Our data showed that after initial impairment due to cancer treatment, the further decline of AMH levels in long-term female CCS is not accelerated. Moreover, no treatment modality caused an increased extra risk for accelerated depletion of the primordial follicle pool. Of CCS with an initial AMH level in the clinically normal range, 88% were still within the normal range at long-term follow-up. The results from this study can provide improvement of the counselling patients must receive before their treatment starts and at long-term follow-up, regarding their expected fertile lifespan.

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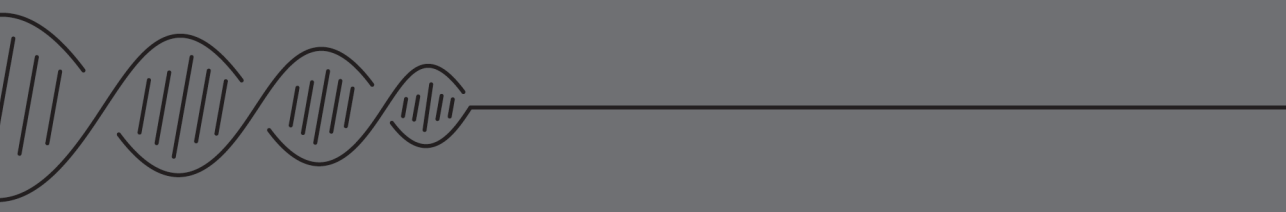
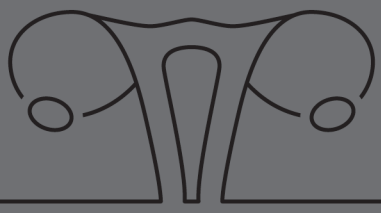
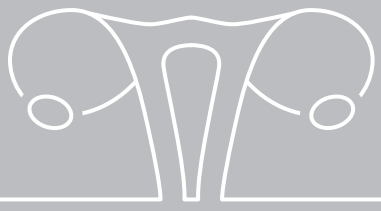




PART II

Genetic aspects of ovarian function



CHAPTER 5

The influence of genetic variation on late toxicities in childhood cancer survivors: a review

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ABSTRACT

Introduction: The variability in late toxicities among childhood cancer survivors (CCS) is only partially explained by treatment and baseline patient characteristics. Inter-individual variability in the association between treatment exposure and risk of late toxicity suggests that genetic variation possibly modifies this association. We reviewed the available literature on genetic susceptibility of late toxicity after childhood cancer treatment related to components of metabolic syndrome, bone mineral density, gonadal impairment and hearing impairment.

Methods: A systematic literature search was performed, using Embase, Cochrane Library, Google Scholar, MEDLINE, and Web of Science databases. Eligible publications included all English language reports of candidate gene studies and genome wide association studies (GWAS) that aimed to identify genetic risk factors associated with the four late toxicities, defined as toxicity present after end of treatment.

Results: Twenty-seven articles were identified, including 26 candidate gene studies: metabolic syndrome (n=6); BMD (n=6); gonadal impairment (n=2); hearing impairment (n=12) and one GWAS (metabolic syndrome). Eighty percent of the genetic studies on late toxicity after childhood cancer had relatively small sample sizes (n<200), leading to insufficient power, and lacked adjustment for multiple comparisons. Only four (4/27=15%) candidate gene studies had their findings validated in independent replication cohorts as part of their own report.

Conclusion: Genetic susceptibility associations are not consistent or not replicated and therefore, currently no evidence-based recommendations can be made for hearing impairment, gonadal impairment, bone mineral density impairment and metabolic syndrome in CCS. To advance knowledge related to genetic variation influencing late toxicities among CCS, future studies need adequate power, independent cohorts for replication, harmonization of disease outcomes and sample collections, and (international) collaboration.

INTRODUCTION

Survival rates after childhood cancer now approach 80% in developed countries as a result of enhanced stratification, more effective treatment and optimized supportive care[1]. The increasing number of childhood cancer survivors (CCS) has led to the growing awareness of chronic health effects resulting from treatment for childhood cancer[2,3]. Examples of long-term consequences include hearing impairment, gonadal impairment and cardiotoxicity. The inter-individual variability in the number and magnitude of health problems in similarly treated CCS suggests that genetic variation modifies the association between treatment and risk of late toxicity.

To identify such genetic variants two common approaches have been applied: a candidate gene approach, and more recently, the genome wide association study (GWAS) approach. Candidate gene studies focus on associations between genetic variation within pre-specified genes of interest and specific outcomes, while GWASs are hypothesis-free searches that can identify novel single-nucleotide polymorphisms (SNPs) that potentially modify the risk of a late toxicity.

After completion of the Human Genome Project (HGP)[4] in 2003 and the International HapMap project, GWASs have discovered many thousands of genetic variants associated with a variety of diseases[5], which catalyzed research on genetic variation underlying late toxicity among cancer survivors[6]. Except for cardiotoxicity[7], the resulting number of genetic variation studies in CCS have not produced unambiguous evidence in this field. The lack of strong evidence has impeded translation into clinical practice, such as patient counseling or dose-reduction trials. In contrast, genotyping of childhood cancer patients in order to risk-adapt treatment based on risk models predicting susceptibility to specific (direct and late) toxicities is expected to become standard of care. A comprehensive review of genetic aspects of acute toxicity was recently published[8]. However, a recent overview of genetic susceptibility studies concerning late toxicities in CCS is not yet available.

An international collaboration is currently working on the identification of genetic determinants associated with hearing impairment and female gonadal impairment, in a large cohort of CCS (European Union's Seventh Framework programme project PanCareLIFE). In the current study, we summarize the results of a systematic literature search and evaluate the results and quality of available literature on genetic susceptibility of these two late toxicities (hearing impairment and female gonadal impairment) and three hormone-related late toxicities (male gonadal impairment, metabolic risk factors and bone mineral density impairment).

METHODS

Search strategy

To provide an overview of the established genetic susceptibility factors associated with late toxicities in childhood cancer survivors, we identified relevant articles, published up until September 2017, by systematically searching Embase, Cochrane, Google Scholar, MEDLINE and Web of science. Details of the full search strategy for each database are included in Appendix I. The computer-based searches were conducted by a medical information specialist at the university medical library in the Erasmus Medical Center.

Definitions

The majority (>80%) of the cohort in every article had to be diagnosed with cancer ≤21 years of age. As we were specifically interested in 'late' toxicity, defined as toxicity still apparent at follow-up after end of treatment, we only included studies that evaluated metabolic risk factors, bone mineral density, gonadal impairment or hearing impairment in CCS present after end of treatment regardless of follow-up time. Definition of endpoints used by the authors were extracted from the corresponding papers and assembled in tables.

Study selection

Two independent investigators (EC and ALFvdK) reviewed all titles and abstracts, and independently selected potentially eligible studies. Case series, case reports, abstracts or reviews were excluded. Only studies published in English were selected for the analysis. Disagreements were resolved through consensus. Full text papers were retrieved to assess fulfilment of the selection criteria (Figure 1). Cross reference check was performed to identify additional studies that were potentially overlooked during the initial search. Authors were contacted to clarify or supplement their results where necessary.

RESULTS & DISCUSSION

The search strategy yielded 2,762 unique records (Figure 1). After screening titles and abstracts, 156 articles were selected for detailed evaluation of full texts. After full-text review, 56 articles remained that reported on late toxicities. For the purpose of the current review we focused on gene-association studies of metabolic syndrome, low bone mineral density, and gonadal impairment and hearing impairment. As a result, 27 articles were considered in this review, including seven studies on metabolic syndrome (six candidate gene studies and one GWAS), six candidate gene studies of low bone mineral density, two candidate gene studies of gonadal impairment, and 12 candidate gene studies of hearing impairment (Table 1-4).

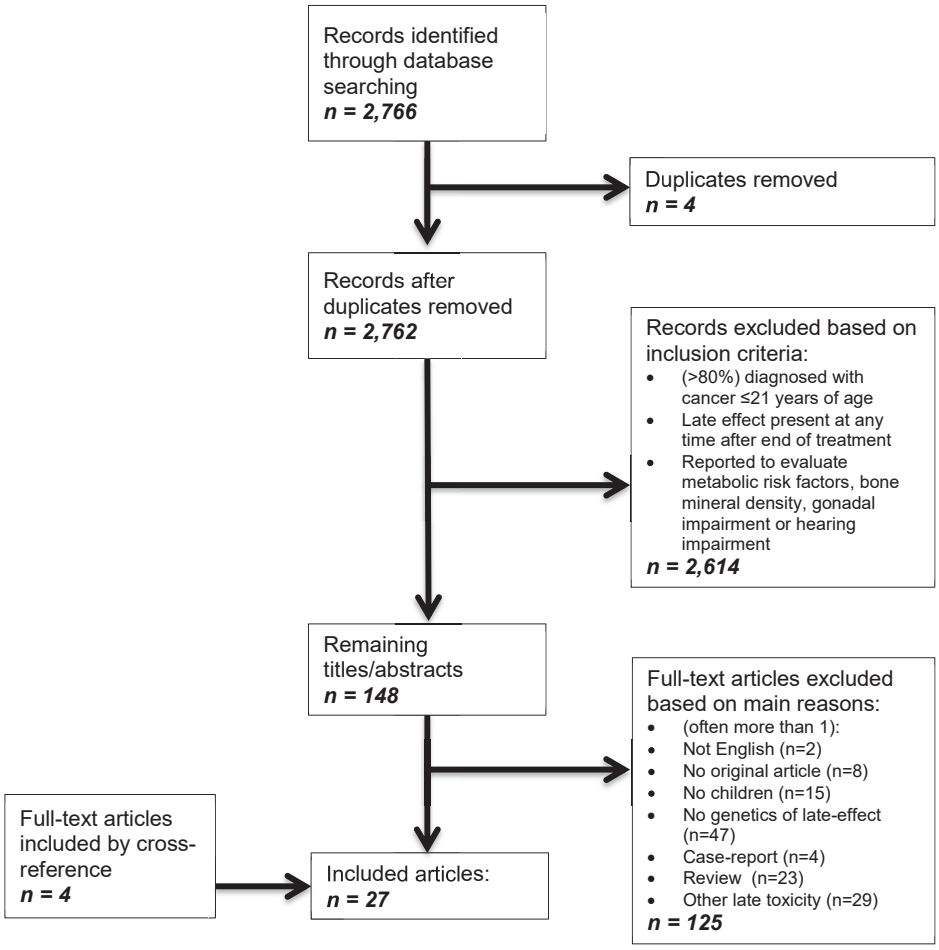

Figure 1. Flowchart study selection process Review Genetics of Late Effects

Of the candidate gene studies, 50% (13/26) had less than 100 participants while 80% (21/26) had less than 200 participants. Only two included a cohort of more than 500 CCS (n=532 and n=600)[9,10]. Only six of the candidate studies (23%) adjusted for multiple testing to reduce the chance of type I error (false positive results), which would take into account the multiple models tested[11-16]. One candidate study investigated both metabolic syndrome and bone mineral density[9]. Where possible, the multivariable analysis of the combined results of the discovery and replication cohort are reported (Tables 1-4). Where applicable, the adjusted p-value corrected for multiple testing was reported.

Table 1. Overview of studies on the influence of genetic variation on components of metabolic syndrome in CCS

		Study population					Analyses							
Study	**Method**	**Cohort size (cases/ control)***	**Country of origin; ethnicity**	**Gender (% males)**	**Tumor type**	**Treatment**	**Replication**	**Definition endpoint**	**Studied no of SNPs (adj for multiple testing)**	**Gene / region**	**Variant**	**Effect allele/ genotype**	**Multivariate analysis adjust for:**	**OR**	**P-value**
Wilson et al. 2015	GWAS	1996 (723/1273)	USA; 86.5% white, 12.5% black	51	Solid and hematological	CRT	Yes	Obesity BMI ≥ 30 kg/m^2	N/A	SOX11	rs4971486	G	race; age at follow-up, age at diagnosis, chest/abdominal/ pelvic radiation, glucocorticoid, alkylating agents, obesity at diagnosis	2.01 (1.50-2.71)	**3.5E-6**
						CRT				GLA3	rs4530610	C		0.55 (0.40-0.76)	**0.0004**
						CRT				CDH18/ BASP1	rs2923762	G		1.78 (1.40-2.26)	**2.6E-6**
						CRT				FAM155A	rs3566997	G		0.57 (0.44-0.74	**2.8E-6**
						No CRT				VPS45	rs12073359	C		1.56 (1.24-1.96)	**0.0008**
Sawicka-Zkowska et al. 2013	Cand. gene	74	Poland; 100% Caucasian	61	ALL and lymphoma	22% CRT	No (no replication in another CCS cohort)	leptin levels (linear)	1	LEPR	rs1137101	GG	total BMD SDS, spinal BMD SDS, lean mass SDS, cranial radiotherapy	NA	0.0952
Van Waas et al. 2013	Cand. gene	532	Netherlands; 100% Caucasian	55	Solid and hematological	16% CRT	No	Hypertension: blood pressure ≥ 140/90 mmHg. MetS: two of the following: blood pressure ≥ 140/90 mmHg; BMI ≥30 kg/m2; self-reported prevalence of diabetes or medication; serum total cholesterol ≥ 5.2 mmol/l or medication	7 (no multiple testing)	ATP2B1	rs2681472	CT vs TT	Age, gender, educational level, follow-up time, abdominal and cranial irradiation	None significant	n.s.
								Hypertension: blood pressure ≥ 140/90 mmHg. MetS		ATP2B1	rs2681492	GA vs AA			
								Waist circ: female above/ below 88 cm, males above/ below 102 cm. MetS		TFAP2B	rs987237	AG vs AA			
								Waist circ, Mets		MSRA	rs7826222	CG vs GG			
								Diabetes: using medication for diabetes, MetS		JAZF1	rs864745	AG vs AA			
								Diabetes, MetS		THADA IRSI	rs758597 rs2943641	TC vs TT CT vs CC			

Table 1. Overview of studies on the influence of genetic variation on components of metabolic syndrome in CCS (continued)

		Study population					Analyses								
Study	**Method**	**Cohort size (cases/ control)***	**Country of origin; ethnicity**	**Gender (% males)**	**Tumor type**	**Treatment**	**Replication**	**Definition endpoint**	**Studied no of SNPs (adj for multiple testing)**	**Gene / region**	**Variant**	**Effect allele/ genotype**	**Multivariate analysis adjust for:**	**OR**	**P-value**
Surapolchai et al. 2011	Cand. gene	131 (IR 40/91 and IGT 10/121)	Thailand; (ethnicity not specified)	59	127 (97%) ALL and 4 (3%) Lymphoblastic lymphoma	low/standard/ high ALL risk stratification	No	Impaired glucose tolerance: fasting plasma glucose level of 101 to 126 mg/dL and a 2-hour plasma glucose level of 140 to 200 mg/dL. Insulin resistance: whole body insulin sensitivity index < 5^27	6 (no multiple testing)	PAX4 TCF7L2	rs2233580 rs12255372	AG vs GG GT vs GG	age at follow-up	5.28 (1.06-26.40) n.s.	**0.043** n.s.
Skoczen et al. 2011	Cand. gene	77 (24/53)	Poland; (ethnicity not specified)	55	ALL	BFM/NewYork treatment regimens	No	BMI (≥ 85th percentile)	3 (no multiple testing)	LEPR LEPR Leptin gene	- 	Gln/Gln Arg/Arg vs Arg/Gln Gln/Gln G>A	None	NA	n.s.
Skoczen et al. 2011	Cand. gene	191 Polish ALL CCS (40/151)	Poland; (ethnicity not specified)	48	ALL	BFM/NewYork treatment regimens	No	BMI (≥ 85th percentile)	1 (no multiple testing)	FTO	rs9939609	AA	Stratification for treated with CRT (12-24 Gy) yes/no	0.24 (0.08-0.7)	**0.016**
Ross et al. 2004	Cand. gene	600 (278/322)	USA; non-hispanics	51	ALL	≥20 Gy CRT (females)	No	BMI ≥ 25 kg/m^2	1 (no multiple testing)	LEPR	GlnQ223Arg	Arg/Arg vs Arg/Gln and Gln/Gln	Stratification for treated with CRT ≥ 20 Gy in females, adjusted for age at diagnosis	6.1 (2.1-22) (effect only in females)	**0.002**

Abbreviations: CRT: cranial radiotherapy; HT: hypertension; IGT: impaired glucose tolerance; MS: metabolic syndrome (defined by blood pressure ≥140/90 mmHg, BMI ≥30 kg/m^2, self-reported prevalence of diabetes or serum total cholesterol ≥5.2 mmol/l); N/A: not applicable; NA: not available

*indicated is the cohort size (cases and controls), as defined by the authors of the original article

P-values in bold are considered statistically significant by the authors of the original article. Ethnic race is stated if reported in original article.

Where applicable, the multivariable analysis of the combined results of the discovery and replication cohort are reported. If no replication cohort was included, multivariable analysis of the discovery cohort is reported, or univariate analysis of the discovery if multivariable analysis was missing

Where applicable, the adjusted p-value corrected for multiple testing was reported

Metabolic syndrome components

The prevalence of components of the metabolic syndrome, including obesity, hypertension, dyslipidemia and type 2 diabetes (or specifically hyperglycemia or hyperinsulinemia), has been reported to be higher in CCS compared to the general population[17-20]. Six candidate gene studies and one GWAS investigated polymorphisms associated with different aspects of metabolic syndrome. The polymorphisms in the candidate gene studies had been identified previously in GWASs performed in the general population or were based on the genes coding for hormones (or its receptor) associated with obesity. No studies addressed the genetic susceptibility of dyslipidemia. The only variants that had been investigated in multiple independent cohorts (Table 1) were variants within the gene coding for the leptin receptor (*LEPR)* which were evaluated because of their hypothesized functional contribution to obesity.

Leptin, a hormone secreted in adipocytes, has a key role in increasing satiety and energy homeostasis[21]. Leptin insensitivity has been reported to be associated with obesity, leading to the hypothesis that obesity in CCS may be influenced by a carrier status of polymorphisms in the leptin receptor (*LEPR*)[10]. Only one[10] of the three independent candidate gene studies in CCS that investigated the leptin pathway found a statistically significant correlation between a polymorphism in *LEPR* (GlnQ223Arg) and higher odds of being obese[10,22,23]. The effect was sex-dependent and after stratification on sex, it was only significant in females (n=294, OR 2.5 95% CI 1.3-4.8) and not in males (n=306). In addition, in the female subgroup, a significant interaction with cranial radiation (>20 Gy)[10] was observed, suggesting that the impact of the polymorphism is especially prominent in female survivors who were treated with cranial irradiation. The impact of cranial irradiation can for a large part be attributed to the subsequent increased risk for growth hormone deficiency. The association between the GlnQ223Arg *LEPR* polymorphism and obesity has not been validated in the other two candidate gene studies[22,23], although cranial radiotherapy did amplify the association with leptin levels[22,23]. These two studies were small (77 and 74 survivors, respectively) as compared to the study by Ross (600 survivors)[10], which suggests that this inconsistency may be due to lack of power, especially considering the possible need for stratification for sex, which both studies did not carry out[22,23]. Alternatively, this discrepancy in results could be due to a false positive result in the initial study by Ross et al, which did not include an independent replication cohort.

Using a candidate gene approach based on polymorphisms identified in GWASs in the general population, the association of seven polymorphisms (rs2681472, rs2681492, rs987237, rs7826222, rs864745, rs758597, and rs2943641) with respect to hypertension, waist circumference, diabetes and metabolic syndrome (defined as blood pressure ≥140/90 mmHg; BMI ≥30 kg/m^2; self-reported prevalence of diabetes, or serum total cholesterol ≥5.2 mmol/l) was investigated[9]. None of these SNPs were associated with the development of any single parameter of metabolic syndrome among CCS[9], including the presence of diabetes, and adjustment for cranial and abdominal radiotherapy did not change these

results. In contrast, cranial and abdominal radiotherapy were strongly associated with the presence of, or components of, metabolic syndrome. This may suggest that the impact of treatment, mainly radiotherapy, is more dominant than the influence of the tested variants on the components of metabolic syndrome[9].

The most recent genetic study was a GWAS in CCS of the St. Jude Lifetime Cohort, performed to identify genetic variants associated with obesity[24]. In this GWAS, the cohort was stratified on cranial radiation exposure. Next, 70% of the strata was used as discovery cohort and 30% as replication cohort. Neither strata showed polymorphisms in the *LEPR* gene to be associated with obesity[24]. Polymorphisms in regions near or within the *SOX11* and *CDH18* genes, regulators of neuronal growth, repair, and connectivity[25,26] increased the risk of obesity among cranial radiated CCS[24]. On the other hand, a polymorphism in *FAM155A*, thought to disrupt the hypothalamic-pituitary axis[24,27], decreased the likelihood of obesity in cranial radiated CCS. These findings have not yet been investigated in independent cohorts. Nevertheless, it is important to stress that the observed genetic variation will only partly explain the total variation in obesity, as other environmental factors such as cancer treatment and lifestyle are of major importance. In this GWAS, the pseudo R^2 (a measure for the amount of variability explained) in the cranial radiated strata was 0.174 for the clinical risk factors model, and 0.303 for the clinical risk factors combined with the SNPs model[24]. Despite a significant increase, it also shows that this complex human trait deserves further research to understand its pathophysiological mechanism and its genetic components. Although a polymorphism in the *LEPR* gene would be a logical genetic determinant of metabolic risk factors, the evidence to date for the association is limited.

Gonadal impairment

Two candidate gene approach studies examined gonadal impairment; one in female and one in male CCS, and neither included a replication cohort.

The candidate gene study in female CCS explored the association between genetic variation and gonadal impairment based on high or low AMH levels[11] (Table 2). Seven polymorphisms, each in a different gene, were evaluated. The polymorphisms had previously been identified in GWASs as associated with age at natural menopause in the general population[28,29]. In this study in CCS, females with a heterozygous genotype for rs1172822 in the *BRSK1* gene had higher odds of having a low AMH value (OR=3.15, 95% CI 1.35-7.32, p=0.008). A modifying effect of the SNPs on the impact of treatment was not specifically evaluated, but the OR was adjusted for alkylating agents score and abdominal radiotherapy. *BRSK1* is expressed in the human forebrain and to a lesser extent in mammalian ovaries. Overexpression of the *BRSK1* gene has been hypothesized to disturb hypothalamic-pituitary-ovary axis regulation by affecting the secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus[30] or to influence cell-cycle progression since it is essential for centriole duplication.

Table 2. Overview of studies on the influence of genetic variation on gonadal impairment in CCS

		Study population					Analyses								
Study	**Method**	**Cohort size (cases/ controls)***	**Country of origin; ethnicity**	**Gender (% males)**	**Tumor type**	**Treatment**	**Replication**	**Definition endpoint**	**Studied no of SNPs (adj for multiple testing)**	**Gene/ region**	**Variant**	**Effect allele/ genotype**	**Multivariate analysis adjust for:**	**OR**	**P-value**
Van Dorp et al. 2013	Cand. gen	176 (61/115)	Dutch; 100% Caucasian	0	Solid and hematological	Miscellaneous, with and without alkylating agents and abdominal radiation	No	AMH level below/ above 1 µg/L	7 (multiple testing)	IGF2R	rs9457827	CT	Age at measurement, AAD score and abdominal radiotherapy	0.75 (0.24-2.40)	0.633
										MCM8	rs236114	CT		0.96 (0.44-2.11)	0.919
										ARHGEF7	rs7333181	GA		1.14 (0.46-2.83)	0.777
										PCSK1	rs271924	TT		1.40 (0.40-4.91)	0.602
										TNF	rs909253	GG		1.46 (0.47-4.49)	0.510
										BRSK1	rs1172822	CT		3.15 (1.35-7.32)	**0.008**
Romerius et al. 2011	Cand. gene	127 (23/104)	Sweden; 100% Caucasian	100	Not specified	Miscellaneous, with and without alkylating agents and radiation	No	azoospermia: no sperms found in 40 microscopic fields of semen sediment at 400x magnification	51 (no multiple testing)	ER Alpha	rs2207396	AG vs GG	only univariate analyses, but stratified on high risk group (high doses alkylating agents or radiotherapy)	8.8 (2.1-36)	**0.004**
										ER Alpha	rs9340958	CT vs CC		16 (2.1-100)	**0.008**
										ER Alpha	rs9340978	AG vs GG		8.1 (1.1-56)	0.091

Abbreviations: RT: radiotherapy; TBI: total body irradiation

*indicated is the cohort size (cases and controls), as defined by the authors of the original article.

P-values in bold are considered statistically significant by the authors of the original article. Ethnic race is stated if reported in original article.

Where applicable, the multivariable analysis of the combined results of the discovery and replication cohort are reported. If no replication cohort was included, multivariable analysis of the discovery cohort is reported, or univariate analysis of the discovery if multivariable analysis was missing

Where applicable, the adjusted p-value corrected for multiple testing was reported

In male non-CCS, estrogen receptor deficiencies and polymorphisms are associated with infertility, although the exact mechanism remains to be elucidated[31-33]. The only genetic study addressing estrogen receptor polymorphisms in 127 CCS examined 51 SNPs. This study did not adjust for multiple comparison and had no replication cohort, increasing the risk of type 1 errors. Only SNPs in the estrogen receptor α gene were associated with increased risk of developing azoospermia, and this effect was stronger in the subgroup treated with high cumulative doses of alkylating agents/cisplatin or lower doses with additional radiotherapy[34]. Other polymorphisms, coding for androgen receptors and estrogen receptor β, were not found to be associated with infertility in these CCS.

In both male and female CCS only one candidate gene study has been performed to evaluate the genetic component of variation in long-term gonadal impairment. This variation needs further investigation, preferably in large GWASs with a replication cohort.

Bone mineral density impairment

Genetic variation in low bone mineral density (BMD) in CCS has been studied in six candidate gene studies (Table 3), of which one candidate gene study included up to 100 SNPs and adjusted for multiple comparisons[12]. The most recently published study[35] included a replication cohort, which failed to corroborate any of the earlier associations from the discovery cohort.

The *CRHR1* gene has previously been found to be associated with impaired lung function in asthma patients[36] and it has been suggested that *CRHR1* gene variants may also explain differences in susceptibility to exogenous corticosteroid therapy, thereby influencing lung function, but also BMD. The G allele of a polymorphism (rs1876828) in the *CRHR1* gene was associated with lower BMD in male survivors of acute lymphoblastic leukemia (ALL) (p=0.02), while, in contrast, a non-significant higher BMD was observed in female ALL survivors (p=0.09)[37]. As previously indicated for obesity, stratification by gender can be valuable, which again stresses the need for adequately sized cohorts.

Te Winkel and colleagues investigated 69 and 83 ALL survivors for respectively two and seven polymorphisms of six candidate genes and published this in two articles that in previous studies had shown an association between BMD impairment in the general population[38-41]. ALL survivors who were carriers of the vitamin D receptor (*VDR*) 5'-end haplotype 3 had an increased risk for lower lumbar spine BMD[42]. Similarly, the *MTHFR* gene T-allele (rs1801133) was also identified as a risk factor for lower total body BMD[43]. These studies also showed that carrier status of both *VDR* and *MRHFR* polymorphisms were associated with low BMD at diagnosis, before any treatment had been administered. However, the subsequent rate of BMD decline during treatment did not differ between carriers and non-carriers. Also, parameters of body composition were not different between carriers and non-carriers of the *MTHFR* and *MTRR* polymorphisms at diagnosis, nor during treatment or

Table 3. Overview of studies on the influence of genetic variation on bone mineral density in CCS

		Study population					Analyses								
Study	Method	Cohort size	Country of origin; ethnicity	Gender (% males)	Tumor type	Treatment	Replication	Definition endpoint	Studied no of SNPs (adj for multiple testing)	Gene/region	Variant	Effect allele/ genotype	Multivariate analysis adjust for:	OR	P-value
Den Hoed et al. 2016	Cand. gene	334	Netherlands; caucasian	59	ALL, AML, lymphoma, brain tumor, renal tumor, sarcoma, neuroblastoma	45% glucocorticoid; 17% CRT	No	lumbar spine bone mineral density (standard deviation score)	12 (no multiple testing)	VDR	rs4516035	Haplotype 1-2	Height	NA	-
										VDR	rs11568820	Haplotype 3			**0.02**
										ESR1	rs2504063	GG vs AG or AA			**0.03**
										LRP5	rs599083	TT vs TG or GG			**0.02**
										MTHFR	rs1801133	CC vs. CT or TT			0.21
										MTRR	rs1801394	GG vs Ga or AA			0.26
Park et al. 2016	Cand. gene	59	USA (73% white; 27% other)	52	ALL	Glucocorticoid	No	lumbar spine bone mineral density (z-scores)	100 (multiple testing)	RAPGEF5	rs6461639	Ref. allele homozygote	age, gender, height, BMD, Z-score, height, Tanner stage and vitamin D level measured at baseline	NA (lower BMD)	**0.015**
Sawicka-Zkowska et al. 2013	Cand. gene	74	Poland; caucasian	61	ALL, lymphoma	22% CRT	No	total bone mineral density (standard deviation score)	1 (no multiple testing)	LEPR (Q223R)	rs1137101	GG	No	NA	0.423
Te Winkel et al. 2011	Cand. gene	83	Netherlands	57	ALL	Glucocorticoid	No	bone mineral density total body (standard deviation score)	2 (no multiple testing)	MTHFR	rs1801133	T	No	NA (lower BMD)	**0.01**
										MTRR	rs1801394	G		-	-
Te Winkel et al. 2010	Cand. gene	69	Netherlands	57	ALL	Glucocorticoid	No	bone mineral density lumbar spine(standard deviation score)	7 (no multiple testing)	VDR 5'-end (haplotype 3, bAT)	rs4616035	G	No	NA (lower BMD)	**0.01**
Jones et al. 2008	Cand. gene	309	USA (87% white, 12% black, 1% other)	51	ALL	Glucocorticoid	No	bone mineral density (z-scores)	9 (no multiple testing)	CRHR1	rs1876828	G	Ethnicity, weight, treatment	NA (lower BMD)	Males: **0.02**; female: 0.09

Abbreviations: CRT: cranial radiotherapy; N/A: not applicable; NA: not available

P-values in bold are considered statistically significant by the authors of the original article Ethnic race is stated if reported in original article.

Where applicable, the multivariable analysis of the combined results of the discovery and replication cohort are reported. If no replication cohort was included, multivariable analysis of the discovery cohort is reported, or univariate analysis of the discovery if multivariable analysis was missing

Where applicable, the adjusted p-value corrected for multiple testing was reported

after treatment. This suggests that while genetic variation may play a role in BMD variation, it does not modify the effect of treatment on BMD in ALL patients[44-46].

Hearing impairment

Hearing impairment is commonly observed after treatment of CCS with the platinum agents cisplatin and carboplatin, or after cranial radiation[47]. The effect of these treatments could be modified by genetic polymorphisms.

Twelve candidate gene studies have been performed, none of which included a discovery cohort larger than 250 subjects (Table 4). Only three of the studies included an independent replication cohort and to date, no GWAS has been published on CCS after completion of treatment.

In a study by Ross et al, in 53 CCS subjects almost 2,000 SNPs in 220 key pharmacogenetic genes involved in the absorption, distribution, metabolism and elimination of drugs were genotyped. This study included an independent replication cohort of 109 CCS. They identified *COMT* and *TPMT* as genetic determinants of variation in hearing impairment between CCS[16]. Catechol O-methyltransferase (*COMT*) is involved in the metabolism of catechol drugs and is highly expressed on hair cells of the mouse[48]. However, its role in auditory function remains unclear. Thiopurine S-methyltransferase (*TPMT*), involved in the metabolism of thiopurine drugs, has not yet been linked to cisplatin metabolism in the general population, although it has been demonstrated in murine inner ear cells to play a role in cisplatin metabolism and detoxification[49,50]. Four additional studies aimed to replicate the previously identified associations of hearing impairment with *COMT* and *TPMT*. While one study confirmed these associations[51], albeit with smaller effect sizes, the other three did not[13,52,53]. One small study in a population of 63 children with hearing function measured during cisplatin treatment, did not detect a significant association of *TMPT* and *COMT* polymorphisms in children with hearing impairment[54]. Despite the functional validation of the *TMPT* marker in murine inner ear cells[49], uncertainty remains regarding whether *COMT* or *TPMT* polymorphisms are genetic risk factors for hearing impairment[54]. Lack of replication may be due to different methods for defining hearing impairment (e.g., Brock classification, Münster grading system, SIOP Boston criteria, CTCAE classification, Chang grading), heterogeneity of the study cohorts in regards to treatment exposure and age at diagnosis, or small sample sizes. In the study by Yang et al, nearly all patients (91%) received the otoprotectant amifostine and all had cranial radiotherapy, both of which might mask genetic susceptibility[53]. However, in their small underpowered cohort of 41 survivors who did not receive amifostine or cranial radiation, the association between *TMPT* and hearing impairment are in line with the other studies[16,51]. This highlights the importance of a homogenous population with a large sample size, in order to avoid type 2 errors.

Polymorphisms in the low density lipoprotein-related protein 2, or megalin (*LRP2*) gene, which is expressed in the marginal cells of the stria vascularis in the inner ear, have been

postulated to predispose to cisplatin-induced hearing impairment. Three studies investigated the association between the *LRP2* gene polymorphism (rs2075252) and hearing impairment, of which one study showed that the prevalence of hearing impairment was higher in CCS who carried the A allele of this polymorphism[15,16,55]. However, this study did not include a replication cohort.

Another variant in this gene (rs2228171) was investigated in 68 CCS and was found to be significant, but has not been replicated in subsequent studies[15].

The association between hearing impairment and *GSTT1* and *GSTP1* loci, members of the glutathione S-transferases (*GSTs*) superfamily, was first described in survivors of adult cancer[56]. *GSTs* are known to play an important role in cell protection by scavenging free radicals caused by cisplatin by conjugating it with glutathione[57,58]. In CCS, the association between cisplatin-induced hearing impairment and polymorphisms in the GST gene family (*GSTP1, GSTT1, GSTM1, GSTM3, GSTZ1*) was investigated in four studies[15,16,57,59]. One study of 39 survivors identified the *GSTM*B* allele to be associated with a lower risk of hearing impairment (OR: 0.11, 95% CI not given, p-value: 0.02)[57] and a larger study of 86 medulloblastoma survivors found that survivors with the *GSTP1* AG or the GG genotype had a greater risk of hearing impairment (OR 4.0, 95% CI: 1.2-13.6, p=0.03) than survivors with the AA genotype[59]. However, the latter finding may be false positive since the study by Ross et al[16], in 162 subjects had 99.9% power to detect a similar effect at $p \leq 0.05$, but did not find a significant association between the *GSTP1* genotype and hearing impairment.

While no GWAS examining hearing impairment has been performed in CCS after completion of therapy, one GWAS in 238 subjects reported on susceptibility to cisplatin-induced hearing impairment measured during childhood cancer treatment[60]. This study identified one significant SNP in the *ACYP2* gene[60], which codes for an acylphosphatase that can influence Ca^{2+} homeostasis in the cochlea and is involved in hair cell development[61]. This finding was replicated in an independent cohort of 156 CCS after treatment, although pooling of the results from both studies was needed to reach statistical significance[62]. This stresses the need not only for replication in independent studies, but also for adequately sized studies. The replication indicates there is no difference in genetic susceptibility in the cohorts with hearing impairment measured during or after treatment, which is in line with current knowledge concerning the irreversibility of hearing impairment. However, recent data suggests that in some survivors, cisplatin-induced hearing impairment manifests later in life, suggesting that some cases of cisplatin-induced hearing impairment might be missed if hearing function is only measured during treatment[63]. Up until now, no GWAS has been published to study the effect of genetic variation on hearing impairment in long-term CCS. In summary, the following genes were associated with hearing impairment in at least two independent sets of CCS subjects: *COMT* (rs4646316 and rs9332377, five reports, two significant[16,51]), *TPMT* (rs12201199, rs1142345, rs1800460, five reports, two significant[16,51]) and *ACYP2* (rs1872328, two reports, two significant[13,62]). Although large cohorts and

replication cohorts are requirements for solid genetic research, many studies on hearing impairment do not meet these criteria. The functional significance is not fully understood for all SNPs and the clinical implication of polymorphisms in *TPMT* in hearing impairment has only been recently demonstrated in murine inner ear cells[49]. The functional significance of polymorphisms in *COMT* in hearing impairment is still unclear.

FUTURE DIRECTIONS

Among childhood cancer survivors the heterogeneity of late toxicities is broad, even in survivors who have been treated with the same protocols. This suggests a role for genetic variation. However, the evidence for an association between genetic variation and late toxicities after childhood cancer is largely insufficient or inconclusive to date, with few exceptions such as the reported associations between *ACYP2* and hearing impairment. The inconclusive evidence is mainly due to a lack of well-designed, adequately powered studies. To date, in the reported late effects, only one GWAS has been performed. Especially in candidate gene studies, a) cohorts are small, b) replication cohorts are often lacking, c) the definitions used for biological endpoints are inconsistent across studies, and d) there are differences in study design across studies which hinders comparability. The lack of consistent associations across studies can be largely explained by methodological factors. In addition, variations in biological factors play an important role, since most of the outcomes studied are known to have multi-factorial etiologies, which include differences in genetic background, environment, behavioral factor, as well as co-morbidity. Moreover, clinical feasibility to collect data in a sufficiently powered and homogeneous cohort may play a role. Future research studies in this field could therefore benefit from considering the following principles.

Firstly, future studies need to include adequately sized cohorts in order to have sufficient power to identify low risk variants, which are the expected risk variants in common traits such as the evaluated late toxicities (i.e., common disease, common variant hypothesis). Several studies highlight the need for stratification or sub-analyses[10,37], which again require larger study populations. Power calculations and adjustment for multiple testing are essential tools to minimize type 1 and 2 errors. GWASs are becoming more popular and are evaluating hundreds of thousands to millions of SNP markers at the same time and require a multiple testing adjustment to $p<5*10^{-8}$. Therefore large sample sizes are required to achieve sufficient statistical power[64]. The number of SNPs to be included can increase exponentially when the sample size increases and studies with larger sample sizes are able to detect smaller associations as a result of higher power[65]. This highlights the need for international collaboration to assure sufficient sample sizes to identify genetic associations. Moreover, a large sample size is important as the focus of genetic studies in late

Table 4. Overview of studies on the influence of genetic variation on hearing impairment in CCS

		Study population					Analyses								
Study	**Method**	**Cohort size (cases/ controls)***	**Country of origin; ethnicity**	**Gender (% males)**	**Tumor type**	**Treatment**	**Replication**	**Definition endpoint**	**Studied no of SNPs (adj for multiple testing)**	**Gene/ region**	**Variant**	**Effect allele/ genotype**	**Multivariate analysis adjust for:**	**OR**	**P-value**
Thiesen et al. 2017	Cand. gene	116	UK; 88% white, 5% Asian, 3% African	64	Medulloblastoma, hepatoblastoma, osteosarcoma, neuroblastoma, and other solid tumours	Cisplatin alone, combined cisplatin and carboplatin, or carboplatin after cisplatin; CRT (34%); vincristine (54%)	No	CTCAE and Chang	6 (multiple testing)	ACYP2	rs1872328	GG	Age at diagnosis, gender, CRT, cumulative dose cisplatin, exposure to carboplatin and vincristine	NA	**0.027**
										TPMT	rs12201199	AA		NA	0.34
										TPMT	rs1142345	TT		NA	1.00
										TPMT	rs1800460	CC		NA	1.00
										COMT	rs9332377	CC		NA	1.00
										COMT	rs4646316	CC		NA	1.00
Vos et al. 2016	Cand. gene	156 (77/79)	Netherlands; 99% European descent	51	Osteosarcoma	Cisplatin (with or without coadministered carboplatin); no CRT; no amifostine	No	Chang	1 (no multiple testing)	ACYP2	rs1872328	A	No	12.06 (0.66-221.98)	**0.027**
Brown et al. 2015	Cand. gene	71 (26/45)	USA; 42% non-Hispanic white, 35% Hispanic, 24% other	73	Medulloblastoma or supratentorial primitive neuroectodermal	Cisplatin and CRT; amifostine (39%)	No	Use of hearing aid	5 (multiple testing)	SOD2	rs1880	C	Age at diagnosis, gender, ethnic group, cumulative cisplatin dose and CRT doses ≥34 Gy	3.06 (1.30-7.20)	**0.040** (FDR)
Hagleitner et al. 2014	Cand. gene	110 (42/68)	Netherlands	50	Osteosarcoma	Cisplatin; no CRT; vincristine (4.5%); no otoprotectants	38 Osteosarcoma CCS; Spain; Cisplatin, no CRT	CTCAE, SIOP Boston	5 (no multiple testing)	TPMT	rs12201199	A	Vincristine exposure	0.65 (0.22-1.91)	0.44
										TPMT	rs1142345	G		0.96 (0.30-3.08)	0.95
										TPMT	rs1800460	A		0.49 (0.12-1.97)	0.31
										COMT	rs4646316	G		0.49 (0.22-1.14)	0.10
										COMT	rs9332377	A		0.80 (0.41-1.55)	0.51

Table 4. Overview of studies on the influence of genetic variation on hearing impairment in CCS (continued)

		Study population					Analyses								
Study	Method	Cohort size (cases/ controls)*	Country of origin; ethnicity	Gender (% males)	Tumor type	Treatment	Replication	Definition endpoint	Studied no of SNPs (adj for multiple testing)	Gene/ region	Variant	Effect allele/ genotype	Multivariate analysis adjust for:	OR	P-value
Yang et al. 2013	Cand. gene	213 (64/149)	USA; 79% white, 21% non-white	66	Medulloblastoma	Cisplatin and CRT; amifostine (91%)	41 USA CCS; Cisplatin, no CRT	CTCAE, Chang	7 (no multiple testing)	TPMT	rs12201199	TT vs TA vs AA	No	NA	0.50
										TPMT	rs1142345	AA vs AG vs GG		NA	0.14
										TPMT	rs1800460	GG vs GA vs AA		NA	0.11
										COMT	rs4646316	GG vs GA vs AA		NA	0.15
										COMT	rs9332377	GG vs GA vs AA		NA	0.78
Rednam et al. 2013	Cand. gene	86	USA; 44% non-Hispanic white, 33% Hispanic, 23% other	70	Medulloblastoma, supratentorial primitive neuroectodermal tumor	Cisplatin and CRT; no otoprotectants	No	Use of hearing aid	1 (no multiple testing)	GSTP1	RS1695	AG/GG vs AA	No	4.0 (1.2-13.5)	**0.03**
Pussegoda et al. 2013	Cand. gene	162 (106/56)	Canada; 80% Caucasian	50	Brain tumor, germ-cell tumor, hepatoma, and other solid tumors	Cisplatin; CRT (19%); tobramycin (29%); vancomycin (22%); vincristine (40%); gentamicin (17%); no otoprotectants	155 Canadian CCS; cisplatin with and without CRT	CTCAE	6 (no multiple testing)	TPMT	rs12201199	A	Age, vincristine treatment, germ cell tumor and CRT	8.9 (3.2-24.9)	**4.0E-5**
										TPMT	rs1142345	G		6.1 (2.1-17.3)	**0.0039**
										TPMT	rs1800460	A		6.6 (2.0-21.8)	**0.00073**
										ABCC3	rs1051640	G		2.0 (1.3-2.9)	**0.0033**
										COMT	rs4646316	G		1.8 (1.2-2.6)	**0.0068**
										COMT	rs9332377	A		1.9 (1.2-3.1)	**0.043**
Choeyprasert et al. 2013	Cand. gene	68 (54/14)	Thailand	59	Fibrosarcoma, germ cell tumor, hepatoblastoma, medulloblastoma, nasopharyngeal carcinoma, neuroblastoma, osteosarcoma, rhabdomyosarcoma	Cisplatin; CRT (29%); aminoglycosides (52%); no otoprotectants	No	Brock	4 (multiple testing)	LRP2	rs2228171	C	No	4.33 (1.01-18.57)	**0.034**
										LRP2	rs2075252	C		NA	0.763
										GSTM1	null	Non-null		NA	0.734
										GSTT1	null	Non-null		10.05 (1.80-56.00)	**0.023**

Table 4. Overview of studies on the influence of genetic variation on hearing impairment in CCS (continued)

		Study population					Analyses								
Study	**Method**	**Cohort size (cases/ controls)***	**Country of origin; ethnicity**	**Gender (% males)**	**Tumor type**	**Treatment**	**Replication**	**Definition endpoint**	**Studied no of SNPs (adj for multiple testing)**	**Gene/ region**	**Variant**	**Effect allele/ genotype**	**Multivariate analysis adjust for:**	**OR**	**P-value**
Ross et al. 2009	Cand. gene	53 (33/20)	Canada	68	Brain tumor, germ cell tumor, hepatoblastoma, neuroblastoma, osteosarcoma, sarcoma	Cisplatin; CRT (17%); vancomycin (4%); vincristine (4%); no otoprotectants	109 CCS; Cisplatin with or without CRT	CTCAE	1,949 (multiple testing)	TPMT	rs12201199	A	Gender and age	16.89 (2.27-125.88)	**0.0318**
										TPMT	rs1142345	G		10.93 (1.44-82.74)	0.221
										TPMT	rs1800460	A		17.96 (1.07-302.66)	0.413
										COMT	rs4646316	G		2.51 (1.48-4.27)	0.076
										COMT	rs9332377	A		5.52 (1.91-15.95)	**0.0261**
										GSTP1	rs1695	GG		0.71	0.61
										LRP2	rs2075252	A		1.2	0.55
										GSTM1	Null	Non-null		0.78	0.51
Riedemann et al. 2008	Cand. gene	50 (25/25)	Germany	54	Ostesarcoma, neuroblastoma, medulloblastoma, germ cell tumor, teratoma, testicle cancer	Cisplatin; no CRT; no ototoxic comedication or otoprotectants	No	Muenster	2 (no multiple testing)	LRP2	rs2075252	A	No	3.45 (1.22-9.76)	**0.016**
Knoll et al. 2006	Cand. gene	11	USA	NA	Osteosarcoma, soft tissue sarcoma, CNS tumor	Cisplatin; CRT (64%); no otoprotectants	No	Clinically apparent hearing loss	5 (no multiple testing)	GJB2	rs80338939	G	No	NA	**0.016**
Peters et al. 2000	Cand. gene	39 (20/19)	Germany	56	Osteosarcoma, germ cell tumor, neuroblastoma, brain tumor	Cisplatin; no CRT; no otoprotectants	No	Muenster	5 (no multiple testing)	GSTM1	*B, *0	*A	No	-	-
										GSTM3	*B	*A		0.11	**0.02**
										GSTT1	*0	*A		-	-
										GSTP1	*B, *C	*A		-	-
										GSTZ1	*B, *C	*A		-	-

Abbreviations: CRT: cranial radiotherapy; N/A: not applicable; NA: not available

*indicated is the cohort size (cases and controls), as defined by the authors of the original article
P-values in bold are considered statistically significant by the authors of the original article. Ethnic race is stated if reported in original article.
Where applicable, the multivariable analysis of the combined results of the discovery and replication cohort are reported. If no replication cohort was included, multivariable analysis of the discovery cohort is reported, or univariate analysis of the discovery if multivariable analysis was missing
Where applicable, the adjusted p-value corrected for multiple testing was reported

toxicities after cancer often is on an interaction between treatment and a polymorphism, and interaction studies require even larger power than regular association studies.

Secondly, future genetic studies will benefit from inclusion of independent replication cohorts, as is common practice in the GWAS field, to strengthen the study design and avoid type I errors. Yet again this needs international collaboration to replicate findings in independent studies.

Thirdly, to ensure that the genetic difference observed between cohorts is related to the disease or condition under study and to rule-out spurious associations, inclusion of cohorts with similar genetic backgrounds (similar ethnicity) is preferred. For study situations, where this is not feasible by design, several methods have been developed to correct for ancestrally distinct populations, such as principal components analysis, based on the variance of the studied genotypes[66]. To date, most genetic studies have been performed in Caucasians. Genetic analyses in all ethnicities are required to avoid disparities in addressing knowledge gaps related to genetic susceptibility to late treatment effects.

In addition, to increase the chance of replication of results, harmonization by consistent definitions of outcomes and evaluation of possible confounders are necessary. Also, sufficient understanding of the molecular mechanisms underlying the disease or condition is important to adequately define cases and controls. In this regard, the proper selection of cases and controls has been extensively discussed within genetic epidemiology[67].

Next, it is essential that collection, processing, storage and retrieval of bio-specimens is conducted under quality control programs using standard operating procedures to guarantee low inter-sample variance and high quality of the samples. Within international collaborations, the establishment of an international biobank could be of value. Biobanks require high ethical practice standards, but offer research and researchers the possibility of cross-collaboration and synergy between different fields which is needed to further advance genetic research.

Finally, genetic technology is continuously improving, resulting in even bigger datasets with higher genetic resolution[6]. Yet, the same principles as described above apply and with even more necessity given the even larger number of genetic variants tested. With the increasing availability of commercially available arrays and increasing affordability of large-scale GWAS, performance, coverage and imputation quality should be considered when choosing an array. While whole genome sequencing and whole exome sequencing have gained considerable attention in genetic epidemiology, and are gaining ground in the diagnostic phase of childhood cancer, none of these approaches have yet been taken in the evaluation of genetic susceptibility to late effects in CCS.

Up until now, evidence-based guidelines for CCS concerning genetic susceptibility testing have only been developed for cardiotoxicity[68]. However, these guidelines are not implemented in clinical practice yet. For other late toxicities after childhood cancer the currently available literature is not robust enough, as yet, to inform reliable prediction

models. However, genotyping childhood cancer patients in order to risk-adapt treatment based on risk models predicting susceptibility to specific late toxicities is likely to become standard of care. International collaboration is critical to advance knowledge of specific genetic risk factors in order to guide the development of scientifically rigorous prediction models. Currently, we are investigating the genetic susceptibility of hearing impairment and female gonadal impairment in an international consortium (European Union's Seventh Framework programme project PanCareLIFE) with replication planned in independent cohorts from North America[69].

CONCLUSIONS

With growing knowledge of genetic determinants of late-effects and the continuation in decreasing genotyping costs, more personalized treatment protocols may become possible in the future. The criteria of 1) adequately sized cohorts and 2) the inclusion of independent replication cohorts are mandatory for well-founded research in genetic variability. International collaboration can ensure adherence to these criteria and thus be beneficial for the quality of research.

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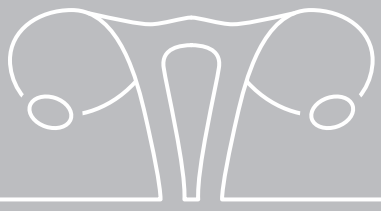

CHAPTER 6

Genetic variation in gonadal impairment in female survivors of childhood cancer: a PanCareLIFE study protocol

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ABSTRACT

Background: Improved risk stratification, more effective therapy and better supportive care have resulted in survival rates after childhood cancer of around 80% in developed countries. Treatment however can be harsh, and 3 in every 4 childhood cancer survivors (CCS) develop at least one late effect, such as gonadal impairment. Gonadal impairment can cause involuntary childlessness, with serious consequences for the well-being of CCS. In addition, early menopause increases the risk of comorbidities such as cardiovascular disease and osteoporosis. Inter-individual variability in susceptibility to therapy related gonadal impairment suggests a role for genetic variation.

Currently, only one candidate gene study investigated genetic determinants in relation to gonadal impairment in female CCS; it yielded one single nucleotide polymorphism (SNP) that was previously linked with the predicted age at menopause in the general population of women, now associated with gonadal impairment in CCS. Additionally, one genome wide association study (GWAS) evaluated an association with premature menopause, but no GWAS has been performed using endocrine measurements as the primary outcome in CCS.

Methods: As part of the PanCareLIFE study, the genetic variability of chemotherapy induced gonadal impairment among CCS will be addressed. Gonadal impairment will be determined by anti-Müllerian hormone (AMH) levels or alternatively by fertility and reproductive medical history retrieved by questionnaire. Clinical and genetic data from 837 non-brain or non-bilateral gonadal irradiated long-term CCS will result in the largest clinical European cohort assembled for this late-effect study to date. A candidate gene study will examine SNPs that have already been associated with age at natural menopause and DNA maintenance in the general population. In addition, a GWAS will be performed to identify *novel* allelic variants. The results will be validated in an independent CCS cohort.

Discussion: This international collaboration aims to enhance knowledge of genetic variation which may be included in risk prediction models for gonadal impairment in CCS.

INTRODUCTION

As a result of continuous improvements in treatment and supportive care, survival rates after childhood cancer have increased over the past decades, now reaching 80% in developed countries. However, the harsh treatment components that have led to increased survival rates can induce serious long-term complications. One in every four childhood cancer survivors (CCS) reveals severe or life-threatening adverse late effects[1], and three in every four survivors report at least one late effect[2,3]. In female CCS, apart from radiotherapy involving the field of the ovaries or pituitary, alkylating agents are important risk factors for fertility impairment[4-7] and damage is dose-dependent[8]. Such toxic agents can damage the ovarian follicle pool severely, leading to impaired fertility illustrated by an absent or substantially shortened reproductive window. Consequently, considering the current tendency in European countries to postpone childbearing, female survivors may find themselves involuntarily childless, leading to an increased use of artificial reproductive techniques. The feasibility to reach parenthood is of great significance to both parents of children with cancer and to CCS, and is an important determinant of quality of life[9-14]. In addition, gonadal impairment or early menopause carries adverse health risks for women, such as an increased risk for cardiovascular disease and osteoporosis, which require intensive and long-term medical attention[15].

Variations in long-term gonadal impairment in CCS who received the same treatment suggest that genetic variation may be an important determinant of gonadal impairment in CCS. Currently, only limited information is available on the role of genetic factors in the development of impaired gonadal reserve after childhood cancer treatment[4]. One single center study has been performed which evaluated seven genetic single nucleotide polymorphisms (SNPs) in 176 female CCS. These SNPs were selected based on the fact that they have been found to be associated with age at menopause in large genome wide association studies (GWAS) in the general female population[16,17]. While one of these allelic variations in the *BRSK1* gene (rs1172822) was found associated with a low anti-Müllerian hormone (AMH) level in CCS[4], replication of this finding has not been reported so far. Meanwhile, many more SNPs have been reported to be associated with reproductive ageing in the general population coming from large-scale collaborative consortia[18,19] but none have yet been investigated in CCS. In order to identify independent genetic determinants for therapy related gonadal impairment, substantially sized cohorts with well-documented clinical as well as treatment data are required. In addition, independent replication cohorts must be available to validate the results. One GWAS[20] has been performed (with Affymetrix 6.0 SNP array) in the St. Jude Lifetime Cohort Study (SJLIFE) among 799 ethnically mixed female CCS, which included an independent replication cohort (genotyped with the Illumina Omni5 SNP array) of 1624 women from the ethnically mixed Childhood Cancer Survivor Study (CCSS). This GWAS did not identify a genome wide significant hit, but found a SNP

(rs9999820) that was borderline significantly associated ($p=3.3*10^{-7}$) with an increased risk of premature menopause, especially in the subgroup of CCS who had undergone ovarian irradiation. This haplotype, consisting of 4 SNPs, is associated with increased hippocampal *NPYR2* gene expression, which is associated with a neuroendocrine pathway[20]. Noteworthy is that this GWAS evaluated the genetic variation in (self-reported) premature menopause, the latest manifestation of gonadal impairment or ageing.

The PanCareLIFE initiative, a 5-year (2013-8) EU Framework 7 Programme in the Health Theme originating from the PanCare project, focuses on the identification of determinants of long-term health of CCS. Specifically, PanCareLIFE will evaluate female gonadal impairment, hearing, and quality of life. Investigators from sixteen partner institutions from ten European countries have prospectively and retrospectively collected data from over 12,000 survivors from cancer diagnosed before they were 25 years of age.

The current study is part of this European wide endeavour and focuses on the identification of genetic factors which play a role in the risk of treatment-induced gonadal impairment among female childhood cancer survivors. Its specific objectives are to validate previously identified genetic polymorphisms associated with gonadal impairment in female childhood cancer survivors, using a candidate gene approach; and to identify *novel* SNPs that are independently associated with chemotherapy induced gonadal impairment in female childhood cancer survivors, using a GWAS.

METHODS

Inclusion criteria

For the current study we included female adult survivors (≥ 18 years) of childhood cancer, diagnosed before the age of 25 years, with a follow-up time of at least 5 years after diagnosis. Eligible survivors had to have been treated with chemotherapy. Exclusion criteria included radiotherapy involving both ovaries, defined as bilateral irradiation of the abdomen below the pelvic crest, or radiotherapy involving the pituitary, defined as cranial or craniospinal irradiation. Furthermore, survivors were not eligible if they had undergone myeloablative allogeneic stem cell transplantation, with or without total body irradiation.

Study cohort

PanCareLIFE consists of 8 work packages of which 5 focus on scientific work. Work package 4 encompasses two parts: WP4a focuses on genetic variation in gonadal impairment, and WP4b focuses on genetic variation in ototoxicity. This study addresses work package 4a. For this work package, adult female CCS were recruited in ten institutions from seven countries (Figure 1).

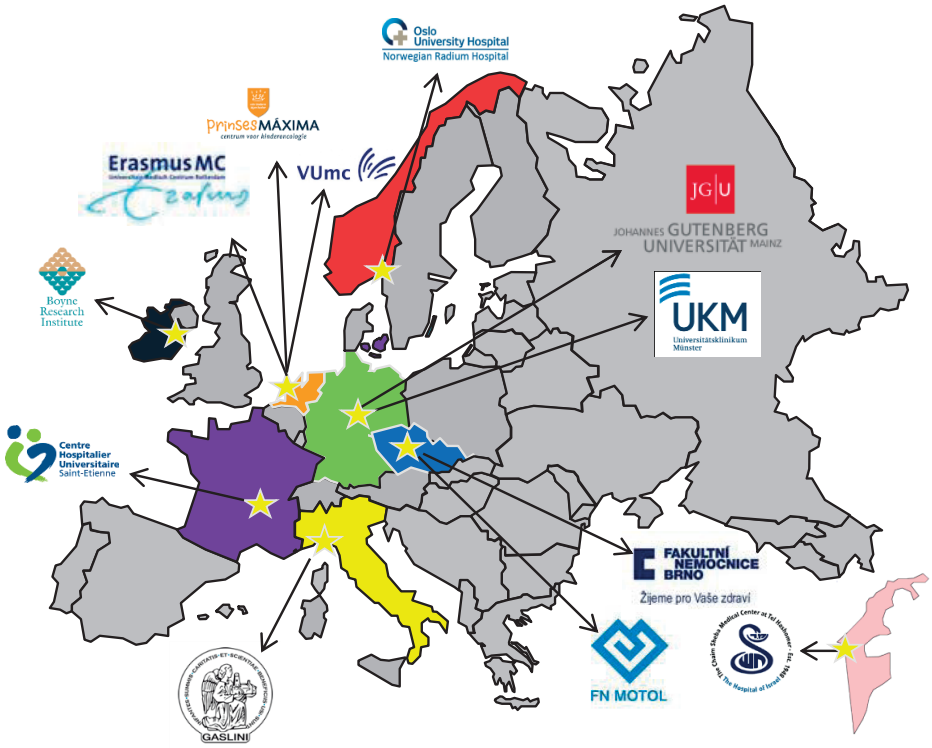

Figure 1. Participating institutions throughout Europe and Israel

The participating institutions and included numbers were: the Dutch Childhood Oncology Group (AMC, EMC, LUMC, UMCG, UMCN, VUmc) (inclusions n=306), Erasmus Medical Center Rotterdam (n=25) and VU Medical Center Amsterdam (n=19) from the Netherlands, Fakultni Nemocnice Brno (n=134) and Fakultni Nemocnice v Motole (n=86) from Czech Republic, Oslo University Hospital Departments of Oncology/ Pediatrics (n=107) from Norway, I.R.C.C.S. Giannina Gaslini (n=67) from Italy, Department of Paediatric Oncology/ University Hospital, St-Etienne (n=64) from France, University Hospital Muenster (n=39) from Germany and Sheba Medical Center (n=18) from Israel. In total 865 CCS were included in this study. DNA samples could not be collected in 28 cases, leaving 837 CCS for analysis (Table 1).

Medical ethics approval for the study was obtained from all relevant local committees and written informed consent was obtained from all participants.

Data collection

Basic demographic data of all participants (month and year of birth and of follow-up), diagnostic data (month and year of diagnosis, type of diagnosis) and full details of cancer

Table 1. Data providing institutions in genetic work package on gonadal impairment

Country	Data provider	Treatment data	DNA samples
The Netherlands	Dutch Childhood Oncology Group	306	298
	Erasmus Medical Center, Rotterdam	25	24
	VU Medical Center, Amsterdam	19	18
Czech Republic	Fakultni Nemocnice Brno	134	132
	Fakultni Nemocnice v Motole	86	81
Norway	Oslo University Hospital	107	107
Italy	I.R.C.C.S. Giannina Gaslini	67	64
France	Center Hospitalier Universitaire de Saint-Étienne	64	58
Germany	University Hospital Muenster, Germany	39	37
Israel	Sheba Medical Center, Tel Hashomer	18	18
TOTAL		**865**	**837**

Dutch Childhood Oncology Group: Academic Medical Center in Amsterdam (AMC), Erasmus Medical Center in Rotterdam (EMC), Leiden University Medical Center (LUMC), University Medical Center Groningen (UMCG), University Medical Center Nijmegen (UMCN), VU Medical Center (VUmc)

treatment were retrospectively collected from medical databases and medical records. Data on cancer treatment comprised of details on surgery, chemotherapy and radiotherapy, including start and stop dates and cumulative dosage. All data will be merged at the central data center in Mainz likewise a former EU funded sister project PanCareSurFup[21], and will finally be pseudonymized for the investigators of this study.

Gonadal function

The primary outcome of this study is AMH level. Serum samples were centrifuged, stored at -20°C and shipped on dry ice to the VUmc Amsterdam where all AMH levels were analysed in the same laboratory using an ultra-sensitive Elecsys AMH assay (Roche Diagnostics GmbH, Mannheim, Germany) at one time point. Data on AMH levels were sent to the central data center in Mainz and merged into the central database and subsequently pseudonymized to the investigators. In addition to the continuous AMH levels, patients will be divided in two groups based on AMH levels considered relevant as a proxy for gonadal impairment, considering data on AMH levels in healthy females measured with the same assay in the reference laboratory in the VUmc Amsterdam. These details will be described in detail in the forthcoming manuscript. In addition, detailed information about menstrual history, and/or FSH level, and/or information on usage of artificial reproductive techniques will be used to evaluate gonadal impairment.

Genotyping

Blood or saliva samples were obtained for DNA isolation. Blood samples (n=781) were stored at ≤ -20°C and shipped on dry ice while saliva kits (n=56) were stored and shipped

at room temperature. Genomic DNA was extracted by the salting-out method. The choice of genotyping array was made after extensive comparison between all currently available arrays. The Infinium® Global Screening Array was chosen based on the rich up-to-date content and its suitability for GWAS including rare variants, while also containing clinically relevant content, including pharmacogenetics.

Statistical considerations

For the GWAS a genetic sample size calculation was performed to estimate the number of cases required in the current study[22]. As it is impossible to estimate the allelic frequencies in our population, the following assumptions were made for the power calculation: 1) a high risk allele frequency of 0.2, 2) a genome-wide significant significance level ($5*10^{-8}$), 3) a cohort size larger than n=800 and 4) a case to control ratio of 1:2. Based on these assumptions, we determined that the number of recruited patients provided statistical power (80%) to identify variants with an odds ratio of at least 1.8.

Quality control and imputations

A quality control (QC) protocol containing multiple filters will be applied to clean the genetic data and to ensure its quality prior to either imputations or analysis[23]. Both a SNP and individual call rate filter of 97.5% will be applied to remove poorly genotyped SNPs and individuals from the data. Furthermore, a Hardy-Weinberg Equilibrium test (significance level $<1*10^{-7}$) will be employed to remove variants containing potential genotyping errors. To ensure sample quality, samples with extreme heterozygosity, gender mismatches, and familial relationships will be assessed and removed. Genetic ancestry of the samples will be assessed and corrected for using principal components (PCs).

Finally, imputations will be performed using the Michigan Imputation Server using default settings[24]. The reference panel chosen for imputations is the Haplotype Reference Consortium (HRC r1.1)[25]. The same approach has previously been used in large-scale population studies such as the Rotterdam Study[26] and Generation R[27].

Association analysis

For the candidate gene approach we will extract the genotypes of a list of predetermined SNPs based on published literature. The Mann-Whitney U test and the Kruskal-Wallis test will be employed to compare the distribution between groups with continuous data. Logistic regression will be performed to calculate the odds ratio and 95% confidence interval of the SNPs to assess their risk of gonadal impairment. This model will adjust for several confounders: principal component analysis (PCA) will be used to correct for population stratification by modelling ancestry differences between cases and controls[28]. PCA is a common tool that has been widely used for the combined analysis of correlated phenotypes in genetic linkage and association studies[29]. Furthermore, the model will adjust for cyclo-

phosphamide equivalent dose (CED). This measure enables comparison of alkylating agent exposure independent of drug dose distribution within a particular cohort (as the formerly used alkylating agent dose), permitting comparison across different cohorts[30]. In addition, linear regression will be performed to calculate the effect of the SNPs on continuous AMH levels. This model will include age, in addition to the principal components and CED. The modifying effect of genetic predisposition on the association between CED and gonadal impairment will be also explored.

To identify relevant SNPs from the GWAS that may be important but do not reach genome-wide significance, we will use a suggestive significance level of $p=5.10^{-6}$. After GWAS analysis, we will use the R script EasyQC[31] to clean the association results based, amongst others, on minor allele frequency and imputation quality. The results will then be visualized and the functional annotation for all leading SNPs will be identified using the online platform called Functional Mapping and Annotation of GWAS (FUMA-GWAS)[32].

Replication

For both the candidate gene approach and GWAS, to ensure that associations are not a chance finding or an artifact due to uncontrolled biases, associations will be replicated within a replication cohort, based on the St. Jude Lifetime Cohort Study (SJLIFE) from St. Jude Children's Research Hospital, Memphis USA[33,34].

DISCUSSION

This paper outlines the design of one study within the PanCareLIFE initiative that has two separate research aims. Female CCS from ten different institutions from seven European countries will be included to validate previously identified genetic polymorphisms associated with gonadal impairment and to identify *novel* SNPs that are independently associated with chemotherapy induced gonadal impairment in female CCS.

Sufficiently-sized cohorts are of key importance in genetic association studies in order to have adequate power to identify low-risk variants. This is especially of importance in the evaluation of common traits such as gonadal function, where many common variants may operate with small effect sizes. To this end, we performed a power calculation to estimate the required cohort size for the current study, based on the estimated allelic frequency in our population.

It is standard practice in current genetic association studies to include an independent replication cohort to validate findings from the initial discovery cohort. However, few large cohorts exist that have sufficient numbers of female CCS, let alone with complete data as well as stored DNA and AMH for analysis. For this project, a collaboration with the St. Jude Children's Research Hospital, Memphis USA and CCSS has been initiated. AMH levels will be

measured in the same laboratory with the same AMH assay for the discovery and replication cohort, thus minimizing lab variation. Given the (non-significant) GWAS observations in the St Jude discovery cohort we believe forces must be joined, and we are therefore actively looking for additional cohorts to include in this and future international collaborations. We encourage readers who are aware of such collections to contact the corresponding author.

Gonadal impairment in CCS can be defined in many ways[35-37], and especially in international collaborations a clear consensus on the definition, as objective as possible, is needed. A separate work package within the PanCareLIFE consortium will combine seven criteria and several different questionnaires to assess clinical gonadal status in 20000 subjects. For the current study, the primary endpoint AMH was chosen, which will be evaluated both linear as categorized. The secondary endpoint is gonadal impairment based on detailed information about menstrual history, FSH levels and information on usage of artificial reproductive techniques. AMH has the advantage to be as objective as possible, in comparison to questionnaire data that may be prone to recall bias or incorrect information given by the survivor. In addition, AMH can serve as a reliable surrogate marker for ovarian function while the primordial follicle pool is not yet depleted[38,39]. The only reported GWAS investigating therapy induced fertility impairment in CCS, used premature menopause as primary outcome (clinically assessed in the discovery cohort and self-reported in the replication cohort)[20]. Prior to the clinical manifestation of amenorrhea and increased levels of FSH, impaired gonadal function can be detected by the measurement of lower serum AMH levels[40]. AMH in females is produced solely in the ovary by granulosa cells of small growing follicles and is considered a surrogate marker for ovarian function and ovarian reserve[38,39]. Like the primordial follicle pool, AMH levels decrease from adolescence on, until menopause occurs. Even survivors who do not report premature menopause (or Primary Ovarian Insufficiency, POI, defined as menopause before the age of 40 years) can still have a poor ovarian function, potentially resulting in reduced fertility or a shorter reproductive window (e.g., early menopause or menopause between 40-45 years). This impairment of gonadal function can be identified by the evaluation of AMH levels.

In conclusion, we describe the design of a genetic association study that will evaluate the association of genetic variability with gonadal impairment in a European cohort of childhood cancer survivors, with AMH levels as the primary outcome measure. This international collaboration will enhance knowledge of genetic variation which may be included in risk prediction models for gonadal impairment in CCS. In the future, patients with childhood cancer, parents and survivors may benefit from better individualized counselling concerning future fertility options and necessity for fertility preservation.

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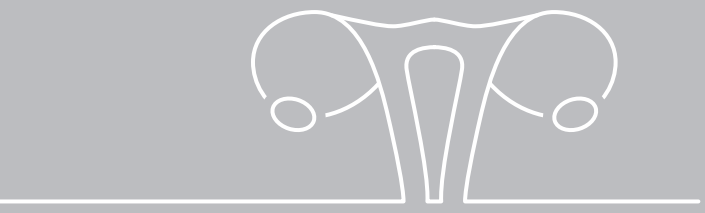

CHAPTER 7

BRSK1 modifies the risk of alkylating chemotherapy-related reduced ovarian function

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Submitted

ABSTRACT

Background: Female childhood cancer survivors (CCS) show large inter-individual variability in the impact of DNA-damaging alkylating chemotherapy, given as treatment of childhood cancer, on ovarian function at adult age. Genetic variants in DNA repair genes affecting ovarian function might explain this variability.

Methods: To evaluate ovarian function, Anti-Müllerian hormone (AMH) levels were assessed in a discovery cohort of female CCS from the Dutch DCOG LATER-VEVO (N=285), and results were validated in the pan-European PanCareLIFE (N=465), and the USA-based St. Jude Lifetime Cohort (N=391). Using additive genetic models in linear and logistic regression, five genetic variants involved in DNA damage response were analyzed in relation to cyclophosphamide equivalent dose (CED) score and their impact on ovarian function.

Results: Meta-analysis across the three independent cohorts showed a significant interaction effect ($p = 3.0 \times 10^{-4}$) between rs11668344 of *BRSK1* (allele frequency = 0.34) among CCS treated with high dose alkylating agents (CED score ≥8,000 mg/m^2), resulting in a 3-fold increased odds of a reduced ovarian function (lowest AMH tertile) for CCS carrying one G allele compared to CCS without this allele (OR genotype AA: 1.8 vs OR genotype AG: 5.3).

Conclusions: Female CCS carrying a common *BRSK1* gene variant appear to be at 3-fold increased odds of a reduced ovarian function after treatment with high doses of alkylating chemotherapy. Genetic testing may inform future individualized counseling regarding treatment-related risks and fertility preservation services in girls with cancer, as well as of young adult survivors of childhood cancer.

INTRODUCTION

Advances in childhood cancer treatment has increased cancer survival rates, leading to a growing population of childhood cancer survivors (CCS)[1]. Abdominal-pelvic radiotherapy and alkylating agents may compromise ovarian function[2-4] and reduce their reproductive window. This may manifest as sub- or infertility[5,6] and a higher risk of premature menopause[7], which in turn may impair quality of life[8-13]. Substantial inter-individual variability in the impact of treatment on ovarian function in similarly treated CCS suggests a role for genetic factors in modifying the association between treatment and the risk of ovarian impairment.

Large-scale genome wide association studies (GWAS) in the general population have identified single nucleotide polymorphisms (SNPs) associated with age at natural menopause or premature ovarian insufficiency (POI)[14-19]. These SNPs include variants associated with the DNA damage response, and account for approximately 30% of the variance in early menopause[19]. Alkylating agents, common chemotherapeutic agents used in childhood cancer treatment, induce apoptosis of cancer cells by damaging DNA and inhibiting cellular metabolisms, DNA replication and transcription[20-23]. We hypothesized that girls and young women with less efficient DNA damage response systems are more vulnerable to the adverse effects of alkylating agents on ovarian function compared to women with a fully efficient DNA damage repair system, leading to ovarian dysfunction later in life.

Serum levels of anti-Müllerian hormone (AMH), produced by the granulosa cells of small growing follicles in the ovaries, are related to age at onset of menopause in healthy women[24] and can detect ovarian dysfunction prior to both detectible changes in FSH/LH or estrogen and clinical manifestations of menopause[25-28]. In addition, AMH, which is stable throughout the menstrual cycle, has been demonstrated as a useful and early surrogate marker of reduced ovarian function in cancer survivors[29-34]. This is convenient since many CCS cohort members are relatively young and have not yet reached menopausal age.

Identifying genetic risk factors for treatment-related reduced ovarian function may have clinical implications for risk assessment and medical decision-making regarding fertility preservation in newly diagnosed girls with cancer[35]. Moreover, this information may inform targeted counseling and surveillance strategies of compromised ovarian function and associated comorbidities in at-risk adult female survivors. The aim of the current study was, therefore, to evaluate whether SNPs in the DNA damage response pathway modify the adverse effect of alkylating agents on ovarian function in CCS.

METHODS

Study participants - Discovery cohort

CCS for the discovery cohort were identified from the Dutch Childhood Oncology Group (DCOG) LATER VEVO-study, a multi-center retrospective cohort study evaluating fertility, ovarian reserve and risk of premature menopause among adult female 5-year survivors of childhood cancer[36]. Data on prior cancer diagnosis and treatments were collected from medical files and information on use of hormones (contraceptives or hormonal replacement therapy (HRT)) and menopausal status at time of study was obtained from the DCOG LATER VEVO-study questionnaire[36]. The timing of serum sampling (menstrual cycle day 2-5, day 7 of hormone-free week, or anytime in case of no menstrual cycle or hormone releasing intrauterine device) was documented. The study was approved by the Medical Ethics Review Committee (IRB protocol number 2006/249, VUmc) and written informed consent was obtained from all participants.

Inclusion and exclusion criteria

Female 5-year CCS, diagnosed with cancer and treated with chemotherapy before the age of 25 years, and aged 18 years or older at time of study were enrolled in the current study. Eligible participants provided a blood sample to quantify AMH levels and extract DNA. To maximize the potential to detect a role of genetic variation, we excluded survivors who received treatments associated with extensive gonadal toxicity including allogeneic stem cell transplantation (SCT), Total Body Irradiation (TBI), bilateral ovary-exposing radiotherapy, cranial and/or craniospinal radiotherapy, or bilateral oophorectomy.

Study participants – Replication cohorts

PanCareLIFE cohort

PanCareLIFE (PCL) is a pan-European research project including 28 institutions from 13 countries addressing ototoxicity, fertility, and quality of life[37]. The first replication cohort included all adult 5-year female survivors from the PanCareLIFE cohort who were treated for cancer before the age of 25 years and fulfilled all inclusion criteria of this study[38]. Approval was obtained from all relevant local review boards and written informed consent from all participants.

St. Jude Lifetime Cohort

The St. Jude Lifetime Cohort Study (SJLIFE) is a cohort study among 10-year CCS in North America coordinated by the St. Jude Children's Research Hospital (Memphis, Tennessee, USA) combining treatment data, patient-reported outcomes and clinical assessment[39]. Participants in SJLIFE who fulfilled the inclusion criteria and had blood samples available for AMH and DNA analysis comprised the second replication cohort. Sex hormone use at time of study was documented.

Outcome and outcome definition

The outcome of this study was ovarian function, primarily determined by serum levels of AMH. AMH levels of all three cohorts were determined in the endocrine laboratory of VU University Medical Center Amsterdam by an ultra-sensitive Elecsys AMH assay (Roche Diagnostics GmbH, Mannheim, Germany) with an intra-assay coefficient of variation (CV) of 0.5% – 1.8%, a limit of detection (LoD) of 0.01 µg/L, and a limit of quantitation (LoQ) of 0.03 µg/L[40].

To account for age-dependency of AMH, participating women in each cohort were divided into four age categories: ≥18-25; ≥25-32; ≥32-40; ≥40 years. In each cohort and for each age category, AMH was divided into tertiles with exception of the last age category in which AMH levels varied too little to adequately define tertiles. CCS with an AMH level in the lowest tertile for their age category were defined as having a reduced ovarian function (case), while those with an AMH-value in the highest tertile for their age category were assumed not to have a reduced ovarian function (control). Women over 40 years of age were not considered a 'case' based on having an AMH-value in the lowest tertile, but on whether or not they had reported a premature menopause (absence of menses for > 12 months before the age of 40) at time of study. No 'control' subjects were defined in this age group due to the inability to identify with sufficient certainty those without a reduced ovarian function.

Candidate gene variant selection

SNPs were selected based on a literature search of recently published GWAS that identified loci associated with age at natural menopause[16,18,19,41]. Five GWAS hits in DNA damage response pathways, specifically in the inter-strand cross-link repair pathway, were selected based on the lowest *p*-value in the largest available GWAS meta-analysis, with the hypothesis that polymorphisms in these regions may increase the gonadotoxic effect of alkylating agents. The selected polymorphisms were in *UIMC1* (rs365132), *FANCI* (rs1054875), *RAD51* (rs9796), *BRSK1* (rs11668344) and *MCM8* (rs16991615). Details concerning the genotype data and quality control protocol are provided in the Supplementary Appendix.

Alkylating agents

For each survivor, the administered cumulative dose of alkylating agents was quantified using the validated Cyclophosphamide Equivalent Dose (CED)-score[42]. To evaluate the effects of no, low, medium and high dose alkylating agent exposure, the CED score was divided into four categories (0; >0 – 4,000 mg/m^2; ≥4,000 – 8,000 mg/m^2; ≥8,000 mg/m^2)[42]. Details on the administered chemotherapeutics, CED score in categories and a fractional polynomial selection procedure for CED score are further discussed in the Supplementary Appendix Tables S1-4.

Statistical analyses

Additive genetic associations, with AMH levels based on imputed allelic dosage, were evaluated by logistic and linear regression analyses based on two models: (1) *a main effect* model; and (2) *an interaction* model. Both models evaluated the association between reduced ovarian function and selected SNPs, adjusted for: ancestry and cohort effects using principle components, CED score (four categories using CED of zero as the reference category)[42], use of sex hormones (replacement or contraception) at time of study (yes/no), age at time of study (linear regression analysis only), and imputed numbers (0-2) of the alternative allele of the investigated variant (additive effects). The *interaction model* additionally included an interaction term (SNP*CED category) for genetic variant and CED score categories to evaluate the modifying effect of the variant on the impact of CED score on low AMH levels. Results of linear and logistic regression analyses are presented as regression coefficients (beta) with standard errors (se) and odds ratios (OR) with a 95% confidence interval (95% CI). For linear regression, AMH-levels were log-transformed to adjust for the skewed residuals distribution. Sensitivity analyses performed to assess the robustness of our findings, choices of the model and linkage disequilibrium (LD) are shown in Supplementary Appendix S5A-B.

SNPs that showed an association with log-transformed AMH levels or reduced ovarian function in either model, or an interaction effect with CED (p-values <0.05) were selected for replication. These analyses were conducted using SPSS (Statistical Package for Social Sciences (SPSS) version 24.0.0.1).

Replication and meta-analysis

Findings from the discovery cohort were assessed in both replication cohorts using identical models, except for sex hormone use at time of study, which was only available in SJLIFE. Data of the discovery and replication cohorts were combined and examined using meta-analytic approaches, in R version 3.5.1, package "rmeta"[43]. Details on the heterogeneity in the meta-analysis are described in the Supplementary Appendix, Tables S11-12. In the meta-analysis, *p*-values <0.01 (0.05/5 gene variants, correcting for multiple testing) were considered statistically significant.

RESULTS

Discovery cohort

In total, 285 CCS from the DCOG LATER-VEVO cohort participated in the current study (Table 1). Allele frequencies of the investigated SNPs are depicted in Table 2. All SNPs were in Hardy-Weinberg equilibrium (significance level $<1*10^{-7}$). Results from logistic regression analyses showed an association between *BRSK1* (rs11668344) and reduced ovarian func-

Table 1. Characteristics of participating CCS in the discovery and two replication cohorts

	Discovery DCOG LATER-VEVO (N=285)	**Replication PanCareLIFE (N=465)**	**Replication St. Jude Lifetime (N=391)**
Age at time of study (years)			
Median (range)	26.1 (18.3 – 52.4)	25.7 (18.0 – 45.0)	31.3 (19.1 – 59.5)
Age at diagnosis (years)			
Median (range)	5.8 (0.3 – 17.8)	10.4 (0.0 – 25.0)	6.9 (0.0 – 22.7)
18-25 years	0 (0)	21 (4.5)	16 (4.1)
Time since diagnosis (years)			
Median (range)	19.7 (6.7 – 41.4)	17.0 (5.0 – 39.1)	23.7 (11.0 – 46.2)
Diagnosis			
Leukaemia	112 (39.3)	109 (23.4)	121 (30.9)
Lymphoma	49 (17.2)	154 (33.1)	70 (17.9)
Renal tumors	37 (13.0)	35 (7.5)	27 (6.9)
CNS tumors	3 (1.1)	12 (2.6)	28 (7.2)
Soft tissue sarcoma	23 (8.1)	31 (6.7)	28 (7.2)
Bone tumors	26 (9.1)	45 (9.7)	34 (8.7)
Neuroblastoma	11 (3.9)	35 (7.4)	36 (9.2)
Other	24 (8.4)	44 (9.6)	47 (12.0)
Radiotherapy			
No	251(88.1)	297 (63.9)	268 (68.5)
Yes[a]	34 (11.9)	170 (36.1)	123 (31.5)
Thorax	22 (7.7)	88 (18.9)	71 (18.2)
Abdomen (above pelvic crest)	3 (1.1)	12 (2.6)	30 (7.7)
Unilateral ovarian[b]	0 (0)	9 (1.9)	3 (0.8)
Other	20 (7.0)	61 (13.1)	51 (13.0)
CED score			
0	106 (37.2)	161 (34.6)	198 (50.6)
> 0 – 4,000 mg/m^2	80 (28.1)	103 (22.2)	21 (5.4)
≥ 4,000 – 8,000 mg/m^2	52 (18.2)	68 (14.9)	78 (19.9)
≥ 8,000 mg/m^2	47 (16.5)	133 (28.6)	94 (24.0)
Hormone use at serum sampling			
No	199 (69.9)	232 (49.9)	263 (67.3)
Yes	86 (30.1)	116 (24.9)	128 (32.7)
Oral contraceptive-free day 7	70 (24.6)	3 (0.6)	n.a.
Anytime during oral contraceptive	n.a.	94 (20.2)	n.a.
HRT stop 7	2 (0.7)	20 (4.3)	n.a.
Anytime, with intrauterine device	14 (4.9)	n.a.	n.a.
Unknown	0 (0)	117 (25.2)	0 (0)

Table 1. Characteristics of participating CCS in the discovery and two replication cohorts (continued)

	Discovery DCOG LATER-VEVO (N=285)	Replication PanCareLIFE (N=465)	Replication St. Jude Lifetime (N=391)
Unilateral ovarian oophorectomy			
No	284 (99.6)	463 (99.6)	391 (100.0)
Yes	1 (0.4)	2 (0.4)	0 (0)
AMH level			
Median (range)	2.5 (<0.01 – 13.1)	2.1 (<0.01 – 18.5)	1.8 (<0.01 – 11.9)
Premature menopause (before age 40) and aged ≥40 years at study,	2 (0.7)	NA	4 (1.0)

Values represent the number (%) of women, unless indicated otherwise. [a]Not mutually exclusive; [b]Likely in radiotherapy field. CNS, central nervous system; CED, Cyclophosphamide Equivalent Dose; HRT, hormonal replacement therapy; n.a., not available

tion (OR 0.56, 95% CI 0.35 – 0.90; p-value = 0.016) in the main effect-model. In addition, a non-significantly modifying effect of *BRSK1* (rs11668344, minor allele frequency 0.34) on the effect of CED ≥8,000 mg/m^2 on reduced ovarian function (OR 5.02, 95% CI 0.76 – 33.08; p-value = 0.09) (Table 2) was observed in the interaction model. A significant modifying effect of a polymorphism in *FANCI* (rs1054875) on the effect of CED in the category >0 – 4000 mg/m^2 (OR 9.93, 95% CI 2.35 – 41.98; p-value = 0.002) was also observed (Table 2). Sensitivity analyses did not change these results (Table S5A-B of the Supplementary Appendix). Linear regression analysis showed a significant main effect of the *BRSK1* gene variant, but not of the other variants (Table S7 in the Supplementary Appendix). The two SNPs within the *BRSK1* and *FANCI* genes were assessed for replication in the two replication cohorts.

Replication and meta-analysis

The PanCareLIFE and SJLIFE replication cohorts included 465 and 391 female CCS, respectively (Table 1). Table 3 shows the combined analysis of both replication cohorts and the final meta-analysis including all three cohorts. Separate findings of the replication cohorts can be found in Table S9-10 in the Supplementary Appendix, full details of the meta-analysis in Tables S11-12. All three single-cohort analyses suggest a consistent modifying effect for the G allele of rs11668344 (*BRSK1*) on the effect of CED ≥8,000 mg/m^2 on reduced ovarian function. The meta-analysis showed an interaction effect of carrying the G allele of rs11668344 in *BRSK1* and an exposure to alkylating agents equivalent to a CED score ≥8,000 mg/m^2 of 3.81 (95% CI 1.85 – 7.86, p = 3.0×10^{-4}). Table 4 shows the cumulative ORs for any genotype per CED category. Female CCS who received alkylating agents equivalent to a CED score ≥8,000 mg/m^2 had a 3-fold higher odds of having an AMH serum level in the lowest tertile for each additional G allele of rs11668344 in *BRSK1* (OR genotype AA 1.82 vs AG 5.27 vs GG 15.26).

Table 2. Association of single nucleotide polymorphisms with reduced ovarian function and CED-score in DCOG LATER-VEVO discovery cohort

Gene	Variant	Chrom	Ref.	Alt.	MAF	Model	Variant, interaction term	OR (95% CI)	P-value
BRSK1	**rs11668344**	19	A	G	0.34	1	rs11668344	0.56 (0.35 – 0.90)	0.016
							CED: 0	1 (ref)	0.001
							– > 0 – 4,000	1.43 (0.65 – 3.11)	0.374
							– ≥ 4,000 – 8,000	4.74 (1.92 – 11.71)	0.001
							– ≥ 8,000	5.04 (1.66 – 15.30)	0.004
							Hormones	2.02 (1.00 – 4.07)	0.049
						2	rs11668344	0.57 (0.25 – 1.31)	0.186
							CED: 0	1 (ref)	0.133
							– > 0 – 4,000	1.94 (0.62 – 6.07)	0.253
							– ≥ 4,000 – 8,000	5.46 (1.32 – 22.66)	0.019
							– ≥ 8,000	1.91 (0.44 – 8.29)	0.386
							SNP*CED: 0	1 (ref)	0.218
							– > 0 – 4,000	0.66 (0.21 – 2.13)	0.489
							– ≥ 4,000 – 8,000	0.85 (0.23 – 3.18)	0.807
							– ≥ 8,000	5.02 (0.76 – 33.08)	0.094
							Hormones	2.01 (0.98 – 4.14)	0.058
FANCI	**rs1054875**	15	A	T	0.36	1	rs1054875	1.01 (0.61 – 1.67)	0.975
							CED: 0	1 (ref)	0.001
							– > 0 – 4,000	1.37 (0.63 – 2.95)	0.425
							– ≥ 4,000 – 8,000	4.17 (1.73 – 10.05)	0.001
							– ≥ 8,000	4.98 (1.66 – 14.91)	0.004
							Hormones	1.79 (0.91 – 3.54)	0.094
						2	rs1054875	0.31 (0.11 – 0.90)	0.032
							CED: 0	1 (ref)	0.009
							– > 0 – 4,000	0.32 (0.10 – 1.06)	0.063
							– ≥ 4,000 – 8,000	2.19 (0.60 – 7.95)	0.235
							– ≥ 8,000	3.71 (0.84 – 16.38)	0.084
							SNP*CED: 0	1 (ref)	0.016
							– > 0 – 4,000	9.93 (2.35 – 41.98)	0.002
							– ≥ 4,000 – 8,000	3.49 (0.78 – 15.57)	0.102
							– ≥ 8,000	2.00 (0.38 – 10.44)	0.413
							Hormones	1.83 (0.90 – 3.73)	0.095
MCM8	**rs16991615**	20	G	A	0.08	1	rs16991615	0.90 (0.38 – 2.15)	0.817
							CED: 0	1 (ref)	0.001
							– > 0 – 4,000	1.37 (0.64 – 2.94)	0.420
							– ≥ 4,000 – 8,000	4.16 (1.74 – 9.97)	0.001
							– ≥ 8,000	4.96 (1.65 – 14.87)	0.004

Table 2. Association of single nucleotide polymorphisms with reduced ovarian function and CED-score in DCOG LATER-VEVO discovery cohort (continued)

Gene	Variant	Chrom	Ref.	Alt.	MAF	Model	Variant, interaction term	OR (95% CI)	P-value
							Hormones	1.80 (0.91 – 3.56)	0.089
						2	rs16991615	0.85 (0.21 - 3.39)	0.820
							CED: 0	1 (ref)	0.005
							– > 0 – 4,000	1.36 (0.59 – 3.14)	0.473
							– ≥ 4,000 – 8,000	4.48 (1.73 – 11.58)	0.002
							– ≥ 8,000	3.82 (1.22 – 11.95)	0.021
							SNP*CED: 0	1 (ref)	0.973
							– > 0 – 4,000	1.07 (0.14 – 8.06)	0.950
							– ≥ 4,000 – 8,000	0.61 (0.05 – 6.74)	0.683
							– ≥ 8,000	NA	NA
							Hormones	1.89 (0.95 – 3.75)	0.069
UIMC1	**rs365132**	5	G	T	0.5	1	rs365132	1.09 (0.70 – 1.69)	0.720
							CED: 0	1 (ref)	0.001
							– > 0 – 4,000	1.35 (0.63 – 2.91)	0.443
							– ≥ 4,000 – 8,000	4.18 (1.75 – 10.00)	0.001
							– ≥ 8,000	5.03 (1.68 – 15.11)	0.004
							Hormones	1.80 (0.91 – 3.54)	0.090
						2	rs365132	0.79 (0.39 - 1.61)	0.518
							CED: 0	1 (ref)	0.017
							– > 0 – 4,000	0.44 (0.11 – 1.82)	0.257
							– ≥ 4,000 – 8,000	4.05 (1.01 – 16.19)	0.048
							– ≥ 8,000	4.83 (0.78 – 29.90)	0.091
							SNP*CED: 0	1 (ref)	0.265
							– > 0 – 4,000	2.89 (0.93 – 8.98)	0.067
							– ≥ 4,000 – 8,000	1.04 (0.32 – 3.39)	0.948
							– ≥ 8,000	1.01 (0.17 – 5.98)	0.988
							Hormones	1.78 (0.89 – 3.57)	0.104
RAD51	**rs9796**	15	A	T	0.42	1	rs9796	0.94 (0.62 - 1.44)	0.787
							CED: 0	1 (ref)	0.001
							– > 0 – 4,000	1.37 (0.64 – 2.94)	0.419
							– ≥ 4,000 – 8,000	4.17 (1.74 – 9.99)	0.001
							– ≥ 8,000	4.98 (1.66 – 14.92)	0.004
							Hormones	1.79 (0.91 – 3.53)	0.092
						2	rs9796	0.92 (0.43 – 1.97)	0.838
							CED: 0	1 (ref)	0.167
							– > 0 – 4,000	1.66 (0.52 – 5.33)	0.397
							– ≥ 4,000 – 8,000	4.33 (1.18 – 15.91)	0.027
							– ≥ 8,000	2.34 (0.48 – 11.42)	0.291

Table 2. Association of single nucleotide polymorphisms with reduced ovarian function and CED-score in DCOG LATER-VEVO discovery cohort (continued)

Gene	Variant	Chrom	Ref.	Alt.	MAF	Model	Variant, interaction term	OR (95% CI)	P-value
							SNP*CED: 0	1 (ref)	0.546
							– > 0 – 4,000	0.81 (0.28 - 2.33)	0.692
							– ≥ 4,000 – 8,000	0.94 (0.29 - 3.16)	0.938
							– ≥ 8,000	2.82 (0.52 – 15.37)	0.230
							Hormones	1.70 (0.85 – 3.39)	0.135

Chrom., chromosome; MAF, minor allele frequency; CCS, childhood cancer survivors; CED, Cyclophosphamide Equivalent Dose; Ref, Reference allele; Alt, alternative allele. Position based on position build 37 on https://www.ncbi.nlm.nih.gov/snp/. Alt is reported as 0/1/2 (recalculated for presentation only, based on allelic dosage) for CCS with and without reduced ovarian function (see Methods section for details). Model 1: adjusted for principal components, use of hormone use and CED-categories. Model 2: additional to Model 1 interaction term of variant*CED category.

The modifying effect of >0 – 4,000 CED in *FANCI* (rs1054875) was non-significant in both replication cohorts. The three-cohort meta-analysis showed no significantly modifying effect on the association between >0 – 4,000 CED and reduced ovarian function (OR 2.76, 95% CI 1.17 – 6.53, p = 0.02) after correction for multiple testing.

DISCUSSION

This is the first study to assess the influence of genetic factors on alkylating chemotherapy-induced reduced ovarian function, using AMH as a biomarker, and incorporating two independent and identically phenotyped replication cohorts and a meta-analysis. We report a strong modifying effect of a common SNP (minor allele frequency 0.34) in the *BRSK1* gene on the toxicity of high dose alkylating agents, resulting in a 3-fold increased odds of a reduced ovarian function for CCS carrying one G allele compared to CCS without this allele (OR genotype AA: 1.8 vs OR genotype AG: 5.3) and a further 3-fold increased odds for CCS carrying two G alleles (OR genotype GG: 15.3).

One previous single center study evaluated the association between ovarian function in CCS with SNPs associated with age at menopause in the general population reporting that the T allele of rs1172822 of the *BRSK1* gene was inversely associated with serum AMH levels[41]. However, this study did not assess interaction between treatment and AMH levels or include validation using replication cohorts. Recently, a SJLIFE GWAS study identified a haplotype associated with an increased risk of premature menopause, especially in the subgroup of CCS who had received pelvic radiotherapy[44]. However, the haplotype is beyond the scope of this study as our population excluded survivors treated with bilateral ovarian radiotherapy due to low inter-individual variation of POI and the haplotype is not associated with DNA damage response genes.

Table 3. Association of single nucleotide polymorphisms with reduced ovarian function and chemotherapy in the meta-analyses

					Replication (PCL+SJLIFE) meta-analysis			Discovery + Replication (VEVO + PCL + SJLIFE) meta-analysis		
Gene	**Variant**	**Ref>Alt**	**Model**	**variant, interaction**	**OR (95% CI)**	**Direction**	**p-value**	**OR (95% CI)**	**Direction**	**p-value**
BRSK1	rs11668344	A>G	2	rs11668344	0.82 (0.54 – 1.24)	-+	0.349	0.76 (0.53 - 1.11)	--+	0.152
				CED: 0	1 (ref)			1 (ref)		
				– > 0 – 4,000	0.58 (0.21 – 1.58)	--	0.284	0.98 (0.46 – 2.09)	+--	0.964
				– ≥ 4,000 – 8,000	3.42 (1.52 – 7.67)	++	2.8×10^{-4}	3.83 (1.90 – 7.74)	+++	1.8×10^{-4}
				– ≥ 8,000	1.77 (0.18 – 17.60)	+-	0.627	1.82 (0.40 – 8.34)	++-	0.442
				SNP*CED: 0	1 (ref)			1 (ref)		
				– > 0 – 4,000	3.27 (1.11 – 9.66)	+-	0.032	1.37 (0.29 – 6.51)	-+-	0.690
				– ≥ 4,000 – 8,000	1.04 (0.44 - 2.48)	+-	0.922	0.98 (0.48 – 2.02)	-+-	0.960
				– ≥ 8,000	3.63 (1.66 - 7.95)	++	1.3×10^{-3}	3.81 (1.85 - 7.86)	+++	3.0×10^{-4}
FANCI	rs1054875	A>T	2	rs1054875	1.01 (0.65 - 1.56)	+-	0.977	0.85 (0.57 - 1.28)	-+-	0.432
				CED: 0	1 (ref)			1 (ref)		
				– > 0 – 4,000	0.88 (0.28 – 2.80)	+-	0.828	0.54 (0.23 – 1.24)	-+-	0.148
				– ≥ 4,000 – 8,000	5.29 (2.08 – 13.50)	++	4.7×10^{-4}	3.91 (1.83 – 8.33)	+++	4.1×10^{-4}
				– ≥ 8,000	3.69 (0.37 – 36.8)	++	0.266	3.70 (0.83 – 16.6)	+++	0.088
				SNP*CED: 0	1 (ref)			1 (ref)		
				– > 0 – 4,000	1.35 (0.46 - 3.96)	++	0.583	2.76 (1.17 – 6.53)	+++	0.021
				– ≥ 4,000 – 8,000	0.64 (0.29 - 1.40)	--	0.264	0.92 (0.46 - 1.86)	+--	0.823
				– ≥ 8,000	1.03 (0.53 - 2.03)	++	0.925	1.14 (0.61 - 2.12)	+++	0.691

PCL, PanCareLIFE cohort; SJLIFE, St. Jude Lifetime Cohort; VEVO, DCOG-LATER VEVO cohort; CED, Cyclophosphamide Equivalent Dose. Model 2: adjusted for principal components, hormone use (only for VEVO, SJLIFE) and CED-categories and the interaction term of variant*CED category. + = positive association of the SNP with reduced ovarian function in PCL and SJLIFE respectively. - = negative association of the SNP with reduced ovarian function in VEVO, PCL and SJLIFE respectively.

Table 4. Estimated cumulative OR per genotype of rs11668344 and CED score on reduced ovarian function, based on meta-analysis point estimates

	genotype AA		genotype AG		genotype GG	
CED in mg/m²	**N (%)**	**Estimated cumulative OR**	**N (%)**	**Estimated cumulative OR**	**N (%)**	**Estimated cumulative OR**
0	51 (40.8)	1 (ref)	36 (40.0)	0.76	14 (31.8)	0.58
> 0 – 4,000	19 (37.3)	0.98	19 (38.8)	1.02	5 (29.4)	1.06
≥ 4,000 – 8,000	36 (69.2)	3.83	36 (66.7)	2.85	7 (43.8)	2.12
≥ 8,000	43 (58.1)	1.82	62 (77.5)	5.27	18 (81.8)	15.26

N (%) represents the number of cases with reduced ovarian function (% of total) within each genotype group. OR, Odds ratio. Estimated ORs calculated by multiplying the corresponding ORs from the full model, for example for the estimate of genotype AG in CED category **≥ 8,000:** 1.82 * 0.76 * 3.81 = 5.27.

Our study revealed a strong *modifying* effect of a G allele of a genetic variant in *BRSK1* (rs11668344 A>G) on alkylating agent related reduced ovarian function. The meta-analysis on reduced ovarian function for the main effect of *BRSK1*, which is associated with an earlier age at menopause in the general population[16,18,19], did not find a significant association as the previous single center study reported[41]. Representing continuous variables such as CED-score in categories could lead to increased type I error for the detection of interaction effects[45]. Supplementary analyses using fractional polynomials (Supplementary Appendix, Tables S4) show that using the available data, estimating more flexible models to potentially avoid these spurious findings offers inconclusive results due to lack of power, while not contradicting the results found using the pre-defined categories.

Rs11668344 is an intronic variant in *THEM150B* and an expression quantitative trait locus that alters *BRSK1* RNA gene expression in whole blood (p-value = 2.4×10^{-19})[46] and has regulatory histone marks, suggesting a regulatory function. Several mechanisms for the modifying effect of *BRSK1* on reduced ovarian function in CCS can be considered. Alkylating agents are known to induce apoptosis of cancer cells by damaging DNA and inhibiting cellular metabolism, DNA replication and DNA transcription[20-23]. We hypothesize that due to a less efficient DNA damage response system, cancer patients carrying the G allele of rs11668344 in *BRSK1* are at an increased risk of the DNA-damaging impact of alkylating agents in healthy tissues most relevant to our outcome studied here, the ovary (Figure 1). It is plausible that the efficiency of the DNA damage response system becomes crucial upon treatment with alkylating agents amounting to high CED scores.

Future research will need to evaluate the relevant expression, which we would expect in granulosa cells or the primordial follicle pool – as opposed to the recruited and selected oocytes that have successfully progressed towards maturation. Several hypothetically relevant mechanisms of action require further research to elucidate causally biological pathways and target tissues involved in the modifying effect of *BRSK1* on alkylating agents-related low AMH levels (Supplementary Appendix).

The identification of this genetic risk factor for alkylating agents-related low AMH levels, if confirmed for other measures of reduced ovarian function, may improve future risk prediction models including more adequate identification of groups with higher or lower risk of chemotherapy-induced ovarian impairment. Upfront fertility preservation programs, including ovarian tissue cryopreservation, will benefit from optimized prediction models as they can be directed to pediatric cancer patients at highest risk for gonadotoxicity for whom the balance of benefits/drawbacks -including ethical considerations- is most beneficial[47]. Moreover, female cancer survivors may also benefit from incorporating genetic testing to risk stratification in current targeted surveillance strategies of ovarian function and family planning counseling[48].

A major strength of this study is the inclusion of two replication cohorts. Yet, there were some differences in age at diagnosis and treatment exposures between the discovery and the replication cohorts. Survivors from the discovery cohort were younger at diagnosis, and were less often treated with alkylating agents amounting to CED score ≥8,000 mg/m^2. We therefore performed multiple sensitivity analyses to assess the choices of the model and cohort, but findings did not change our results. Another strength of this study is the measurement of AMH levels, as a marker for reduced ovarian function, with the same assay at one singular laboratory, eliminating between-assay differences. Previous studies demonstrated that alkylating agents are strongly associated with risk of reduced ovarian function as measured by decreased AMH levels in female CCS[4,28,49,50]. By using AMH levels as a marker of ovarian function, this study included a fairly substantial number of cases likely at increased risk of reduced fertility or a shorter reproductive window. However, while low AMH levels can also identify poor responders in assisted reproductive technology[51,52], it needs to be emphasized that AMH remains a *surrogate* marker of ovarian function. Validation using data collected long-term and using more definite and direct endpoints such as age at menopause, POI, or fecundity is needed to facilitate translation into clinical practice. In addition, larger cohorts would benefit the power of statistical tests.

In conclusion, this study shows that high dose alkylating chemotherapy-induced reduced ovarian function in female CCS is strongly modified by a common DNA variant (rs11668344) of the *BRSK1* gene. This is the first time a genetic risk factor has been described to modify the effect of chemotherapy on long-term ovarian function in three independent cohorts. This finding may serve as a starting point for individualized counseling regarding treatment-related risks and fertility preservation services in children with cancer as well as young adult survivors.

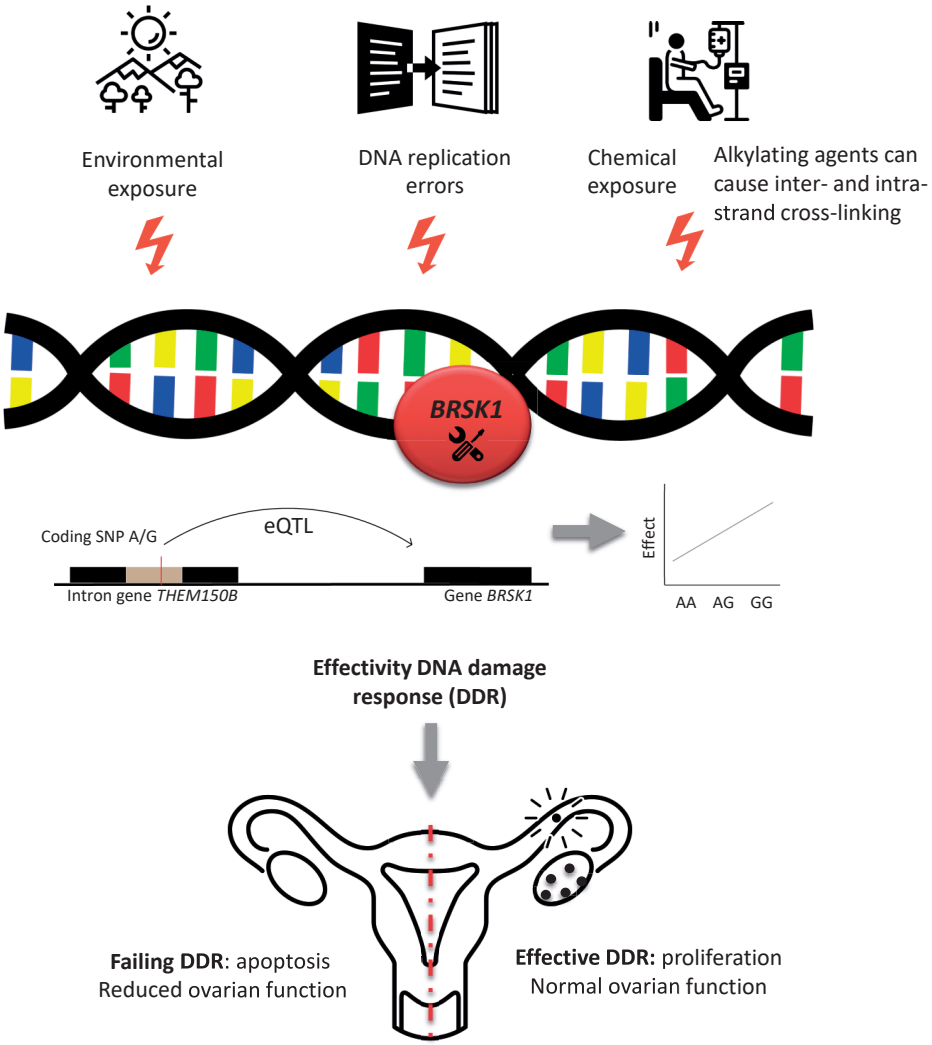

Figure 1. Simplified representation of the hypothesized biological plausibility

Simplified representation of the hypothesized biological plausibility of the effect of BRSK1 on reduced ovarian function. DNA damage can be the result of environmental exposure, DNA replication errors but also of chemical exposure. Alkylating agents are known to induce apoptosis of cancer cells by damaging DNA and inhibiting cellular metabolisms and DNA replication and transcription[20-23]. DNA damage response genes (BRSK1 is known to act as a DNA damage checkpoint) have previously been associated with age at natural menopause. Owing to a less efficient DNA damage response system, childhood cancer survivors carrying the G allele of rs11668344 (BRSK1) may be at an increased risk of the DNA-damaging impact of alkylating agents.

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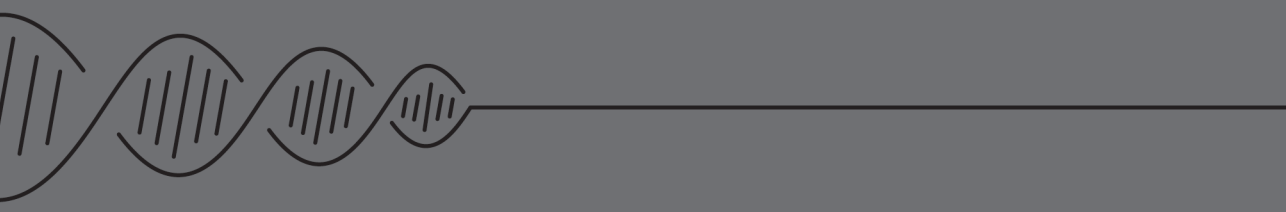
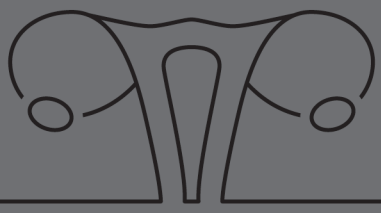
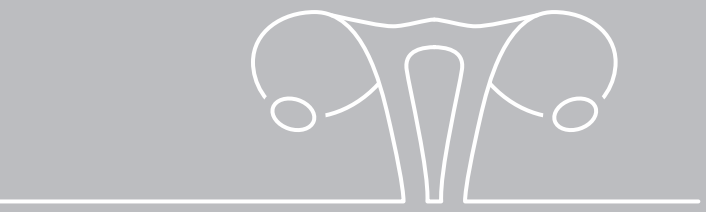




PART III

Obstetric risks



CHAPTER 8

Perinatal risks in female cancer survivors: a population-based analysis

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ABSTRACT

Background/Objectives: Advances in cancer management have resulted in improved survival rates, particularly in children and young adults. However, treatment may adversely affect reproductive outcomes among female cancer survivors. The objective of this study was to investigate their risk of adverse perinatal outcomes compared to the general population.
Design/Methods: We performed a population-based analysis, including all female cancer survivors diagnosed before the age of 40 years between 1981 and 2012. Pregnancy and perinatal complications were identified through linkage of the Scottish Cancer Registry with hospital discharge records based on the Community Health Index (CHI) database. We compared 1,629 female cancer survivors with a first ever singleton pregnancy after diagnosis, with controls matched on age, deprivation quintile, and year of cancer diagnosis selected from the general population (n= 8,899). Relative risks and 95%-confidence intervals of perinatal risks were calculated using log-binomial regression.
Results: Survivors were more likely to give birth before 37 weeks of gestation (relative risk [RR] 1.32, 95%-CI 1.10 – 1.59), but did not show an increased risk of low birth weight (<2.5kg: RR 1.15, 95%-CI 0.94 – 1.39), and were less likely to give birth to offspring small for gestational age (RR 0.81, 95%-CI 0.68 – 0.98). Operative delivery and postpartum haemorrhage were more common but approached rates in controls with more recent diagnosis. The risk of congenital abnormalities was not increased (RR 1.01, 95%-CI 0.85 – 1.20).
Conclusion: Cancer survivors have an increased risk of premature delivery and postpartum haemorrhage, but their offspring are not at increased risk for low birth weight or congenital abnormalities. In recent decades there has been a normalisation of delivery method in cancer survivors, nevertheless careful management remains appropriate particularly for those diagnosed in childhood.

INTRODUCTION

Advances in cancer management have resulted in improved five year survival rates in children and young adults[1]. The impact on later health of survivors is high: quality of life is consistently lower in breast cancer survivors as compared to women without a history of cancer[2,3], 75% of cancer survivors develop at least one health problem[4], and childhood cancer survivors are 8.2 times more likely to have a severe or life-threatening chronic condition such as premature gonadal failure in comparison to their peers[5,6]. Fertility is an important issue for survivors[7,8] but concerns about risks of pregnancy can be a reason to avoid pregnancy[9].

Female survivors of cancer who successfully conceived have been identified to be at risk of premature delivery[10-14] and their offspring have in some studies, but not consistently, been found to be at increased risk of low birth weight[10-13,15,16]. Reassuringly, there does not appear to be an increased risk of congenital abnormalities in their offspring[16-22]. Two small studies, one including childhood cancer survivors[23] and one including survivors of cervical cancer treated with cervical conisation[14], did not identify survivors to be at additional risk of caesarean section as mode of delivery. However, in two large population based studies, a British cohort of childhood cancer survivors[10] and a Finnish cohort of survivors of childhood and young adult cancer diagnosed between 0-35 years[24], the rate of elective caesarean section was increased, while the risk of emergency caesarean section was not increased.

The adverse impact of cancer treatment on pregnancy outcomes has to date been investigated in selected patient groups based on diagnosis or age at diagnosis. Reports from the British Childhood Cancer Survivors Study (BCCSS) and the US Childhood Cancer Survivors Study (CCSS) are confined to long-term survivors diagnosed with cancer between 0-14 years from 1940-1991 in Britain (BCCSS) and 0-21 years at diagnosis from 1970-1986 in the 25 participating institutions in Canada and the United States (CCSS)[10,15,25,26]. Other studies excluded the youngest age group and included adolescent and young adult cancer survivors diagnosed with cancer between ages such as 15-39[11,20] or 16-45 years[21]. Studies focusing on young adults surviving breast cancer[12,27], colorectal cancer[28] or cervical cancer[14] have provided insight into perinatal risks in these specific patient groups but their results cannot with confidence be extrapolated to survivors of other types of cancer. Inference of conclusions to current cohorts is limited by the relatively old cohorts often reported. Survivors in both the CCSS and BCSS were diagnosed several decades ago[10,15,25,26], and the treatment regimens administered may no longer be used[8]. Furthermore, reports based on self-reported questionnaires[13,14,29,30] or from specialist paediatric oncology centres such as the CCSS, may under- or overestimate the prevalence of certain events as a result of recall or selection bias, especially when the event was a substantial time ago**.** Population registries are less prone to recall bias and offer the opportunity to study pregnancy outcomes and perinatal risks at a population level in comparison to the background risk. The objective

of this study is to evaluate the perinatal risks among all female survivors from cancer in Scotland diagnosed before 40 years of age in the time period 1981-2012.

MATERIALS AND METHODS

The Scottish Cancer Registry contains data on cancer diagnoses for all patients in Scotland. All females diagnosed with cancer between 1981 and 2012 before the age of 40 years were identified. They were linked to national general and maternity hospital discharge records to ascertain subsequent first pregnancies leading to delivery of a live, singleton infant up until the end of 2014, using the Community Health Index (CHI) number, a unique identifying number from the CHI database, a population-based register of all patients registered to receive care from the NHS in Scotland. Deliveries occurring less than 6 months following the date of cancer diagnosis were excluded. Population-weighted fifths of Carstairs deprivation scores were assigned to each individual based on census-derived Carstairs scores from 1991 and 2001 for the periods of diagnosis 1981-1995 and 1996-2012, respectively[31]. A comparison group was created from the general population, using the CHI database. For every cancer survivor, three controls were selected matched on age at date of cancer diagnosis/matching and deprivation quintile. Controls had no pregnancies before the date of matching: subsequent first pregnancies leading to delivery of a live singleton infant (at least 6 months after the date of matching) were identified for comparison to deliveries among cancer cases. Only live singleton births in controls and cancer survivors were included in the analyses.

Maternal outcomes that were evaluated included antenatal haemorrhage, postpartum haemorrhage, and mode of delivery: spontaneous vaginal, assisted vaginal, elective caesarean section or emergency caesarean section. Infant outcomes included birthweight, gestational age, small for gestational age (SGA), admission to neonatal unit and congenital abnormalities (ICD codes in S1 Table). Low birthweight was defined as a birthweight <2500 grams, premature delivery as delivery before 37 weeks of gestation and SGA as <10th centile birthweight for gestational age and gender based on the UK90-WHO growth reference[32].

Age at diagnosis of cancer (and its treatment) may affect perinatal risks, therefore data were stratified based on age at diagnosis; 0-14 years; 15-24 years; 25-29 years; 30-34 years; 35-39 years. To evaluate possible effects of socio-economic circumstances, data were stratified based on deprivation fifth. Finally, to investigate possible differences in risk patterns over time, data were stratified into 7-year periods of diagnosis: 1981-1988; 1989-1996; 1997-2004; 2005-2012. P-values for the observed difference were calculated from the two-sample z-test for comparing proportions or t-test for comparing means, and log-binomial regression was employed to calculated risk ratios and 95% confidence intervals. Statistical analyses were conducted in Stata version 14 MP.

The study was approved by the Privacy Advisory Committee of the National Health Service (NHS) National Services Scotland (NSS) – study reference number XRB13215.

RESULTS

A total of 10,271 nulliparous women diagnosed with cancer before 40 years of age between 1981 and 2012 were identified, of whom 1,629 subsequently delivered a first singleton live birth by end 2014. Of 30,811 nulliparous matched control women, 8,899 delivered a first singleton live birth. The 1,629 survivors and the 8,899 matched control women formed the final cohorts. Half of the cancer survivor cohort had been diagnosed before 25 years of age (48%). The most common malignancies were melanoma and non-melanoma skin cancers (36.7%) followed by Hodgkin lymphoma (11.0%) (Table 1).

Cancer survivors were slightly older at first pregnancy than controls (30.1 vs 28.5 years $p<0.001$) (Table 1); body mass index (BMI) at booking was similar with 25.5 kg/m^2, although there were substantial missing data (59.9% in survivors and 72.1% in controls). Smoking was less prevalent in survivors, especially in those diagnosed during childhood (15.1% vs 28.0%, $p<0.001$) and adolescence (11.0% vs 15.5%, $p=0.005$) (Table 1).

Table 1. Diagnostic characteristics of 1,669 included female cancer survivors with a subsequent live singleton first ever birth after diagnosis

	Number	% of included cohort
Type of first cancer		
Colorectal	22	1.4
Liver	5	0.3
Bone	27	1.7
Skin (melanoma and NMSC)	598	36.7
Connective and soft tissue	30	1.8
Breast	112	6.9
Cervix uteri	118	7.2
Ovary	105	6.4
Kidney	20	1.2
Eye	8	0.5
Brain, CNS	66	4.1
Thyroid	128	7.9
Hodgkin lymphoma	179	11.0
Non-Hodgkin lymphoma	48	2.9
Leukaemia	81	5.0
Other	82	5.0

NMSC, non-melanoma skin cancers; CNS, central nervous system

Table 2. Differences in lifestyle factors between female survivors of cancer and a matched control group

	Live singleton births (n)		Mean age at 1st pregnancy (years)			Smoking during pregnancy (%)				
	controls	survivors	controls	survivors	p-value[1]	controls		survivors		p-value[2]
						yes	missing	yes	missing	
Total	8,899	1,629	28.5	30.1	<0.001	13.7	30.9	10.2	18.0	<0.001
Age-group at onset of cancer/match (years)										
0-14	1,292	186	21.2	23.5	<0.001	2.8	16.8	15.1	5.4	<0.001
15-24	2,849	588	24.9	27.2	<0.001	15.5	37.7	11.0	22.4	0.005
25-29	2,367	457	30.0	31.3	<0.001	8.7	33.9	9.2	19.7	0.759
30-34	1,781	306	34.2	35.2	<0.001	8.4	29.8	6.9	15.0	0.376
35-39	610	92	38.4	38.7	0.204	9.2	21.3	10.9	17.4	0.605
Period of diagnosis of cancer/match										
1981-1988	2,700	336	26.3	28.3	<0.001	8.1	71.7	7.1	53.9	0.543
1989-1996	2,690	453	27.9	29.1	<0.001	19.5	19.6	11.5	9.1	<0.001
1997-2004	2,063	480	30.2	31.0	0.010	16.3	8.6	12.7	10.2	0.051
2005-2012	1,446	360	31.3	31.6	0.280	9.5	7.7	8.0	6.4	0.376
Deprivation fifth										
1 – Least deprived	1,833	328	30.2	31.4	<0.001	7.8	30.6	5.8	14.0	0.199
2	1,684	315	28.8	30.8	<0.001	11.3	30.4	8.3	15.6	0.112
3	1,808	320	28.0	29.9	<0.001	14.4	30.3	8.4	20.0	0.004
4	1,880	356	28.2	29.5	<0.001	15.9	30.5	12.9	18.8	0.153
5 – Most deprived	1,694	310	27.1	28.8	<0.001	19.2	33.0	15.4	21.9	0.118

Female cancer survivors compared to a control group matched on age, diagnosis date and deprivation quintile. [1]p-value obtained from t-test. [2]p-value obtained from z-test.

Survivors were more likely to deliver prematurely (RR 1.32, 95% CI 1.10 – 1.59), but did not show a significantly increased risk of low birthweight (RR 1.15, 95% CI 0.94 – 1.39) (Table 2). Offspring of cancer survivors were less likely to be small for gestational age (RR 0.82, 95% CI 0.68 – 0.98) than offspring from the general population (Table 3). This difference in gestational adjusted birthweight was not observed in the more recently diagnosed groups or in more deprived quintiles (S2 Table).

Table 3. Relative risk of perinatal outcomes among female survivors of cancer

	Controls n (%)	Survivors n (%)	RR	LCI	UCI
Premature birth	548 (6.2%)	113 (8.2%)	1.32	1.10	1.59
Low birthweight	548 (6.2%)	115 (7.1%)	1.15	0.94	1.39
Small for gestational age	811 (9.2%)	121 (7.5%)	0.82	0.68	0.98
Admission to neonatal unit	1,090 (12.2%)	207 (12.7%)	1.03	0.90	1.19
Congenital abnormalities	8,746 (8.4%)	1,593 (9.5%)	1.01	0.85	1.20

Relative risks as compared to a control group matched on age, diagnosis date and deprivation quintile. RR, relative risk; LCI, lower confidence interval; UCI, upper confidence interval. Low birthweight is defined as <2.5 kg; Premature birth is defined as before 37 weeks of gestation; Small for gestational age is defined as under 10th centile for gestational age.

A spontaneous vaginal delivery was less common in survivors than in the general population (RR 0.72, 95% CI 0.65 – 0.79) (Table 4). Elective caesarean section was more common in cancer survivors than in the general population (RR 1.59, 95% CI 1.35 – 1.88), as was emergency caesarean section (RR 1.20, 95% CI 1.08 – 1.34). The risk of an elective caesarean section was most increased in women who had been diagnosed aged 0-14 years (RR 3.15, 95% CI 2.04 – 4.88). There were marked changes by period of diagnosis, with the frequency of operative delivery converging with controls with more recent diagnosis (Fig 1, panel B and E). This was most strikingly seen in the elective caesarean section rate, which declined in cancer survivors while increasing in controls (Fig 1, panel B). In those diagnosed between 1981-1988 the elective caesarean section rates were 10.4% vs 3.5% in controls (p<0.001), while the rates for those diagnosed in 2005-2012 were 7.2% vs 6.8% (p=0.8) (S3 Table). While in both the survivor and control group the emergency caesarean section rates rose by period of diagnosis, the absolute difference in prevalence remained constant (Fig 1, panel E). Survivors in the lowest and highest quintile of deprivation were more likely to have an emergency caesarean section than their matched peers, while there was no difference in risk for survivors in the middle quintiles (Fig 1, panel F).

There was no marked increased risk of antepartum haemorrhage (RR 1.13, 95% CI 0.86 – 1.50) for the cancer survivors. Postpartum haemorrhage occurred more often in cancer survivors (RR 1.42, 95% CI 1.29 – 1.55) (Table 4). The prevalence of postpartum haemorrhage increased in the control general population over time from 9.3% to 33.1%, while

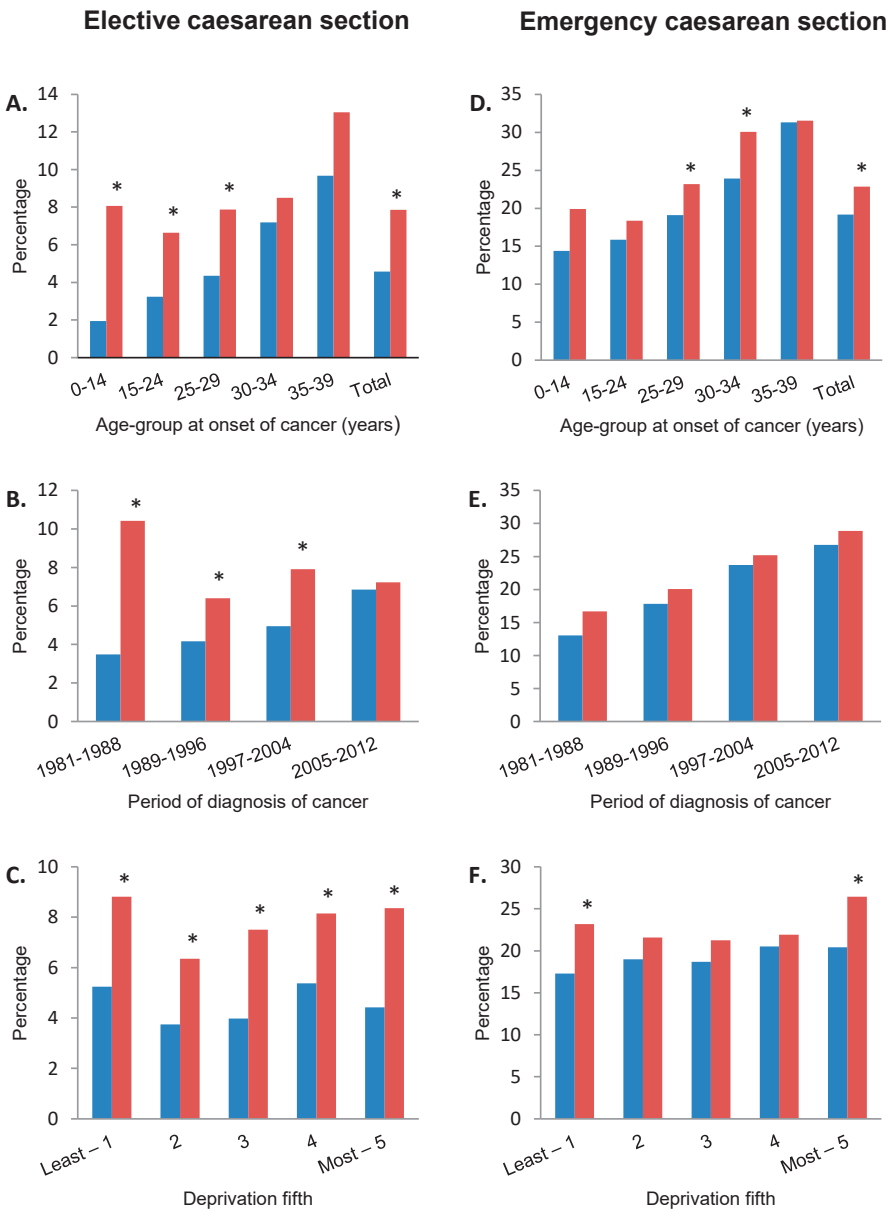

Figure 1. Likelihood of caesarean section in female cancer survivors compared to a matched control group

Panels A, B and C: difference of likelihood on elective caesarean section in female cancer survivors as compared to a matched control group, stratified by age-group at onset of cancer (A), period of diagnosis of cancer (B), and deprivation fifth (C). Panels D, E and F: difference of likelihood on emergency caesarean section by age-group at onset of cancer (D), period of diagnosis of cancer (E), and deprivation fifth (F). Significant differences (p-value < 0.05) between female survivors of cancer and controls are depicted with * per stratified group. Blue bars depict the control group, red bars the cancer survivors.

in the cancer survivors the prevalence increased from 15.8% to 38.3% over time with the prevalence of postpartum haemorrhage being similar to the control general population for later treated cohorts. (S4 Table). The risk of postpartum haemorrhage was most increased in women who had been diagnosed aged 0-14 years (RR 1.62, 95% CI 1.23 – 2.13), while no increased risk was observed in women diagnosed between 35-39 years (RR 1.30, 95% CI 0.92 – 1.83).

Offspring of cancer survivors were equally likely to be admitted to a neonatal unit (RR 1.03, 95% CI 0.90 – 1.19) and showed no increased risk of congenital abnormalities (RR 1.01, 95% CI 0.85 – 1.20) (Table 3).

Table 4. Relative risk of vaginal delivery and haemorrhage among female survivors of cancer

	Spontaneous vaginal			Assisted vaginal or breech			Antepartum haemorrhage			Postpartum haemorrhage		
	RR	**LCI**	**UCI**	**RR**	**LCI**	**UCI**	**RR**	**LCI**	**UCI**	**RR**	**LCI**	**UCI**
Total	0.72	0.65	0.79	1.14	1.00	1.29	1.13	0.86	1.50	1.42	1.29	1.55
Age-group at onset of cancer (years)												
0-14	0.63	0.47	0.83	1.25	0.87	1.79	0.55	0.24	1.24	1.62	1.23	2.13
15-24	0.72	0.61	0.84	1.11	0.89	1.39	1.31	0.81	2.13	1.28	1.08	1.53
25-29	0.74	0.62	0.89	1.12	0.88	1.41	1.47	0.86	2.49	1.65	1.40	1.96
30-34	0.65	0.52	0.82	1.25	0.94	1.65	1.35	0.69	2.66	1.33	1.09	1.61
35-39	0.87	0.58	1.32	0.98	0.56	1.70	1.21	0.43	3.42	1.30	0.92	1.83
Period of diagnosis of cancer												
1981-1988	0.56	0.45	0.69	1.07	0.76	1.48	0.73	0.23	2.37	1.70	1.29	2.23
1989-1996	0.72	0.60	0.87	1.09	0.82	1.45	0.87	0.50	1.50	1.31	1.07	1.61
1997-2004	0.88	0.74	1.04	1.04	0.83	1.29	0.91	0.57	1.47	1.24	1.06	1.45
2005-2012	0.89	0.74	1.08	1.04	0.84	1.30	1.54	0.93	2.55	1.16	1.00	1.35
Deprivation fifth												
1 – Least deprived	0.65	0.53	0.80	1.10	0.84	1.45	1.40	0.79	2.48	1.62	1.36	1.93
2	0.74	0.60	0.92	1.13	0.84	1.50	0.85	0.39	1.87	1.34	1.08	1.67
3	0.76	0.61	0.94	1.07	0.79	1.44	1.34	0.74	2.42	1.30	1.03	1.64
4	0.80	0.65	0.97	1.11	0.85	1.46	0.81	0.40	1.61	1.45	1.18	1.78
5 – Most deprived	0.65	0.52	0.80	1.33	1.00	1.76	1.25	0.71	2.21	1.33	1.06	1.67

Relative risks as compared to a control group matched on age, diagnosis date and deprivation quintile.
RR, relative risk; LCI, lower confidence interval; UCI, upper confidence interval.

DISCUSSION

Main Findings

This population-based study compared the frequency of adverse perinatal outcomes in cancer survivors diagnosed in Scotland before 40 years of age between 1981-2012 and non-cancer controls matched from the general population. Survivors were more at risk of a preterm delivery but their offspring were not at increased risk of low birthweight and had a decreased risk of SGA. Elective caesarean section was more common in cancer survivors as was emergency caesarean section, but there were marked changes by period of diagnosis, with the frequency of both elective and emergency caesarean section converging with controls among those with a more recent diagnosis. Similar findings of increased but converging risk were found for postpartum haemorrhage. The risk of congenital abnormalities in offspring of cancer survivors was not increased.

Strengths and Limitations

The major strengths of this study include the population-based approach using national registry data, which allowed evaluation of all first singleton pregnancy outcomes in female survivors from cancer, diagnosed at an age under 40 years. A large age matched non-cancer control group was identified from the general population. Pregnancy outcomes were accurately recorded and free of recall bias, but this study lacks cancer treatment information including radiotherapy to the abdomen and pelvis as this is not routinely collected in the databases used for this study. We report on the perinatal risks of female survivors from all cancers, which results in a heterogeneous cohort with regard to their diagnosis and treatment. The most common malignancies were melanoma and non-melanoma skin cancers which are more commonly treated with local therapy with lesser likelihood to impact future perinatal risks. Although all presented relative risks are compared to an age and period matched control group, the follow-up for patients diagnosed in the most recent period of diagnosis is still relatively short, especially for childhood cancer survivors. This may have influenced the observed trends.

Interpretation

Cancer survivors achieve fewer pregnancies in comparison to the general population, with an overall reduction in likelihood of pregnancy after diagnosis of 38%[33]. Concerns about risks of pregnancy are sufficient reason to avoid pregnancy for some survivors[9]. Overall, our results are reassuring to cancer survivors who wish to become pregnant. We observed no increased risk of congenital abnormalities, which is consistent with previous studies of risk of congenital malformations in offspring of cancer survivors, which also found no an associations with radiotherapy or chemotherapy treatment[16-22,34]. Our results of increased risk of premature delivery among cancer survivors agree with previously reported studies[10-14]. This

has been particularly linked to radiotherapy to fields which include the uterus, particularly in pre-pubertal girls[35], which can lead to reduced uterine volume and elasticity[36,37]. In addition, uterine vascularisation may be impaired, with potential detrimental consequences for fetal-placental blood flow causing fetal growth restrictions. Our results of no increased risk of low birthweight are consistent with earlier findings in cohorts with survivors from cancer at a young[10,13] and adult[38] age, although in the BCCSS cohort there was an increased risk in the subgroup that received radiation to a field that included the abdomen[10]. Other reports in cohorts with women diagnosed at a young age however, did show an increased risk of low birthweight[16], as did cohorts of women surviving breast cancer[12] and women diagnosed aged 15-39 years[11]. Maternal smoking is a well-recognised risk factor for low birthweight[39] and having a small for gestational age baby[40]. In our study, cancer survivors were less likely to smoke during pregnancy than the control general population, especially those diagnosed in childhood and adolescence. As the prevalence of smoking decreased in the general population in later periods, the differences between cancer survivors and the control general population converged. This may suggest that cancer survivors were more aware of the harmful risks of smoking than the control general population and more inclined to stop or not to start smoking. This supports the value of ongoing health surveillance in this group[41]. The z-score of mean birthweight also converged by period of diagnosis, illustrating that offspring of cancer survivors diagnosed in the eighties and nineties had a higher birthweight than their control peers, a difference that diminished in the offspring of survivors diagnosed after 1997. The lower prevalence of smoking during pregnancy in cancer survivors in our study population may have in part counteracted the negative effects that treatment strategies such as uterine radiation have on uterine elasticity. Their earlier adoption of a healthier lifestyle may have been beneficial to the risk of delivery of offspring that were small for gestational age. Unfortunately, information on smoking during pregnancy was missing in a substantial proportion of the cancer survivors, and in an even larger proportion of the control general population. These non-randomly missing data prohibited adjustment for this possible confounder, as excluding those with missing data from the analysis may lead to biased results[42]. Offspring of the least deprived survivors also had slightly higher birthweights than their matched controls.

Previous studies have indicated that cancer survivors are at increased risk of postpartum haemorrhage, but only after abdominal radiation[10,23] although other studies have reported no increased risk[20,24,43]. We show a higher risk of postpartum haemorrhage in cancer survivors overall, but as the incidence of postpartum haemorrhage has increased more rapidly in the control general population, the difference has diminished in the most recent decade. It is possible that better recording of haemorrhage in routine records (reporting bias), as a result of intensified surveillance, may have played a role in the higher reported incidence of postpartum haemorrhage in cancer survivors.

A lower threshold for intervention may at least in part explain the higher incidence of both emergency and elective caesarean section in the cancer survivors, although rates of elective section in particular converged with controls by period of diagnosis. The rate of emergency caesarean section rose by period of diagnosis in both the survivor and control groups, and there was no significant difference in risk for any single period of diagnosis. The over three-fold increased risk of an elective caesarean section in the women diagnosed aged 0-14 is substantially larger than the impact observed (RR 1.38 in those not treated with radiotherapy and RR 1.46 in those treated with abdominal radiotherapy) in the BCCSS[10]. In that study 40% of survivors were diagnosed in the most recent time period included (1985-1991), with pregnancy outcomes between 1997 and 2012, thus the pregnancy outcomes may be more comparable to the more recently diagnosed (and more recently pregnant) cohorts in the present data.

Inequalities by deprivation were found in the prevalence of operative delivery, where only the most and least deprived show an increased risk of emergency caesarean section, whereas survivors in all deprivation quintiles were at increased risk of elective caesarean section. Deprivation is known to be a major factor in increasing health inequalities[44]. It is possible that survivors from the most deprived group may experience greater medical intervention in their obstetric care due to the presence of more co-morbidities whereas less deprived survivors may be more empowered to influence their obstetric care. However these differences require specific investigation to confirm and determine their basis.

As with the normalized risk of postpartum haemorrhage over time, the impact of a cancer diagnosis on the risk of an operative delivery also diminished in the later periods, resulting in equal risks of all modes of delivery for those diagnosed in the most recent cohort. The reduced impact of a cancer diagnosis on the risk of an intervention during delivery may be a result of better targeted treatment strategies, and of a reduction of therapeutic exposures known to be associated with organ toxicity, e.g. radiotherapy in Hodgkin lymphoma[45]. This observation is also in line with decreased late mortality among survivors of childhood cancer as a result of reduced radiotherapy and chemotherapy exposure[46].

CONCLUSION

Cancer survivors are at increased risk of premature delivery and postpartum haemorrhage, but not of small for gestational age or congenital abnormalities when compared to a non-cancer control population. It is reassuring that the impact of a cancer diagnosis on postpartum haemorrhage and mode of delivery has been greatly reduced in most recently diagnosed cohorts of survivors, although heightened alertness and careful management in cancer survivors remains appropriate.

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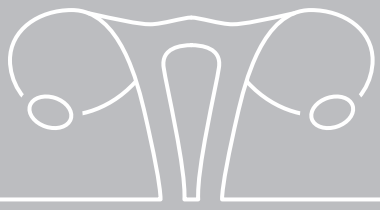

CHAPTER 9

Perinatal complications in female survivors of cancer: a systematic review and meta-analysis

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ABSTRACT

Background: Observational studies have suggested that perinatal outcomes are worse in offspring of cancer survivors. We conducted a systematic review and meta-analysis to examine the risks of perinatal complications in female cancer survivors diagnosed before the age of 40 years.

Methods: All published articles on pregnancy, perinatal or congenital risks in female cancer survivors were screened for eligibility. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines were followed.

Results: Twenty-two studies met the inclusion criteria. Meta-analysis indicates that offspring of cancer survivors are at increased risk of prematurity (relative risk [RR]: 1.56; 95% confidence interval [CI] 1.37 – 1.77) and low birth weight (RR 1.47; 95% CI 1.24 – 1.73) but not of being small for gestational age (RR 0.99; 95% CI 0.81 – 1.22). Cancer survivors have higher rates of elective (RR: 1.38; 95% CI 1.13 – 1.70) and emergency caesarean section (RR: 1.22; 95% CI 1.15 – 1.30) as well as assisted vaginal delivery (RR: 1.10; 95% CI 1.02 – 1.18) and are at increased risk of postpartum haemorrhage (RR: 1.18; 95% CI 1.02 – 1.36). The risk of congenital abnormalities also appears increased (RR 1.10; 95% CI 1.02 – 1.20) but this is likely to be an artefact of analysis. Although meta-analysis of the effects of radiotherapy was not possible for all outcomes, there was an increased risk of prematurity (RR 2.27; 95% CI 1.34 – 3.82) and consistent findings of low birth weight (RR 1.38-2.31). Risk of being small for gestational age was increased only after high uterine radiotherapy dosage.

Conclusion: The increased perinatal risks warrant a proactive approach from health care providers in both counselling and management of perinatal care for cancer survivors.

INTRODUCTION

Around 5% of all cancers are diagnosed before the age of 40 years[1], and survival rates after cancer in children and young adults are relatively high with approximately 80% being alive 5 years after the diagnosis[2]. Building a family may be part of their future, and as societal changes have led women to delay childbirth, an increasing number of survivors have not started a family at the time of diagnosis. Future fertility prospects may be affected by the administered cancer treatment, and pregnancy chances are about a third lower in cancer survivors compared with the general population[3]. Nevertheless, many female survivors have the wish and the potential to become pregnant[4-7].

Several studies have evaluated complications during pregnancy and labour in female cancer survivors in comparison to siblings or the general population. Increased risks for preterm birth were reported in the US Childhood Cancer Survivors Study (CCSS) and the British Childhood Cancer Survivors Study (BCCSS)[8,9], as well as in other large populations with survivors diagnosed in their reproductive life[10,11]. However, contrasting findings were observed for the risk of offspring being small for gestational age[8,11,12]. Despite being an important landmark in pregnancy planning for psychological reasons, less is known about the method of delivery in cancer survivors. Nonetheless, the largest studies showed decreased rates of spontaneous vaginal delivery and increased rates of caesarean section[9,12-14]. Some early studies suggested an increased relative risk (RR) of congenital abnormalities in the offspring of cancer survivors[15,16]. These findings have not been confirmed in more recent analyses[9,12,17,18]. Owing to the low prevalence of both cancer in children and young adults and of some pregnancy and labour complications, evaluation of these data benefits from large number of subjects being involved, giving increased statistical power. To synthesise the available data across studies, we performed a systematic review and meta-analysis.

METHODS

This review and meta-analysis was registered in PROSPERO (CRD42017078007) and the Preferred Reporting Items for Systematic Reviews and Meta-Analyses were followed[19].

The databases Embase, MEDLINE (via OvidSP), Web of Science, Cochrane and Google Scholar were used for the systematic search. Details of the full search strategy for each database are included in Appendix A (online only). In brief, we searched for articles reporting on any perinatal outcomes (maternal and foetal/neonatal) in survivors of any cancer until the age of 40 years. The search was limited to the following criteria: reported between 1990 and September 2018 and published in English. All titles and abstracts were reviewed to select potentially eligible studies by two independent reviewers (ALFvdK and TWK). Full-text articles were retrieved to assess fulfilment of the selection criteria. Studies reporting

on pregnancies and/or births of less than 50 cancer survivors and cohort studies that did not include a control group were excluded, as well as opinion articles or reviews. Cross-reference check of the retrieved studies was performed to identify additional studies that were overlooked during the initial search.

The critical appraisal skills programme (CASP, https://casp-uk.net/) provides tools for a structured approach to find evidence and appraise the evidence based on methodology and validity. The standardised checklist for cohort studies consists of 11 questions within three parts: 'Are the results of the study valid' (section A, focusing on bias and confounding), 'What are the results' (section B, on strength and precision), and 'Will the results help locally' (section C, on generalizability). This assessment was performed by three independent authors (ALFvdK, TWK and RAA) and disagreements were discussed and resolved among them.

Outcome measures that were included were the following: low birth weight (<2500g), preterm birth (<37 weeks gestation), small for gestational age (<10^{th} percentile), spontaneous vaginal delivery, assisted vaginal delivery, elective caesarean section, emergency caesarean section, antepartum haemorrhage (as defined by the authors of included studies, including placenta praevia, placental abruption and other bleeding), postpartum haemorrhage and congenital abnormalities.

For all outcomes, incidence or prevalence numbers were extracted for both the cancer survivor group and the control group. In addition, incidence or prevalence numbers from survivors treated with abdominal radiotherapy were extracted or 'any radiotherapy' if no more details were available. Heterogeneity between the eligible studies was assessed using the I^2 statistic, with I^2 > 80% indicating high variation between included studies, I^2 between 50% and 80% indicating moderate variation and I^2 <50% indicating sufficient similarity between the studies to ensure that pooling was valid. When heterogeneity was considerable (i.e., I^2 ≥50% and $p<0.05$), pooled estimates based on the random effects model were presented. Otherwise, pooled fixed effects were presented. Meta-analysis was only performed if more than two studies were available for the meta-analysis. Funnel plots were created to evaluate the possibility of publication bias. This type of graph plots each study's precision against its result. In this way, studies with high precision are plotted near the average and studies with lower precision are spread to the side in a funnel-shaped manner. Asymmetry of the resulting scatterplot can be a result of publication bias or other study heterogeneity and warrants further investigation. Summary measures of RR and 95% confidence intervals (95% CIs) were obtained using standard meta-analysis in the R package meta[20, 21].

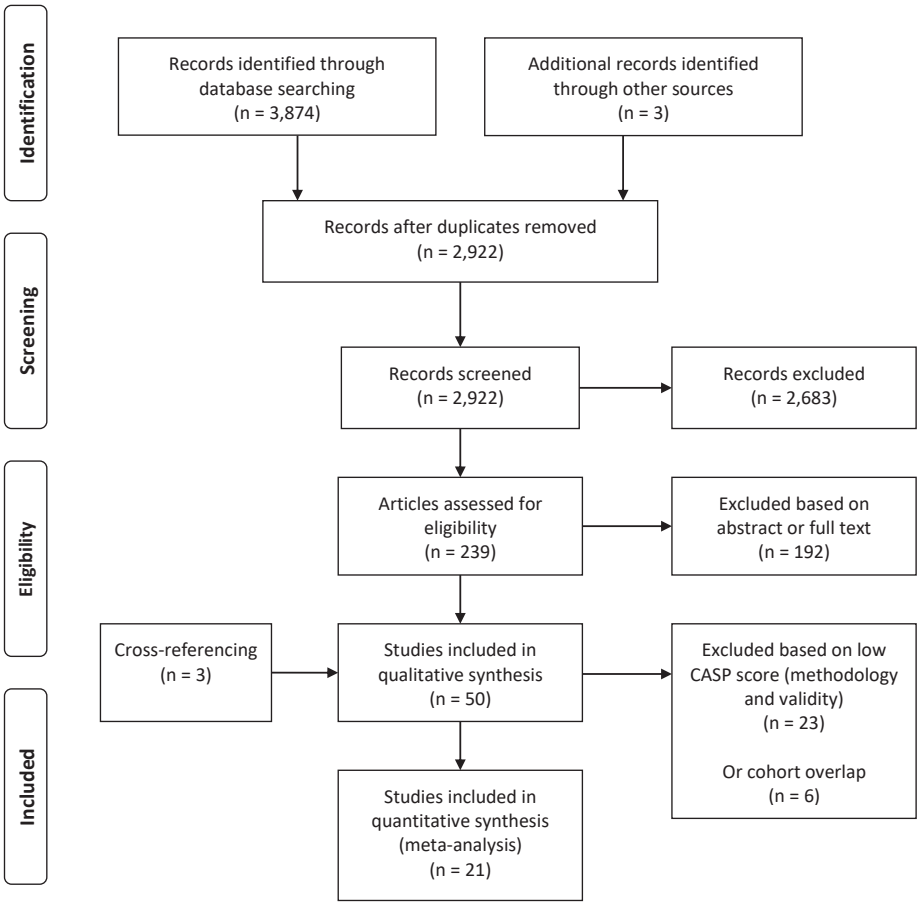

Figure 1. PRISMA flowchart showing selection of studies. PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analysis.

RESULTS

After exclusion of duplicates, the search yielded 2,922 citations. After screening of titles, 239 remained of which 192 could be excluded based on abstract or full-text, while three other publications were identified from cross-reference checking. The remaining 50 studies were included for CASP scoring, in which ≥9 of 11 points were required for inclusion in the meta-analysis. Studies reporting on cohorts from the same region were examined for overlapping data, and in these cases, the oldest reports were excluded. A total of 22 studies were included for the meta-analysis[6,8-14,18,22-34]. The list of included and excluded studies and their assigned CASP scores can be found in Appendix B (online only).

All 22 included studies were retrospective cohort studies. Most studies (n=15), especially the most recently reported, had obtained data by population registry linkage. One study was based on medical records[24], and six studies were based on questionnaire data[6,22,27,31-33].

While all studies included survivors of cancer, age at diagnosis varied. Eight studies had included only survivors of childhood cancer[8,9,28,29,31-34], the largest cohorts being the CCSS and the BCCSS, confined to survivors diagnosed before the age of 21 and 15 years respectively[6,9]. Eight studies included adults until the age of approximately 40 years[10,22-27,30,35] and the remaining five studies included survivors diagnosed with cancer between 0-40 years[12-14,18,36]. Five studies reported on the risks after a specific cancer diagnosis: cervical cancer[22,27], Hodgkin lymphoma[30] or breast cancer[10,23].

Outcomes

Prematurity

Fourteen studies reported the incidence of prematurity (gestational age less than 37 weeks)[8-13,22-27,30,31] For this outcome, in total 17,495 cancer survivors were compared with 6,070,504 controls. The RR in the random effects model of a preterm delivery for cancer survivors was 1.56 (95% CI 1.37 – 1.77), with moderate to high heterogeneity (I^2 = 82%, p <0.01) (Figure 2A). The funnel plot did not suggest publication bias (supplementary Figure, online only). Prematurity in high-risk groups, e.g., after radiotherapy or (if available) after abdominal radiotherapy, was reported in eight of these studies. The random effects meta-analysis of the four studies which also provided incidence data showed an RR of 2.27 (95% CI 1.34 – 3.82) (Figure 6A)[9,30,31,36]. Four studies reported only ratios but not the exact number, of which two showed similar effect sizes[8,35], one did not find an increased risk[13] and one found an increased risk in those treated with radiotherapy only, but not in survivors treated with radiotherapy in combination with chemotherapy[25] (Appendix C, online only).

Low birth weight

Twelve of the studies reporting on prematurity also reported the incidence of low birth weight (<2.500g), comparing in total 19,073 cancer survivors with 6,099,456 controls[8-13,22,24-27,31]. Meta-analysis showed a significantly higher risk of having a baby with a low birth weight in cancer survivors when compared with controls (RR 1.47; 95% CI 1.24 – 1.73). Owing to the high heterogeneity (I^2 = 86%, p <0.01), the random effects model was used (Figure 2B). The funnel plot did not reveal publication bias (Supplementary Figure, online only). Low birth weight after high-risk treatment was reported in six studies[8,9,13,25,31,35], but only two studies reported incidence numbers, which prohibited meta-analysis (Appendix C, online only). RR ranged from 1.38 (95% CI 1.03 – 1.85) after any radiotherapy versus controls[8] to 2.31 (95% CI 1.50 – 3.55) after abdominal radiotherapy in comparison to survivors not treated with radiotherapy[9] (Appendix C, online only).

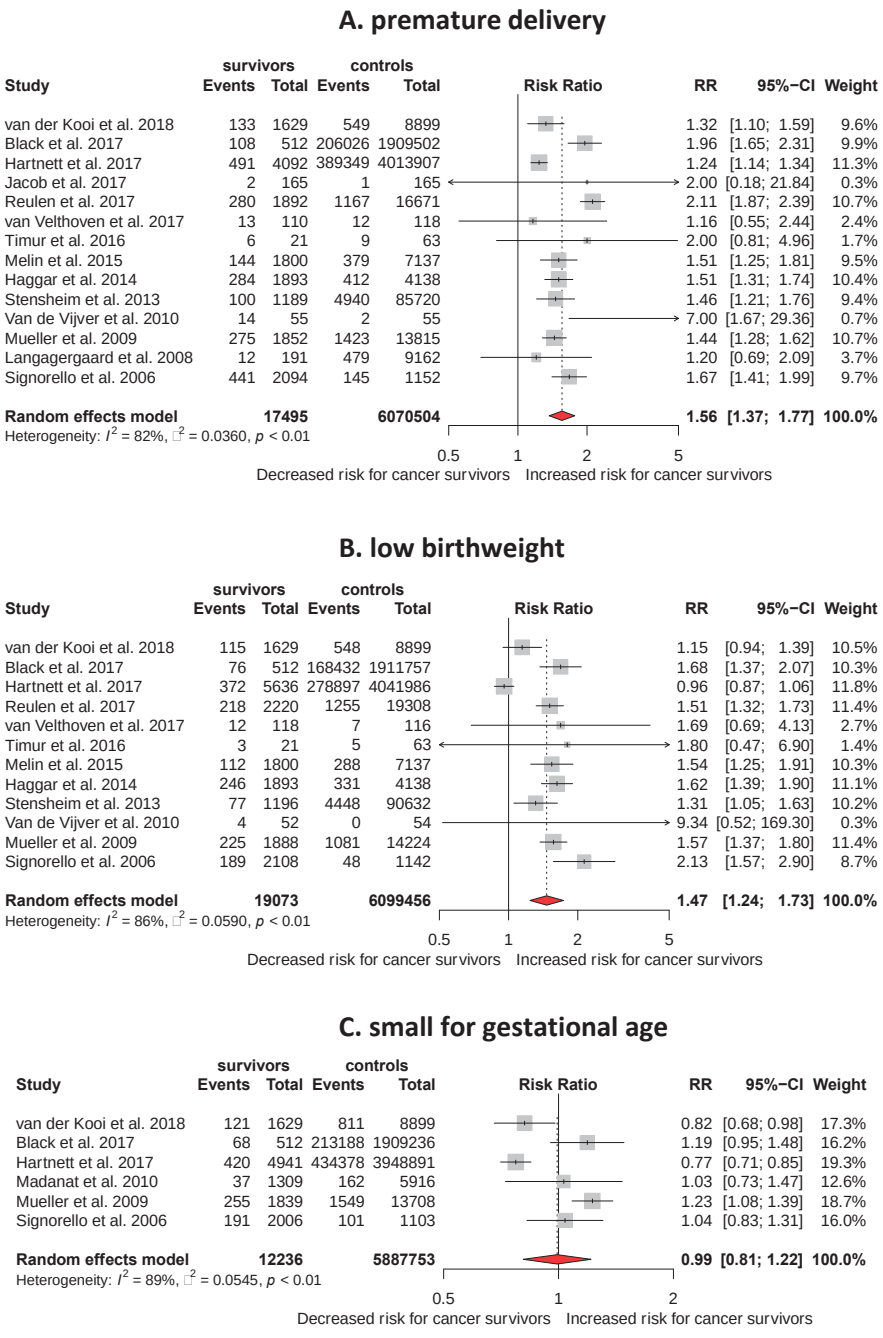

Figure 2. Pooled relative risk (RR) of premature delivery (<37 weeks of gestation; A), low birth weight (<2,500 gram; B) and being small for gestational age (<10th percentile; C) of cancer survivors compared with controls. CI, confidence interval.

Small for gestational age

Six studies (comparing in total 12,236 cancer survivors with 5,887,753 controls) reported on the outcome of being small for gestational age, defined as a weight less than the 10th percentile for that gestational age in the reference population[8,10-12,31,36]. The risk of having a small-for-gestational-age baby was not statistically significantly different for cancer survivors compared with controls (RR 0.99; 95% CI 0.81 – 1.22) in the random effects model. There was high heterogeneity among the studies (I^2 = 89%, p <0.01) (Figure 2C). The funnel plot did not reveal any significant publication bias (supplementary Figure, online only). Two studies reported on the risk on being small for gestational age after radiotherapy: one did not detect any increased risk after radiotherapy alone or in combination with chemotherapy[35] and the other found an increased odds ratio (4.0, 95% CI 1.6 – 9.8) after a radiation dose of >500cGy to the uterus but no significant effect at lower doses[31] (Appendix C, online only).

Spontaneous vaginal delivery

There were five studies that reported on the incidence of spontaneous vaginal deliveries, in total reporting on 3,497 cancer survivors and 24,370 controls[12,13,23,24,28]. In the random effects model, cancer survivors were equally likely to have a spontaneous vaginal delivery: RR was 0.95 (95% CI 0.84 – 1.07) (Figure 3A). Heterogeneity was high (I^2 = 82%, p <0.01) and the funnel plot showed a deviation, a study of breast cancer survivors, which showed that breast cancer survivors were more likely to have a spontaneous vaginal delivery (Supplementary Figure, online only)[23].

Assisted vaginal delivery

Six studies reported the incidence of assisted vaginal deliveries, in 10,710 survivors and 1,771,131 controls[12-14,23,27,28]. The RR of an assisted vaginal delivery was 1.10 (95% CI 1.02 – 1.18) (Figure 3B). Heterogeneity was low to moderate (I^2 = 49%, p = 0.08) and the funnel plot showed a deviation with overrepresentation of studies on the left side of the plot, presenting small studies not showing a significant increase in the risk (supplementary Figure, online only). The risk of assisted vaginal delivery after abdominal radiation was only assessed in one sub study with six survivors[28], and one study reported no increased risk after treatment with (any) radiotherapy[13] (Appendix C, online only).

Emergency caesarean section

Five studies with in total 5,471 survivors and 45,593 controls reported the incidence of emergency caesarean sections in their cohorts[9,12,13,27,28]. The relative risk was 1.22 (95% CI 1.15 – 1.30) (Figure 3C). There was no heterogeneity (I^2 = 0%, p = 0.46) and the funnel plot did not suggest publication bias (supplementary Figure, online only). The two studies that reported on the risk on an emergency caesarean section after radiotherapy[13] or abdominal radiotherapy[9] showed no increased risk (Appendix C, online only).

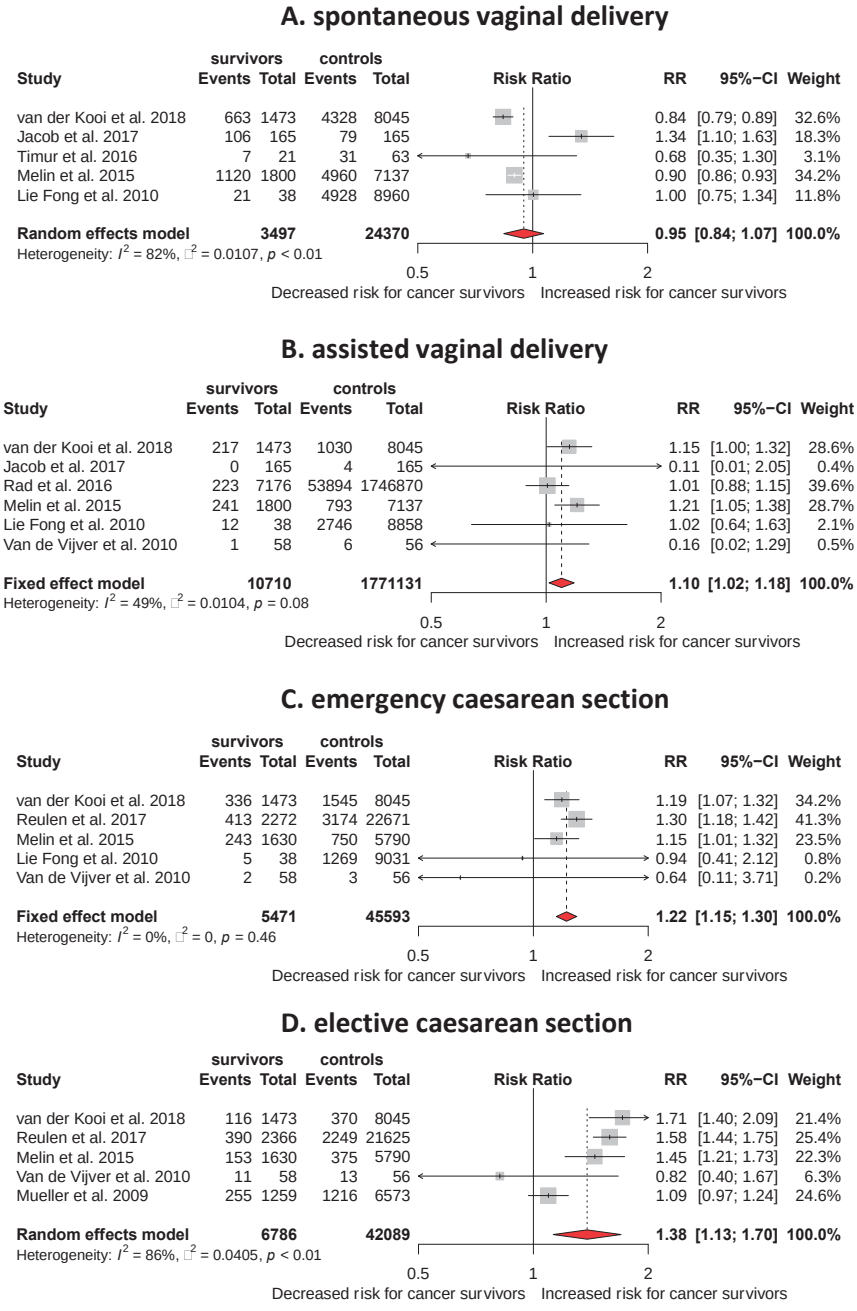

Figure 3. Pooled relative risk (RR) of the mode of delivery of cancer survivors compared with controls. CI, confidence interval.

Elective caesarean section

An elective caesarean section occurred more often in cancer survivors than in controls. Five studies reported on 6,786 survivors and 42,089 controls[8,9,12,13,27]. The RR of elective caesarean section was 1.38 (95% CI 1.13 – 1.70). Heterogeneity was high (I^2 = 86%, p <0.01), therefore the random effects model was used (Figure 3D). The funnel plot suggested no significant publication bias (supplementary Figure, online only). The risk in survivors treated with radiotherapy to the abdomen was only reported in the BCCSS cohort, showing an increased risk of 1.46 (1.07 – 1.99). The risk from any radiotherapy was reported to be not elevated in two other studies[8,13] (Appendix C, online only).

Antepartum haemorrhage

Three studies reported the incidence of antepartum haemorrhage[12,14,25]. The definition of antepartum haemorrhage varied between the studies. Hagger *et al.* defined it as occurrence of placental abruption, placenta praevia or other excessive bleeding during labor and delivery[25]. In contrast, Rad *et al.*[14] and Van der Kooi *et al.*[12] based their outcome on the International Classification of Diseases (ICD) 10, where 'antepartum haemorrhage' does not include placenta praevia or abruptio placentae, as those outcomes were separately reported.

For this outcome, in total 10,505 cancer survivors were compared with 1,759,869 controls. The RR of antepartum haemorrhage for cancer survivors was not significant with an RR of 1.06 (95% CI 0.88 – 1.29), while there was no heterogeneity of this RR (I^2 = 0%, p = 0.86) (Figure 4A). The funnel plot did not suggest publication bias (supplementary Figure, online only). None of the studies reported on the risk in a high-risk survivor population, e.g., after abdominal radiotherapy.

Postpartum haemorrhage

Postpartum haemorrhage was reported in six studies[9,12-14,25,28]. Three studies[9,12,14] based postpartum haemorrhage on O72 of the ICD 10 which defines postpartum haemorrhage as blood loss >500 mL after vaginal delivery or >1000 mL after caesarean delivery. In contrast, Melin *et al.*[13] and Lie Fong *et al.*[28] defined postpartum haemorrhage as >1000 mL while Hagger *et al.*[25] defined it as >500 mL.

The incidence of postpartum haemorrhage was compared between in total 14,314 cancer survivors and 1,795,524 controls. Cancer survivors were at increased risk of postpartum haemorrhage (RR: 1.18; 95% CI 1.02 – 1.36) (Figure 4B). Heterogeneity across studies was substantial (I^2 = 77%, p <0.01); therefore, the random effects model is presented; the funnel plot did not suggest publication bias (Supplementary Figure, online only). Adjustment for parity and maternal age had reduced the effect sizes in some of the original articles[9,13]. Postpartum haemorrhage after (abdominal) radiotherapy was reported in three studies; in one, it is described not to have an increased risk but without numerical data[13]; therefore, a

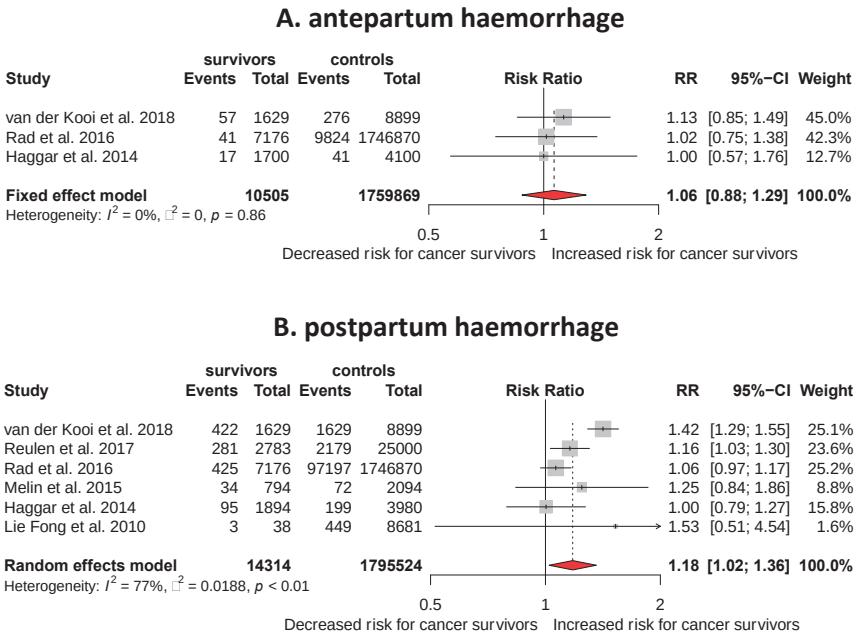

Figure 4. Pooled relative risk (RR) of antepartum (A) and postpartum haemorrhage (B) of cancer survivors compared with controls. CI, confidence interval.

meta-analysis was not feasible. One small study found an increased risk in the subgroup of six abdominally radiated survivors[28], and one analysis from the BCCSS found no increased risk after adjustment for confounding (RR 1.33; 95% CI 0.84 – 1.07) compared with survivors not treated with any radiotherapy[9] (Appendix C, online only).

Congenital abnormalities

Twelve studies reported the prevalence of congenital abnormalities in a total cohort of 23,099 cancer survivors and 254,264 controls[8,12,18,24-26,28-30,32-34]. The definition of congenital abnormalities ranged from 'coded as ICD diagnoses (ICD8 740-760)' to 'presence of any malformation'. All reported anomalies are pooled in this meta-analysis. The resulting pooled RR of congenital abnormalities appears to be higher in the cancer survivor group, with an RR of 1.10 (95% CI 1.02 – 2.20) (Figure 5). There was moderate observed heterogeneity (I^2 = 45%, p = 0.05) and the funnel plot did not suggest publication bias (supplementary Figure, online only). Five studies also reported incidence numbers of congenital abnormalities after high-risk radiation[18,28-30,32,33]. The fixed effect model showed a non-significant RR of 1.15 (95% CI 0.76 – 1.75) in keeping with the statistically non-significant reported risks or odds ratios in all the source articles (Appendix C, online only).

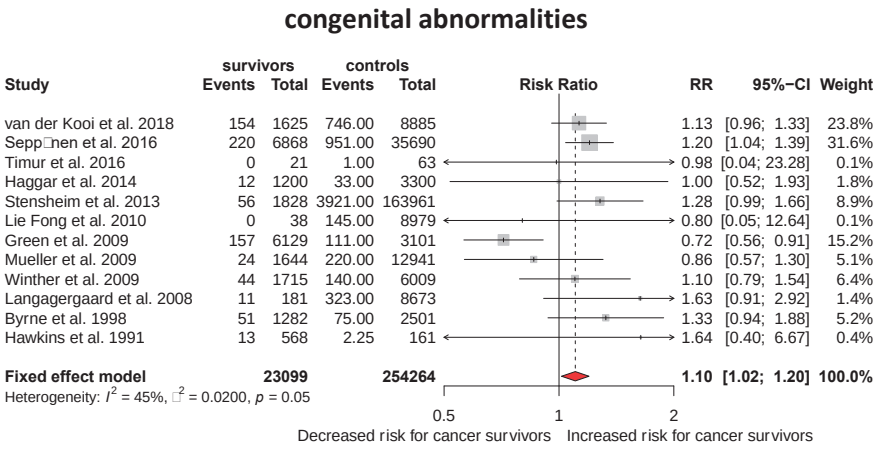

Figure 5. Pooled relative risk (RR) of congenital abnormalities of cancer survivors compared with controls. CI, confidence interval.

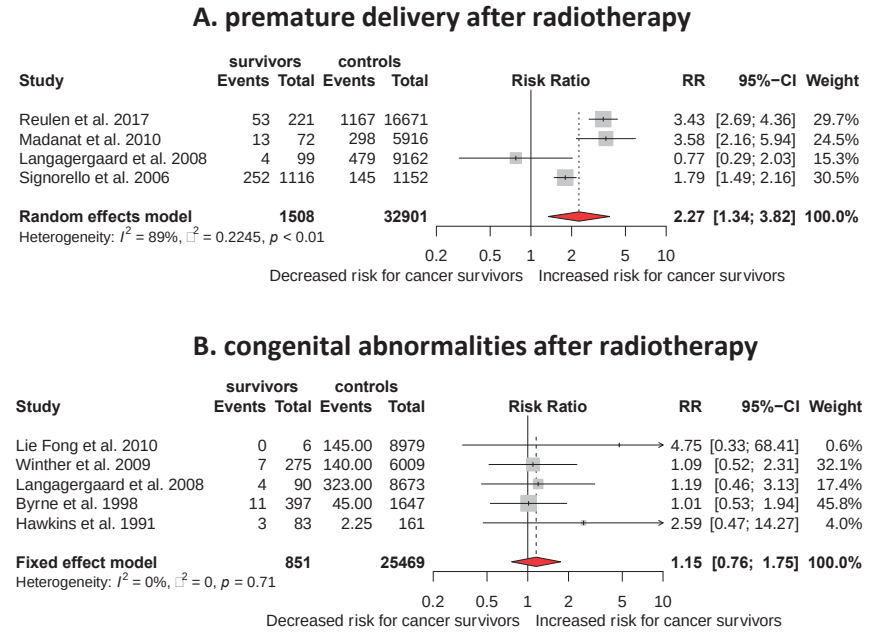

Figure 6. Pooled relative risk (RR) of premature delivery and congenital abnormalities after treatment with radiotherapy (A and B, respectively) of cancer survivors compared with controls. CI, confidence interval.

DISCUSSION

Principal findings

This systematic review and meta-analysis summarises the evidence for risks in perinatal outcomes in female cancer survivors. Outcome measures investigated were low birth weight, preterm birth, being small for gestational age, mode of delivery, antepartum haemorrhage, postpartum haemorrhage and congenital abnormalities. Offspring of cancer survivors are at increased risk of prematurity and a low birth weight, but do not face an increased risk of being small for gestational age. Cancer survivors are at increased risk of elective and emergency caesarean section as well as assisted vaginal delivery, and postpartum but not antepartum haemorrhage.

Cancer treatment protocols can include chemotherapy and radiotherapy. Irradiation of the abdomen can damage the uterine vasculature and the muscular development of the uterus[37]. Endometrial function, possibly partly due to impaired blood supply, has also been postulated to be defective. Impairment of decidualisation could interfere with normal placentation and trophoblast invasion. In addition, impairment of uterine vasculature leading to impaired foetal-placental blood flow may cause fetal growth restriction, and reduced uterine elasticity and volume could lead to preterm delivery or postpartum haemorrhage[37,38]. Smaller uterine volumes can also be the result of hormonal deficiency as a consequence of ovarian failure[38].

Although the risks of a premature birth and low birth weight were increased, the pooled estimates showed no evidence for increased risks of offspring being small for gestational age. Despite this reassurance, future research on very premature deliveries, such as before 32 weeks of gestation instead of the 37 weeks of gestation that is now most often evaluated, may be of value. Very premature birth may be of a greater consequence for future health and well-being[39], even if the offspring is not small for gestational age. One study reported the risk of being small for gestational age to be increased only after a high radiation dose[31]. The effect of radiation dose to the uterus has not been sufficiently examined to review, but it is likely that a distinction between higher and lower dosages of radiotherapy will reveal an increased risk currently obscured by pooling all dosages.

There was a markedly increased risk (38%) in elective caesarean section, although one study showed that this risk may have reduced in more recent years[12]. There was also an increased risk of an emergency caesarean section (by 22%), and the need for assistance during a vaginal delivery (by 10%). These increased risks may be the reflection of an increased awareness and pro-active management of women treated for cancer, specifically after treatment with abdominal radiotherapy. This analysis showed an increased risk of postpartum haemorrhage, indicating that a proactive approach to prevention may be warranted.

The meta-analysis indicates an increased risk of congenital abnormality. Congenital abnormalities could be a result of germ cell mutagenicity cause by chemotherapy or irradiation of the ovarian follicle pool. Most evidence on radiation and chemical induced mutations is based on germ cells of mice[40]. In humans however, long-term follow-up studies of the offspring of Japanese atomic bomb survivors did not indicate an increased risk of congenital abnormalities as a result of parental radiation exposure[41,42]. The apparent increased risk of congenital abnormalities is likely to be an example of Simpson's paradox, a statistical phenomenon in which certain effects observed in different groups or cohorts disappear or reverse when the groups are combined. In such cases there is often an unidentified confounding variable introduced either by the recruitment of subjects, by the analysis for studies forming the pool, or by the analysis of pooled results[43,44]. In the case of congenital abnormalities, the definition varies greatly – with large fluctuations in prevalence rates ranging from 1.4%[8] to 9.5%[12]. In the separate studies, only one of the 12 studies reporting on congenital abnormalities reported a higher prevalence in cancer survivors[18]. In that study, the unadjusted prevalence ratio was 1.21 (95% CI 1.03 – 1.40) but after adjustment for maternal age at birth of child, parity, sex of child and birth decade of child, the adjusted prevalence ratio was 1.07 (95% CI 0.91 – 1.25). This study accounted for 31.6% of weight in the meta-analysis. The apparent increased effect is therefore likely to be biased (or paradoxical), introduced by a heterogeneous definition of congenital abnormalities resulting in large variation in prevalence rates and the absence of adjustment for possible confounders such as maternal age, or genetic predisposition/hereditary disease.

Strengths and limitations

This systematic review offers an inclusive overview of relevant publications and meta-analyses of eleven outcomes, which facilitate the interpretation of the summarised literature. A choice of relatively frequently evaluated outcomes was made: perinatal risks such as cardiomyopathy after treatment with anthracyclines[45], pregnancy-induced hypertension[9,46], diabetes mellitus or gravidarum[8,9,25] and others were, therefore, beyond the scope of this report. The main limitation is the heterogeneity within the meta-analyses, possibly a result of differences in the diagnostic criteria between the studies. Owing to the varied designs of the observational studies and lack of individual patient data, systematic adjustment for confounders was not possible, so an overestimation or underestimation of the RRs could have occurred. For congenital abnormalities, this is especially striking with a possible example of the Simpson's paradox as a result. In addition, there was no uniformity in subanalysis of potential high-risk groups, such as women who had received radiotherapy to a field that included the uterus. Some studies reported risks after any radiotherapy, some after only radiotherapy and some after certain fields of radiotherapy. Nonetheless, these subgroups can be used as an approximation of high-risk treatment groups, and conclusions can be drawn where the observed risks are consistent.

The increasing numbers of cancer survivors as a result of better treatment protocols, and the increasing possibilities for fertility preservation, will in the future allow more survivors to consider a pregnancy. In the near future, more survivors who otherwise would not have had the possibility of reproduction, who are likely to have been exposed to higher doses of chemotherapy and radiotherapy than those whose fertility was not impaired, may become pregnant as a result of improving fertility preservation techniques such as vitrification of oocytes and ovarian tissue cryopreservation[47-49]. Possible effects of these fertility treatments have not been taken into account in these analyses, but the increase in number of pregnancies in this at-risk population underline the importance of surveillance and supervision of these pregnancies and deliveries.

CONCLUSIONS

This meta-analysis confirms that survivors of cancer are at increased risk of postpartum haemorrhage, especially after abdominal radiotherapy, and of increased rates of elective and emergency caesarean section. In addition, offspring of cancer survivors are at increased risk of prematurity and a low birth weight, but not for being small for gestational age. Our results show a likely Simpson's paradox regarding the risk of congenital abnormalities, with the true effect being no increased risk. The magnitude of the perinatal risks warrants a proactive approach from health care providers.

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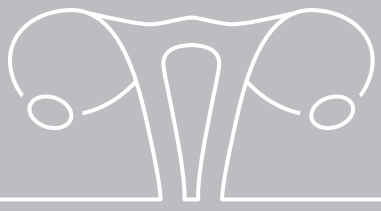

CHAPTER 10

Recommendations for counseling and surveillance of obstetric risks for female survivors of childhood, adolescent, and young adult cancer: a report from the International Late Effects of Childhood Cancer Guideline Harmonization Group

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ABSTRACT

Female survivors of childhood, adolescent, and young adult (CAYA) cancer have an increased risk of adverse pregnancy outcomes (e.g. miscarriage, premature delivery, perinatal cardiomyopathy) related to their cancer or treatment-associated sequelae. Optimal care for CAYA cancer survivors can be facilitated by clinical practice guidelines that identify specific adverse pregnancy outcomes and the clinical characteristics of at-risk subgroups that should be closely monitored. However, national guidelines are scarce and vary considerably in their recommendations. Thus, this guideline from the International Late Effects of Childhood Cancer Guideline Harmonization Group (IGHG) evaluated the quality of available evidence for adverse obstetric outcomes in CAYA cancer survivors (diagnosed before 25 years of age and not pregnant at that time), and formulated recommendations to enhance evidence-based obstetric care and counseling of female CAYA cancer survivors. We recommend that healthcare providers should discuss the risk of adverse obstetric outcomes based on the specific cancer treatment exposures with all female CAYA cancer survivors of reproductive age. Survivors and their health care providers should be aware that there is no evidence to support that there is an increased risk of giving birth to a child with congenital anomalies (high quality evidence). Survivors treated with radiotherapy to volumes exposing the uterus and their health care providers should be aware of the risk of adverse obstetric outcomes including miscarriage (moderate quality evidence), premature birth (high quality evidence) and low birth weight (high quality evidence) and therefore, high risk obstetric surveillance is recommended. Based on the IGHG cardiomyopathy guideline, cardiomyopathy surveillance is reasonable prior to pregnancy or in the first trimester for all female survivors treated with anthracyclines and/or chest radiation. Gaps in knowledge and directions for future research are presented to further refine evidence-based recommendations.

INTRODUCTION

Advances in cancer treatment strategies have resulted in 5-year survival for childhood, adolescent, and young adult (CAYA) cancer patients that approaches 80%[1]. Consequently, increasing numbers of CAYA cancer survivors are at risk for adverse cancer and/or treatment-related complications that may affect both physical and psychosocial functioning. Physical late effects include the development of subsequent malignancies as well as dysfunction of the cardiovascular, pulmonary, hepatic, renal, endocrine and reproductive systems[2]. Among these, reproductive health, and specifically pregnancy and delivery, represents a critical area for long-term follow-up care as having children is an important determinant of quality of life for CAYA cancer survivors[3-7].

Previous research indicates that CAYA cancer survivors can have difficulty conceiving or carrying a pregnancy to term as well as experiencing excess risk of adverse pregnancy outcomes. For example, the risks of premature birth and postpartum hemorrhage are both higher in CAYA cancer survivors compared to women who did not have cancer[8-13], and the risks increase in survivors treated with abdominopelvic radiotherapy[9,11-14]. Evidence-based clinical guidelines on surveillance in pregnancy can identify the type and prevalence of specific obstetric and perinatal complications, characterize the clinical features of those at risk, help survivors make informed decisions, facilitate counseling and timely referral to obstetric care specialized in high risk pregnancies, and facilitate opportunities for interventions to optimize pregnancy outcomes.

Unfortunately, few recommendations for obstetric care of CAYA cancer survivors exist. Obstetric risks in CAYA cancer survivors are generally noted in published clinical practice guidelines by North American and European groups[15-18], but without comprehensive assessment of the risk features of women who may benefit from high-risk obstetric follow-up. In a previous report, the International Late Effects of Childhood Cancer Guideline Harmonization Group (IGHG) developed recommendations for cardiomyopathy surveillance[19], including early detection among women planning to become pregnant. In the current effect, the IGHG summarizes the results of a systematic review and presents a critical appraisal of available evidence on obstetric risks in CAYA cancer survivors (diagnosed before 25 years of age and not pregnant at that time), to synthesize these findings into evidence-based recommendations for surveillance and counseling of CAYA cancer survivors who are at risk for complications during pregnancy and delivery due to their cancer or cancer treatment.

METHODS

The aim of the IGHG is to establish a common vision and integrated strategy for the surveillance of late effects in childhood, adolescent and young adult cancer survivors. Methods

of the IGHG have been described previously[20]. This guideline focuses on the identification of 'at risk' CAYA cancer survivors diagnosed with cancer before age 25 years who would benefit from preconception counseling and high-risk surveillance during pregnancy. This guideline is focused on facilitating timely identification and referral of CAYA survivors at high-risk of obstetric complications. Management of obstetric complications is beyond the scope of the present guideline, which should defer to standards established by local/national health systems. Standardized definitions as used in this guideline are presented in Appendix 1, available upon request or online.

The obstetric guideline panel consisted of 33 experts from the United States of America, United Kingdom, Denmark, France, New Zealand, Australia, Japan and the Netherlands who represent relevant disciplines, including gynecology, obstetrics, midwifery, endocrinology, pediatric oncology, radiation oncology, epidemiology, and guideline methodology, as well as CAYA survivor/family representatives.

Concordances and discordances across existing survivorship guidelines of the North American Children's Oncology Group (COG)[15], the Dutch Childhood Oncology Group (DCOG)[16], the Scottish Intercollegiate Guidelines Network (SIGN)[18], and the UK Children's Cancer and Leukaemia Group (UKCCLG)[17] were evaluated. We defined the major outcomes for obstetric problems in survivors and congenital problems in offspring (Appendix 1). For all discordances and relevant outcomes, focused clinical questions were formulated to determine whether specific preconception consultation or surveillance was indicated. Four working groups evaluated the following topics: 1) adverse fetal outcomes in pregnancy (such as miscarriage); 2) adverse maternal outcomes in pregnancy; 3) delivery outcomes; and 4) congenital anomalies of the neonate.

A systematic literature search was performed in MEDLINE (through PubMed) to identify all available evidence published between January 1990 and December 2018, using the search terms "childhood cancer", "survivors", "late effects" and "obstetric problems". Details of the full search strategy are included in Appendix 2. All study designs with a sample size larger than 40 pregnancies in female childhood cancer survivors were eligible. Studies published in English were selected for analysis. All abstracts were screened by two independent reviewers (ALLFK and one member of the working groups). Disagreements were resolved through consensus. Cross-reference checking was performed to identify additional studies that were potentially overlooked during the initial search. Each relevant article was summarized in one evidence table drafted by two reviewers (ALLFK and one member of the working groups), which also included a critical appraisal of risks of bias (Appendix 3). The evidence tables were subsequently assembled into summary of findings tables (ALLFK) and revised where necessary (RLM, LCMK). Next, we assessed the quality of the body of evidence for every clinical question according to criteria based on Grading of Recommendations Assessment Development and Evaluation (GRADE)[21] (Appendix 4).

Translating evidence into recommendations

Recommendations were drafted considering the level of the evidence, other effects of the expected risks (such as unnecessary medicalization), and the need to maintain flexibility across health care systems[22]. Terminology employed can be found in Appendix 5. Decisions were made through group discussion and final recommendations were discussed until unanimous consensus was reached. The strength of the recommendations was graded according to published evidence-based methods (Appendix 4). Recommendations were classified into strong or moderate recommendations, and based on high quality evidence, moderate quality evidence or expert opinion[20,22,23]. Pregnancy care-related recommendations from the IGHG cardiomyopathy guideline were adopted in this guideline in order to provide a complete overview of recommendations for pregnancy surveillance. The final harmonized recommendations were critically appraised by four independent external experts in the field and two survivor representatives.

FINDINGS

Discordances across existing LTFU guidelines

Identification of concordances and discordances amongst existing surveillance recommendations is displayed in Appendix 6, showing many discordant guideline areas for which we searched the evidence. The literature search yielded 2,772 abstracts for pregnancy and delivery related risks and 2,492 abstracts for congenital anomalies. In total, 98 full texts were reviewed and 28 articles were included (Figure 1, included articles in Appendix 7). The evidence tables and summary of findings are presented in Appendix 8. The conclusions of evidence tables including GRADE assessment are summarized in Table 1 and Appendix 9, and depicted in a color scheme in Appendix 10.

Who needs preconception consultation or specific obstetric surveillance?

Evidence for risks during pregnancy

Miscarriage

There is moderate level evidence that CAYA cancer survivors are at increased risk of miscarriage after radiotherapy to volumes exposing the uterus in comparison to the general population[9,14,24-30], although this association was only borderline statistically significant in a large cohort from the British Childhood Cancer Survivor Study[27] and not significant in two smaller studies[25,29]. There is only low level evidence for a dose-response relationship[30,31]. The evidence indicated no significant effect due to chemotherapy[9,27,31,32].

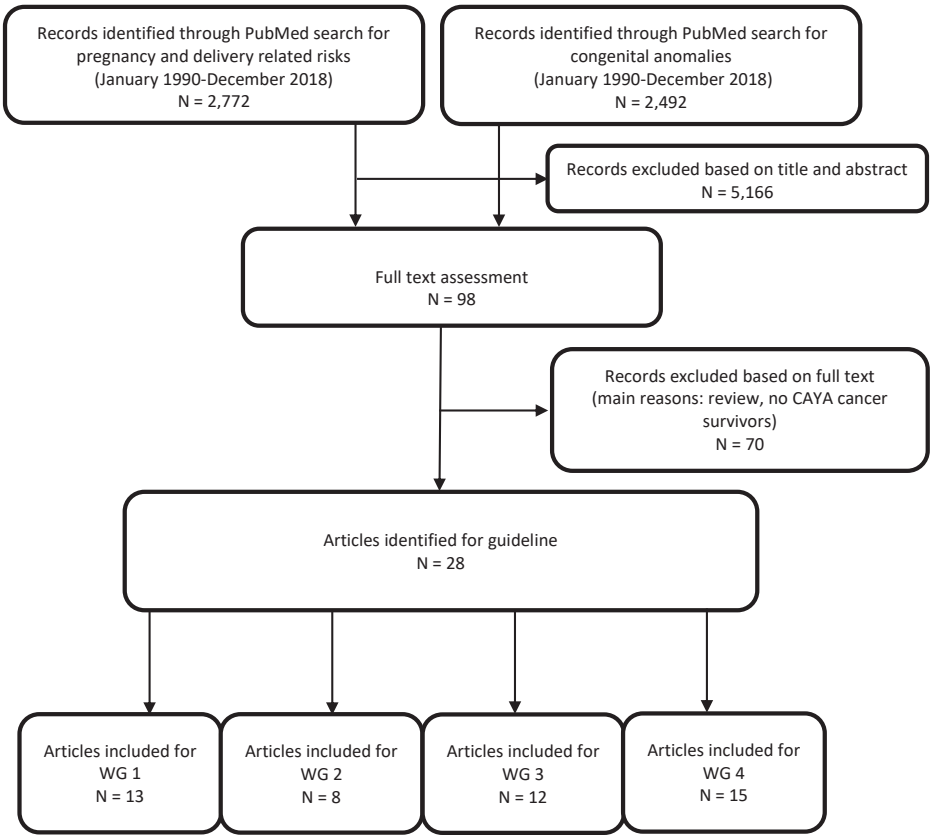

Figure 1. Flowchart of selected studies. Articles could be included for multiple working groups (WG). Four working groups respectively evaluated the following topics: 1) adverse fetal outcomes in pregnancy (such as miscarriage); 2) adverse maternal outcomes in pregnancy; 3) delivery outcomes; and 4) congenital anomalies of the neonate.

Termination of pregnancy

In the relevant articles, termination of pregnancy was defined as 'medically induced abortions' or 'not further defined', limiting, for instance, a distinction between medical and elective termination of pregnancy. In general, there is no suggestion for an increased risk of medically-induced terminations (very low level evidence)[14,24,27,30,33] in CAYA cancer survivors. However, there is (very) low level evidence for an increased risk of termination of pregnancy after any radiotherapy[14, 27] and chemotherapy[14,27].

Still birth

There is no suggestion for an increased risk of still birth (moderate level evidence) in CAYA cancer survivors in general[9,30], and low level evidence for increased risk of still birth after moderate to high doses ovarian-uterine radiotherapy (>10 Gy)[34] or abdominopelvic radiotherapy (>25 Gy)[31].

Table 1. Overall conclusions of evidence for obstetric risks in female childhood and adolescent cancer survivors (key outcomes)

Who needs preconception counseling? Who needs high-risk pregnancy surveillance?	
Risk of miscarriage in female cancer survivors diagnosed before age 25 years	**Level of evidence***
No increased risk in CAYA cancer survivors vs controls.	⊕⊕⊕⊖ MODERATE[9, 25, 26, 28, 30, 33]
Increased risk after (*abdominopelvic) radiotherapy* vs. no radiotherapy.	⊕⊕⊕⊖ MODERATE[9, 14, 24-30]
Increased risk with increasing *doses of abdominopelvic and pituitary radiotherapy* vs. no radiotherapy.	⊕⊕⊖⊖ LOW[30, 31]
No significant effect of *chemotherapy* vs. no chemotherapy.	⊕⊕⊕⊖ MODERATE[9, 14, 26, 27, 31]
Increased risk after *chemotherapy and radiotherapy* (no specific field) vs. no chemotherapy and radiotherapy.	⊕⊕⊖⊖ LOW[9, 14, 25, 26, 31]
No significant effect of *age at diagnosis*.	⊕⊕⊖⊖ LOW[9]
Risk of terminations in female cancer survivors diagnosed before age 25 years	**Level of evidence**
No increased risk in CAYA cancer survivors vs controls.	⊕⊖⊖⊖ VERY LOW[30, 33]
Increased risk after *radiotherapy* vs. no radiotherapy.	⊕⊕⊖⊖ LOW[14, 27]
Increased risk after *chemotherapy* vs. no chemotherapy.	⊕⊖⊖⊖ VERY LOW[14, 27]
Increased risk after chemotherapy and/or radiotherapy (to any field or gonadal) vs. no chemotherapy and radiotherapy.	⊕⊕⊖⊖ LOW[14, 24]
Risk of still birth in female cancer survivors diagnosed before age 25 years	**Level of evidence**
No increased risk in CAYA cancer survivors vs controls.	⊕⊕⊕⊖ MODERATE[9, 30]
No significant effect of *radiotherapy* vs. no radiotherapy.	⊕⊕⊖⊖ LOW[9, 14, 27, 31, 42]
Increased risk after *high-dose ovarian-abdominal radiotherapy* vs. no radiotherapy.	⊕⊕⊖⊖ LOW[31, 34, 42]
Increased risk after *abdominopelvic radiotherapy (>1.00 Gy)* given before menarche vs. no radiotherapy, but no significant effect when given after menarche	⊕⊕⊖⊖ LOW[34]
No significant effect of *chemotherapy* vs. no chemotherapy.	⊕⊕⊖⊖ LOW[9, 14, 27, 31]
No significant effect of *alkylating agent dose*.	⊕⊕⊖⊖ LOW[34]
No significant effect of *alkylating agents in combination with abdominal-pelvic radiation* vs. no alkylating agents and abdominal-pelvic radiation.	⊕⊕⊖⊖ LOW[14, 24, 31]
Risk of gestational hypertension in female cancer survivors diagnosed before age 25 years	**Level of evidence**
No increased risk in CAYA cancer survivors vs controls.	⊕⊖⊖⊖ VERY LOW[13, 36]
Increased risk after *abdominopelvic radiotherapy* vs. no radiotherapy.	⊕⊖⊖⊖ VERY LOW[13, 35, 36]
Increased risk with *increasing doses of flank radiotherapy* in CAYA Wilms tumor survivors.	⊕⊖⊖⊖ VERY LOW[45]
No significant effect of *chemotherapy* vs. no chemotherapy.	⊕⊖⊖⊖ VERY LOW[36]
No significant effect of age at diagnosis.	⊕⊕⊖⊖ LOW[35]
Risk of pre-eclampsia in female cancer survivors diagnosed before age 25 years	**Level of evidence**
Increased risk in CAYA cancer survivors vs controls.	⊕⊕⊖⊖ LOW[9, 11, 13]
No significant effect of *abdominopelvic radiotherapy* vs. no radiotherapy.	⊕⊖⊖⊖ VERY LOW[13]

Table 1. Overall conclusions of evidence for obstetric risks in female childhood and adolescent cancer survivors (key outcomes) (continued)

Who needs preconception counseling? Who needs high-risk pregnancy surveillance?	
Risk of maternal anemia in female cancer survivors diagnosed before age 25 years	**Level of evidence**
No increased risk in CAYA cancer survivors vs controls.	⊕⊕⊕⊖ MODERATE[9, 11]
Increased risk after *(abdominopelvic) radiotherapy* vs. no radiotherapy.	⊕⊕⊖⊖ LOW[11, 35]
Increased risk after *chemotherapy* vs. no chemotherapy.	⊕⊕⊖⊖ LOW[11]
No significant effect of *radiotherapy and chemotherapy* vs. controls.	⊕⊕⊖⊖ LOW[11]
No significant effect of age at diagnosis.	⊕⊕⊕⊖ MODERATE[11, 35]
Risk of gestational diabetes in female cancer survivors diagnosed before age 25 years	**Level of evidence**
Increased risk in CAYA cancer survivors vs controls.	⊕⊕⊖⊖ LOW[9, 11, 36]
Increased risk after (*abdominopelvic) radiotherapy* vs. no radiotherapy.	⊕⊕⊖⊖ LOW[9, 11, 35, 36]
No significant effect of *chemotherapy* vs. no chemotherapy.	⊕⊕⊕⊖ MODERATE[9, 11, 36]
Increased risk after chemotherapy in combination with radiotherapy vs. controls.	⊕⊖⊖⊖ VERY LOW[9, 11]
No significant effect of age at diagnosis.	⊕⊕⊕⊕ HIGH[9, 11, 35]
Risk of malposition in female cancer survivors diagnosed before age 25 years	**Level of evidence**
No increased risk in CAYA cancer survivors vs. controls.	⊕⊖⊖⊖ VERY LOW[10]
No significant effect of *radiotherapy* vs. no radiotherapy.	⊕⊕⊖⊖ LOW[35]
Increased risk with *increasing doses flank radiation*.	⊕⊖⊖⊖ VERY LOW[45]
No significant effect of age at diagnosis.	⊕⊕⊕⊕ HIGH[10, 35]
Risk of postpartum hemorrhage in female cancer survivors diagnosed before age 25 years	**Level of evidence**
Increased risk in CAYA cancer survivors vs controls.	⊕⊕⊖⊖ LOW[8-10, 13, 35]
Increased risk after *abdominopelvic radiotherapy* vs. no radiotherapy.	⊕⊖⊖⊖ VERY LOW[13, 35]
No significant effect of age at diagnosis.	⊕⊕⊖⊖ LOW[35]
Risk of premature birth in female cancer survivors diagnosed before age 25 years	**Level of evidence**
Increased risk in CAYA cancer survivors vs. controls.	⊕⊕⊕⊖ MODERATE[9-13, 28, 36]
Increased risk after (*abdominopelvic) radiotherapy* vs. no radiotherapy.	⊕⊕⊕⊕ HIGH[9, 11, 13, 29, 35, 36]
Increased risk with *increasing doses of ovarian-abdominal radiotherapy* (>5/15 Gy).	⊕⊕⊖⊖ LOW[12, 45]
Increased risk after *chemotherapy* vs. no chemotherapy.	⊕⊕⊖⊖ LOW[9, 11, 36]
No significant effect of *alkylating agent dose.*	⊕⊕⊖⊖ LOW[12]
Increased risk after *radiotherapy and chemotherapy* vs. no radiotherapy and chemotherapy.	⊕⊕⊕⊖ MODERATE[9, 11]
Increased risk in *survivors aged >5 yrs at cancer diagnosis* vs. controls, but no significant effect in survivors aged <5 yrs at cancer diagnosis	⊕⊕⊖⊖ LOW[9, 11, 35]

Table 1. Overall conclusions of evidence for obstetric risks in female childhood and adolescent cancer survivors (key outcomes) (continued)

Who needs preconception counseling? Who needs high-risk pregnancy surveillance?	
Risk of low birth weight in female cancer survivors diagnosed before age 25 years	**Level of evidence**
Increased risk in CAYA cancer survivors vs controls.	⊕⊕⊕⊖ MODERATE[9-13, 28, 36]
Increased risk after (*abdominopelvic) radiotherapy* vs. no radiotherapy.	⊕⊕⊕⊕ HIGH[9, 11, 13, 29, 31, 35, 36]
Increased risk after *increasing doses of abdominopelvic radiotherapy* (>2.5/25 Gy)	⊕⊕⊕⊖ MODERATE[12, 28, 31, 45]
Increased risk after *chemotherapy* vs. no chemotherapy.	⊕⊖⊖⊖ VERY LOW[9, 11, 31, 36]
No significant effect alkylating agent dose.	⊕⊖⊖⊖ VERY LOW[12]
Increased risk after *radiotherapy and chemotherapy* vs. no radiotherapy and chemotherapy.	⊕⊖⊖⊖ VERY LOW[9, 11, 31]
Increased risk in *survivors aged ≥20 yrs at cancer diagnosis* vs. controls, but no significant effect in survivors aged <20 yrs at cancer diagnosis	⊕⊖⊖⊖ VERY LOW[9, 11, 35]
Risk of delivery of a child small for gestational age in female cancer survivors diagnosed before age 25 years	**Level of evidence**
No increased risk in CAYA cancer survivors vs. controls.	⊕⊕⊖⊖ LOW[11, 12, 36]
No significant effect of (*abdominopelvic) radiotherapy* vs. no radiotherapy.	⊕⊕⊖⊖ LOW[13, 29, 31, 36]
Increased risk after *increasing doses of abdominopelvic radiotherapy*.	⊕⊕⊖⊖ LOW[12, 31]
No significant effect of *chemotherapy* vs. no chemotherapy.	⊕⊖⊖⊖ VERY LOW[36]
No significant effect of alkylating agent dose.	⊕⊕⊖⊖ LOW[12]
No significant effect of radiotherapy and chemotherapy vs. surgery only.	⊕⊖⊖⊖ VERY LOW[31]
Risk of intrauterine growth restriction in female cancer survivors diagnosed before age 25 years	**Level of evidence**
No increased risk in CAYA cancer survivors vs. controls.	⊕⊖⊖⊖ VERY LOW[9]
Likelihood of vaginal delivery in female cancer survivors diagnosed before age 25 years	**Level of evidence**
Decreased likelihood of vaginal birth in in CAYA cancer survivors vs. controls.	⊕⊕⊕⊕ HIGH[8, 10]
Likelihood of assisted vaginal delivery in female cancer survivors diagnosed before age 25 years	**Level of evidence**
No increased likelihood of in CAYA cancer survivors vs. controls.	⊕⊕⊕⊖ MODERATE[8, 10, 13]
No significant effect of *radiotherapy* vs. no radiotherapy.	⊕⊖⊖⊖ VERY LOW[13]
No significant effect of age at diagnosis.	⊕⊕⊖⊖ LOW[10]
Risk of any cesarean section in female cancer survivors diagnosed before age 25 years	**Level of evidence**
Increased likelihood of any cesarean section in in CAYA cancer survivors vs controls.	⊕⊕⊖⊖ LOW[9-11, 36]
Increased likelihood after *radiotherapy* vs. no radiotherapy.	⊕⊕⊖⊖ LOW[9, 36]
Increased likelihood after *chemotherapy* vs. no chemotherapy,	⊕⊕⊖⊖ LOW[9, 36]
Significant effect of age at diagnosis (increased effect if 0-14 yrs at diagnosis)	⊕⊖⊖⊖ VERY LOW[9, 10]

Table 1. Overall conclusions of evidence for obstetric risks in female childhood and adolescent cancer survivors (key outcomes) (continued)

Who needs preconception counseling? Who needs high-risk pregnancy surveillance?	
Likelihood of an elective/primary cesarean section in female cancer survivors diagnosed before age 25 years	**Level of evidence**
Increased likelihood in CAYA cancer survivors vs controls.	⊕⊕⊕⊕ HIGH[8, 10, 11, 35]
Increased likelihood after *radiotherapy* vs. no radiotherapy, specifically after abdominal radiotherapy in Wilms survivors.	⊕⊕⊕⊖ MODERATE[35]
No significant effect of age at diagnosis.	⊕⊕⊕⊕ HIGH[35]
Likelihood of an emergency/secondary/urgent cesarean section in female cancer survivors diagnosed before age 25 years	**Level of evidence**
No increased likelihood in CAYA cancer survivors vs controls.	⊕⊕⊕⊖ MODERATE[8, 10, 13, 35]
No significant effect of *radiotherapy* vs. no radiotherapy.	⊕⊕⊕⊕ HIGH[13, 35]
No significant effect of age at diagnosis.	⊕⊕⊕⊖ MODERATE[8, 35]
Risk of congenital anomalies/abnormalities in female cancer survivors diagnosed before age 25 years	**Level of evidence**
No increased risk in CAYA cancer survivors vs controls.	⊕⊕⊕⊕ HIGH[9, 11, 13, 33, 37-41]
No significant effect of (*ovarian-abdominal) radiotherapy* vs. no radiotherapy.	⊕⊕⊕⊕ HIGH[13, 31, 37, 39, 40, 42, 43]
No significant effect of radiotherapy dose.	⊕⊕⊕⊖ MODERATE[31, 37, 42, 43, 45]
No significant effect of *alkylating agents* vs. no alkylating agents.	⊕⊕⊕⊖ MODERATE[31, 39, 40, 42, 43, 52]
No significant effect of alkylating agent dose.	⊕⊖⊖⊖ VERY LOW[43]
No significant effect of *alkylating agents in combination with abdominal-pelvic radiation* vs. no alkylating agents and abdominal-pelvic radiation.	⊕⊕⊕⊖ MODERATE[24, 31, 42]
No significant effect of age at diagnosis.	⊕⊖⊖⊖ VERY LOW[40]
Rate of supervision of high-risk pregnancy in female cancer survivors diagnosed before age 25 years	**Level of evidence**
No increased rates in CAYA cancer survivors vs controls.	⊕⊕⊖⊖ LOW[35]
No significant effect of *radiotherapy* vs. no radiotherapy.	⊕⊕⊖⊖ LOW[3]
Risk of retained placenta/manual removal of the placenta in female cancer survivors diagnosed before age 25 years	**Level of evidence**
No increased risk in CAYA cancer survivors vs. controls.	⊕⊕⊖⊖ LOW[9, 13]
Risk of placental pathologies in female cancer survivors diagnosed before age 25 years	**Level of evidence**
No increased risk in CAYA cancer survivors vs. controls.	⊕⊖⊖⊖ VERY LOW[10]
Risk of resuscitation of the neonate born to female cancer survivors diagnosed before age 25 years	**Level of evidence**
Increased risk in CAYA cancer survivors vs. controls.	⊕⊖⊖⊖ VERY LOW[9]
Likelihood of admission to a special care unit in neonates born to female cancer survivors diagnosed before age 25 years	**Level of evidence**
Increased likelihood in CAYA cancer survivors vs. controls.	⊕⊖⊖⊖ VERY LOW[9]

*Citations refer to papers on which the GRADE level of evidence was based on, and do not necessarily support the overall conclusion.

Gestational hypertension

There is very low level evidence for an effect of radiotherapy on the risk of gestational hypertension in CAYA cancer survivors as compared to survivors treated without radiotherapy. The increased risk was only reported in the abdominopelvic irradiated survivors who had been diagnosed with Wilms tumor in the British Childhood Cancer Survivors Study[35], while two smaller studies did not find this association[13,36]. A report from the National Wilms Tumor Study Group observed an increased risk of any hypertensive disorder of pregnancy with increasing doses of flank radiotherapy, but as this was the only identified study assessing radiotherapy dose, the level of evidence is very low.

Pre-eclampsia

There is low level evidence for an increased risk of pre-eclampsia in CAYA cancer survivors as compared to controls, as this association was reported in one large population-based Australian study[9] but not in two other studies[11,13]. One of these studies included a small sub-cohort of 6 CAYA cancer survivors exposed to radiotherapy to the abdomen, none of whom developed pre-eclampsia[13]. No studies were identified that evaluated the risk of pre-eclampsia after alkylating agents.

Maternal anemia

There is low level evidence that abdominopelvic radiotherapy increases the risk of maternal anemia in CAYA cancer survivors as compared to non-irradiated survivors. This is based on increased risks observed in one large study[35] while the effect was not observed in another equally-sized cohort[11].

Gestational diabetes

There is low level evidence for an increased risk of gestational diabetes in CAYA cancer survivors as compared to controls, based on one report that found the association[9] and two that did not show a statistically significant association[11,36]. There is low level evidence for an effect of abdominopelvic radiotherapy[9,11,35,36]. There is moderate level evidence that there is no effect of chemotherapy on the risk of gestational diabetes[9,11,36] and high level evidence that there is no effect of age at diagnosis[9,11,35].

Malposition of the fetus

There is low and very low level evidence that there is no increased risk on malposition of the fetus, and that there is no effect of radiotherapy on this outcome[10,35].

Evidence for gestational length and birth weight

Premature birth

CAYA cancer survivors are at increased risk of premature birth (before 37 weeks of gestation) as compared to siblings and the general population (moderate level evidence)[9-13,28,29,36]. High level evidence showed that exposure to radiotherapy to volumes exposing the uterus increases the risk of premature birth[9,11,13,29,35,36]. Two reports did not include specific radiotherapy volumes, categorizing groups as treated with or without any type of radiotherapy; both also showed increased risk after treatment with radiotherapy[9,11]. We found low level evidence for a dose response relationship with radiotherapy, including one study that showed a trend for increasing risk with increasing flank radiation dose, specifically with doses >15 Gy[14]. Another study showed increased risks specifically with doses >5 Gy to the uterus and in a smaller sub-cohort before the onset of menarche a lower threshold of 2.5 Gy[12]. One study showed that chemotherapy (specific agents not further specified) was associated with an increased risk of premature birth (low level evidence)[11]. However, this effect was not found in a small Japanese study[36] and a large Australian population-based study[9]. One study investigated the effect of alkylating agent dose on the risk of premature birth and did not find a statistically significant effect (very low level evidence)[12].

Low birth weight

There is moderate level evidence for an increased risk of delivering a child with a low birth weight (below 2500 grams) in CAYA cancer survivors as compared to controls[9-13,28,36] and there is high level evidence for an effect of radiotherapy to volumes exposing the uterus[9,11,13,29,31,35,36]. A dose response relationship was observed in survivors of Wilms tumor[32] and a risk increasing effect of radiotherapy was specifically observed after >2.5 Gy[12] to the uterus and >25 Gy[31] abdominopelvic radiotherapy (moderate level evidence)[12,31]. While three studies did not identify chemotherapy as a risk factor for a low birth weight[9,31,36], the association was suggested in one report[11], yielding very low level evidence for this association. There also seems to be no effect of alkylating agent dose (very low level evidence) on the risk of giving birth to a child with a low birth weight[12].

Small for gestational age

There is low level evidence that there is no increased risk of delivering a child small for gestational age (SGA; <10th percentile birth weight for gestational age) among CAYA cancer survivors in general as compared to controls[11,12,36]. Although radiotherapy versus no radiotherapy was not found to be significantly associated with this outcome in four studies[13,29,31,36], two studies showed that patients treated with specific doses of abdominopelvic radiotherapy (>5 Gy and >25 Gy, respectively) had an increased risk of delivering a child small for gestational age (low level evidence)[12,31].

Evidence for mode of delivery

Vaginal delivery

There is high level evidence indicating that rates of spontaneous vaginal births are lower in CAYA cancer survivors compared to controls[8,10]. Regarding assisted vaginal delivery rates, there was no significant difference between survivors and controls (moderate level evidence)[8,10,13], and no significant effect of radiotherapy (very low level evidence)[13] on occurrence of assisted vaginal delivery.

Cesarean delivery

There is low level evidence for higher rates of any cesarean sections (any cesarean section: from reports that did not make a distinction between elective (primary) and emergency (secondary/urgent) cesarean sections) among CAYA cancer survivors as compared to controls[9-11,36], especially after radiotherapy and chemotherapy (low level evidence)[9,36].

High level evidence was specifically identified for an increased rate of an elective cesarean delivery[8,10,11,35], especially after abdominopelvic radiotherapy (moderate level evidence)[35]. No statistically significant increased rate for the occurrence of emergency cesarean delivery (moderate level evidence) was found[8,10,13,35]. There was also no statistically significant effect of radiotherapy and age at diagnosis on rate of cesarean section (high level evidence)[8,13,35].

Evidence for risks related to delivery

Postpartum hemorrhage

There is low level evidence for an increased risk of postpartum hemorrhage in CAYA cancer survivors as compared to controls. An increased risk was observed in one report[8] but not in four others[9,10,13,35]. There is low level evidence for a statistically significant effect of abdominal radiotherapy, based on one small study suggesting an increased risk after this treatment[13], while one larger study did not find an increased risk[35].

Evidence for problems of the neonate

Congenital anomalies

There is high level evidence that there is no increased risk of congenital anomalies among neonates of CAYA cancer survivors as compared to controls. Nine studies, with large heterogeneity in outcome definitions, have reported on the prevalence of congenital anomalies and none showed an increased risk[9,11,13,33,37-41]. There is also high level evidence that there is no statistically significant effect of radiotherapy on the risk of congenital anomalies[13,31,37,39,40,42,43].

Evidence for additional obstetric outcomes

The evidence levels on the risk of retained placenta/manual removal of the placenta, placental pathologies, fetal growth restriction, uterine scar from previous surgery and

perineal laceration/rupture were low to very low, or revealed no increased risk for these outcomes. Concerning the neonate, the evidence levels on the risk of resuscitation and admission to a special care unit were very low. Additional outcomes evaluated in only very limited number of papers are reported in Appendix 6 and also generated only low to very low levels of evidence.

Translating evidence into recommendations

The final recommendations are summarized in Table 2. Recommendations were formulated based on at least moderate levels of evidence for the risk of obstetric outcomes and its determinants (Table 1). There was moderate level evidence for an increased risk of miscarriage after radiotherapy to volumes exposing the uterus, and high level evidence for an increased risk of premature birth (<37 weeks of gestation) and low birth weight (<2500 grams) after radiotherapy to volumes exposing the uterus. In addition, CAYA cancer survivors had higher rates of elective cesarean section (high level evidence). There was high level evidence that there is no increased risk of congenital anomalies in the offspring of CAYA cancer survivors. Lower levels of evidence were included for the identification of gaps in knowledge and future research directions (Panel). Radiotherapy was of specific interest if and where a dose-response relationship was identified. Although low level evidence suggests a dose-response relationship of radiotherapy to volumes exposing the uterus[30,31], too little evidence is available to identify a safe threshold dose.

For every adverse outcome, the balance between benefits and harms of preconception counseling and surveillance, resource use, acceptability to stakeholders and feasibility or barriers for implementation was considered. The panel agreed that all female CAYA cancer survivors have the right to be informed about their potential risk for adverse obstetric outcomes. Therefore, we recommend that healthcare providers should discuss the risk of adverse obstetric outcomes based on the specific cancer treatment exposures with all female CAYA cancer survivors of reproductive age (strong recommendation). Specifically regarding the risk of miscarriage, premature birth and low birth weight, the panel agreed that the benefits of preconception counseling and obstetric surveillance (i.e., early detection of fetal growth restriction or threatened premature delivery requiring intervention to ensure optimal neonatal outcome) clearly outweigh the potential harms (e.g., stress, anxiety and potential higher health care costs) for CAYA cancer survivors treated with radiotherapy to volumes exposing the uterus. The panel recommends that female CAYA cancer survivors treated with radiotherapy to volumes exposing the uterus and their health care providers should be aware of the risk of adverse obstetric outcomes including miscarriage (moderate quality evidence), premature birth (high quality evidence) and low birth weight (high quality evidence). In addition, high risk obstetric surveillance is recommended for this patient group (strong recommendations).

Table 2. Harmonized recommendations for counseling and surveillance in pregnancy

General recommendation
Health care providers should discuss the risk of adverse obstetric outcomes based on the specific cancer treatment exposures with all female CAYA cancer survivors of reproductive age.
Who needs preconception counseling?
Female CAYA cancer survivors and their health care providers should be aware that there is no evidence to support that survivors have an increased risk of giving birth to a child with congenital anomalies (high quality evidence). Female CAYA cancer survivors treated with radiotherapy to volumes exposing the uterus and their health care providers should be aware of the risk of adverse obstetric outcomes including miscarriage (moderate quality evidence), premature birth (high quality evidence) and low birth weight (high quality evidence).
Who needs specific obstetric surveillance during pregnancy?
High risk obstetric surveillance is recommended for CAYA cancer survivors treated with radiotherapy to volumes exposing the uterus due to the risk of premature birth and low birth weight (high quality evidence).
Who needs specific cardiac surveillance during pregnancy? *Based on IGHG cardiomyopathy guideline*[19]
Cardiomyopathy surveillance is reasonable prior to pregnancy or in the first trimester for all female survivors treated with anthracyclines and/or chest radiation (moderate level recommendation, moderate quality evidence)[19]. No recommendations can be formulated for the frequency of ongoing surveillance in pregnant survivors who have normal LV systolic function immediately prior to or during the first trimester of pregnancy (moderate level recommendation, low quality evidence)[19].

Panel: Gaps in knowledge and future directions for research of obstetric outcomes in CAYA cancer survivors

- Risks of medical and elective termination of pregnancy, including standardized definitions of this outcome and its confounders.
- Risks of gestational diabetes, gestational hypertension and pre-eclampsia, giving birth to babies small for gestational age, very premature delivery (<32 weeks of gestation) or postpartum hemorrhage.
- Effect of radiotherapy and dose-response relationships to specific volumes (e.g., uterus) on obstetric outcomes.
- Influence of relatively low doses of radiotherapy (including 10-15 Gy) that reach the uterus on obstetric outcomes.
- Effect of age at cancer diagnosis and pubertal stage at treatment on all obstetric risks.
- The contribution of environmental factors known to affect obstetric outcomes (e.g., BMI, smoking).
- The contribution of obstetric risk associated with artificial reproductive technology (ART), especially as fertility rates after ART (including donor oocytes) increase.
- Development of a risk prediction algorithm for outcomes including miscarriage, premature delivery and low birth weight, taking into account, e.g., age at cancer diagnosis, cancer treatment, maternal age, smoking, parity and ART.
- Methods to optimize timely provision of information about obstetric risk to CAYA cancer survivors in a variety of health care systems and health literacy settings.
- The effect of high risk surveillance on clinical relevant outcomes for survivors at risk.

Regarding the increased likelihood of elective cesarean section, the panel agreed that no recommendations could be drawn from this observation, as this may be attributable to many other factors such as the survivor's or the healthcare provider's concern.

Because the absence of an increased risk of congenital anomalies (high quality evidence) is of great importance to survivors, the panel agreed that female CAYA cancer survivors and their health care providers should be aware that there is no evidence to support that survivors have an increased risk of giving birth to a child with congenital anomalies (strong recommendation).

Based on previous recommendations from the IGHG for cardiomyopathy surveillance for CAYA cancer survivors, cardiomyopathy surveillance is reasonable prior to pregnancy or in the first trimester for all female survivors treated with anthracyclines and/or chest radiation (moderate recommendation)[19]. No recommendations have been formulated for the frequency of ongoing cardiomyopathy surveillance in pregnant survivors who have normal left ventricular systolic function immediately prior to or during the first trimester of pregnancy. However, the IGHG panel recommended that health care providers remain alert for cardiomyopathy in survivors treated with anthracyclines and/or chest-directed radiation who present with commonly reported symptoms such as shortness of breath, fatigue, and ankle swelling[19]. The panel additionally emphasized that CAYA cancer survivors with compromised left ventricular systolic function (<30%) before pregnancy are more likely to have further reduction in cardiac function during pregnancy or post-partum, irrespective of lifetime anthracycline dose[19].

DISCUSSION

This paper presents the IGHG recommendations for counseling and surveillance of female CAYA cancer survivors before and during pregnancy. Evidence-based recommendations for survivor risk groups were formulated to facilitate consistent long-term follow-up care, to optimize the quality of care and to minimize the burden of disease and unnecessary surveillance. The guideline panel, however, stressed the need for future research in larger cohorts to advance understanding about the radiotherapy dose response relationship to adverse obstetric outcomes.

Critical evaluation of the published literature aided by the GRADE methodology yielded moderate level evidence that CAYA cancer survivors are at increased risk of miscarriage after radiotherapy[9,24,25,27,29,30,32]. The definition of a miscarriage was heterogeneous (if reported, mostly pregnancies ending before gestational week 20 but in the British Childhood Cancer Survivors Study (BCCSS) before 24 weeks), and the panel acknowledged the potential for reporting bias in both self-reported and registry-based data on this subject. However, increased risks were observed in three large cohorts, from the North American Childhood

Cancer Survivor Study (CCSS) (self-reported miscarriage, not further specified[14]), Australia (registered threatened miscarriage after 20 weeks of gestation[9]) and Denmark (registered spontaneous abortion, not further specified[30]). Although low level evidence suggests a dose-response relationship with radiotherapy to volumes exposing the uterus[30,31], there is insufficient evidence to identify a safe threshold dose. Even though there is no specific action to reduce this risk, the panel agreed survivors need to be counseled of their potential increased risk of miscarriage.

Broad and overlapping definitions of termination of pregnancy and still birth, in addition to potential reporting bias for these sensitive topics, resulted in a low body of evidence on which to base recommendations, and these outcomes need further investigation (Panel). Still birth has been variably defined as the fetus not surviving after 20 weeks of gestation[9], after 28 weeks[30], or combined with neonatal deaths within the first 28 days of life in others[34]. Likewise the definition of termination of pregnancy has not been stated in some studies[14,30,33] or specifically defined as medically induced abortion in others[27]. Interestingly, a recent study in survivors aged 39 years or less at cancer diagnosis with robust outcome reporting showed a significantly reduced risk of termination of pregnancy[44], stressing the need for further research to more accurately define the prevalence of this outcome.

We identified high level evidence for the increased risks of premature birth and low birth weight after radiotherapy to volumes exposing the uterus[9-14,28,29,31,32,35,36]. The evidence for a dose-response relationship between radiotherapy and miscarriage, premature birth and low birth weight is compelling, but clear evidence to determine a safe threshold dose is lacking. Different approaches have been used to assess radiotherapy dose, giving rise to bias when comparing these studies. For example, doses have been estimated using mathematical phantoms in cohorts from the CCSS and the National Wilms Tumor Study Group[12,45], approximated by determining the theoretic location of the relevant organ (e.g., uterus, ovary) on the dosimetry schemes[28] categorized in occasional very broad ranges such as 1-40 Gy for primary cancer treatment extending below the diaphragm[30] or abstracted from treatment records[31]. Consistent documentation of received organ volume dose distribution, as opposed to reconstructed organ dose, is important to assess more accurately the relationship of radiation dose and obstetric risk and is possible in modern clinical practice.

Radiotherapy to volumes exposing the ovaries is associated with premature ovarian insufficiency[46-49], but if fertility potential is retained, damage to the oocyte does not lead to increased risks of still birth or congenital anomalies as compared to the general population. Mechanisms leading to increased rates of miscarriage, premature delivery and low birth weight have not been completely elucidated, but several hypotheses have been proposed. Radiotherapy to volumes exposing the uterus can damage the uterine vasculature and muscular development[50], and potentially impair endometrial function due to impaired blood supply. This may result in poor implantation of the embryo and poor placental growth which could result in subsequent early miscarriage. The increased risks of premature birth

and low birth weight may result from uterine vasculature injury leading to impaired uteroplacental blood flow, insufficient placental development and hence fetal growth restriction, or may result from a reduced uterine elasticity and volume[50,51]. Additionally, hormonal deficiency as a consequence of ovarian failure may lead to smaller uterine volumes[51].

The panel has balanced the importance of preventing unnecessary consultations, visits and expenses for CAYA cancer survivors with the cost of failing to identify survivors at risk who would benefit from preconception consultation. As the clinical implication of awareness and preconception counseling can be tailored to the individual, the panel considered all CAYA cancer survivors treated with radiotherapy to volumes exposing the uterus to be at increased risk of miscarriage, premature delivery and low birth weight. In addition, CAYA cancer survivors treated with anthracyclines or chest-directed radiotherapy are at risk of perinatal cardiomyopathy. Cancer survivors should be counseled about obstetric risks when developmentally and clinically appropriate. Multimorbidity is often the norm in CAYA cancer survivors, emphasizing the need to understand specific treatment-related risks and how collectively these conditions may impact course of pregnancy. Communication among obstetric and oncology providers and survivors is key in these complicated cases.

Preconception consultation and obstetric surveillance may lead to referral to a specialized obstetric team rather than a midwifery team, and may ensure selection of a hospital for the place of birth rather than a birth center or home. Further clinical management, such as antenatal monitoring for heightened risk of low birth weight or cardiac monitoring, should adhere to established obstetric care guidelines.

No recommendations were formulated based on the high level of evidence concerning the increased likelihood of an elective cesarean section. Although many clinical, cultural and personal factors, which likely vary widely between health care systems, play a role in the decision for an elective cesarean section, health care providers may have been more cautious with this population knowing their increased obstetric risks. Reassuringly, no increased likelihood of an emergency cesarean section after radiotherapy was identified.

A large and consistent body of evidence indicates that neonates of CAYA cancer survivors treated with and without radiotherapy are not at increased risk of congenital anomalies[13,31,37,39,40,42,43]. As this is often a major concern in CAYA cancer survivors; therefore, the panel recommends reassurance of CAYA cancer survivors that there is no indication of such an increased risk.

The recommendations presented here have benefited from the systematic appraisal of bias and transparent implementation of GRADE in assessing the available evidence. Their relevance is further strengthened by the careful considerations that the multidisciplinary and international panel made by extrapolating evidence to recommendations. Some limitations include variability of definitions of outcomes and availability of specifics regarding radiotherapy (dose and site) and chemotherapy (agents and dose), potential study biases without indication of response rates, and the scarcity of studies with multivariable analy-

ses to address confounding clinical issues. In addition, the body of evidence often indicated no increased risk, but few power calculations were presented in the papers to distinguish between absence of evidence and evidence of absence of an association. Another important topic is surveillance of thyroid dysfunction in CAYA cancer survivors, as latent hypothyroidism can impact fetal brain development[15,16]. Recommendations will be formulated in an upcoming IGHG guideline on surveillance of thyroid dysfunction. A periodic update of the obstetric recommendations is planned, and the IGHG thyroid dysfunction surveillance recommendations will then also be included.

The identification of key gaps in knowledge is an important result of the harmonization process (Panel). According to our findings, future studies should focus on the identification of threshold doses of radiotherapy to volumes exposing the uterus, the effect of different environmental factors such as lifestyle factors and the increasing use of assisted reproductive technology. These evidence gaps should be addressed in strong methodical and comprehensive studies from sufficiently large cohorts, or preferably international multicenter collaborative projects to increase generalizability of the results.

CONCLUSION

The presented IGHG effort was initiated to assist in the identification of specific adverse obstetric related outcomes that are increased in CAYA cancer survivors, and to identify the population that will benefit specifically from an individualized preconception consultation and pregnancy surveillance taking into account their treatment history.

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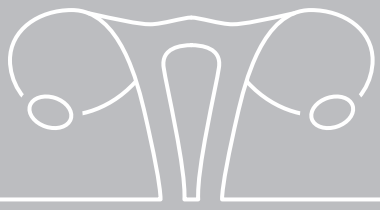

CHAPTER 11

General discussion

Only two generations ago, not many would have imagined the challenges we are struggling with today regarding childhood cancer survivorship. Survival rates used to be so low that essentially all efforts were focussed on survival. Since then, research has made an incredible journey, resulting in dramatic increases in survival rates after cancer treatment. However, with increasing survival rates it became clear that "cure is not enough" – words first coined by Gulio D'Angio[1], since most cancer survivors will also suffer from secondary diseases as a consequence from their previous cancer treatment. This realization made current research focus' grow beyond survival, and into a fully grown and mature field of late effects of childhood cancer.

This thesis builds further on many previous endeavours that have been undertaken to identify late effects, assess risk factors and that started outlining ways in order to minimalize, screen or counsel for these late effects. Reproductive health, being one of the health factors at risk, is the topic of this thesis.

FROM CROSS-SECTIONAL TOWARDS LONGITUDINAL DATA

Ovarian function can be assessed in several ways, some of which are dependent on an active hypothalamic-pituitary-ovarian axis, which is not present yet in prepubertal children. Anti-Müllerian hormone (AMH) is a useful marker of the presence of gonadotropin-independent small growing antral follicles in the ovaries, and therefore constitutes the best marker of ovarian function[2-6], like inhibin B is for gonadal function in boys[7-9]. As was previously reported in girls with newly diagnosed cancer and adults with type 2 diabetes mellitus, metabolic syndrome and systematic lupus erythematous[10-13], we show that boys with newly diagnosed childhood cancer have decreased gonadal function markers at the moment of their diagnosis, indicating that their serious disease already has an impact on the physiology of their gonads[14]. We have also shown that various treatment modalities, specifically abdominal radiation and high dosages of alkylating agents, reduce the gonadal function even further in a large number of the children. Nonetheless, the gonads are resilient as well as dynamic, and our results indicated that around half of the children with low gonadal function directly after the end of their treatment show recovery within the first year[15]. While general markers of gonadal function remain relatively low in long-term adult female cancer survivors, we have also shown that survivors are not at increased risk of a late or sudden drop in their AMH levels[16]. So although their AMH levels declined considerably during treatment, thereafter they might recover and then decline along the normal percentile lines until menopause. Long-term longitudinal data will be needed to assess at which time gonadal function should best be assessed in order to inform survivors about their gonadal function and their expected fertility window.

EXTRAPOLATION OF DATA

This thesis presents two studies with longitudinal data on gonadal function markers, where currently mostly cross-sectional research results have been reported. Further long-term longitudinal data may also help us to better understand the implications of a low AMH level. Even within a healthy population, predicting the cessation of fertility remains challenging based on individual AMH levels[17,18], specifically as for instance a pregnancy can be established with only a severely reduced number of remaining follicles[19,20]. The latter also indicates that although the number of remaining follicles is low, the quality of these remaining follicles are not necessarily low[21]. This is significantly different from women of advanced reproductive age as reduced quantity and quality go hand in hand in ageing women. Hence, ovarian reserve is not a proper term for ovarian capacity and ovarian function is a more adequate description in childhood cancer survivors.

AMH, as a surrogate of remaining ovarian function, has proven to be a valuable predictor of menopause, apart from age[4,22-25]. However, current prediction models have not been designed to predict the extremes of menopausal age[25,26], while the prediction of extreme young menopausal age is exactly what would be of interest to the population of cancer survivors. In addition, the prediction intervals on an individual level remain wide with a variation of around 10 years[25]. Future studies including data on repeated AMH measurements and age at diagnosis in childhood, adolescent and young adult (CAYA) cancer survivors or similar populations with relative low AMH levels, may improve prediction models of age at menopause in these populations.

CHEMOPROTECTANTS

Several courses of action can be undertaken to minimize the impact of childhood cancer treatment on reproductive health. Targeted treatment strategies may include the least amount of radiation and alkylating agent dosage that is still safe for survival. Many steps have already been taken in this regard, and we have shown the reduced impact of a cancer diagnosis on perinatal risks[27]. In young adult women treated for breast cancer, a protective effect of GnRH analogues has been observed [28-31]. Similarly, there are also data suggesting that the use of the oral contraceptive pill might protect the gonads from damage induced by chemotherapy[32], although types of administered estrogen and active cancer increases the risk of venous thromboembolism[33,34]. A recently published mice study showed that administration of AMH resulted in a complete arrest of folliculogenesis, and that AMH prevented chemotherapy-induced overactivation, protecting the ovarian reserve from the burn-out phenomenon[35]. If AMH could be used as a chemoprotectant for ovarian gonadotoxicity, therapeutic indications of AMH could even extent to delaying ovarian aging in

the general population, in analogy to patients with polycystic ovary syndrome with high AMH levels who are known to enter menopause at a relatively late age. To what extent the administration of AMH may be feasible or effective in humans, let alone children, remains speculative at this moment.

OVARIAN TISSUE CRYOPRESERVATION

The administration of chemoprotectants is only one possible course of action in the rapidly growing arena of fertility preservation. For women in their reproductive life, options such as embryo cryopreservation or oocyte vitrification are available[36]. Oocyte donation can be a last resort for women with primary ovarian insufficiency (POI) after gonadotoxic treatment[37]. Recent research has indicated that even in women with POI, harvesting the remains of the exhausted ovary and reimplanting it in the pelvis after it has been dissected and cultured, might rejuvenate follicles and result in pregnancies[38,39]. Success rates of this procedure may be even higher in women with chemotherapy-induced POI, as the quality of the remaining follicles may be better than its quantity suggests[21]. Embryo cryopreservation and oocyte vitrification cannot be offered to prepubertal girls with cancer, mainly due to an inactive hypothalamic-pituitary-ovarian axis and physical restraints. Fortunately, ovarian tissue cryopreservation (OTC), is maturing into an established option for young patients with childhood cancer[40].

Since the first successful pregnancy after OTC was reported in 2004[41], over 130 live births have been reported after harvests in young adults[36,42-45]. The first live births after OTC during childhood have also been reported[46-48]. Beside pregnancies, restoration of ovarian activity with an adequate function of the hypothalamic-pituitary-ovarian axis and restoration of ovulation has been reported[49], with renewed ovarian endocrine function in 95% of women receiving ovarian tissue transplantation with frozen/thawed tissue[50]. In addition, transplantation of cryopreserved ovarian tissue could potentially induce puberty and this practice has been reported[51,52], although given the scarcity of the tissue and the possibility of standard hormonal induction of puberty this may not be the prioritized designation of the valuable cryopreserved ovarian tissue at this moment[53].

The various laparoscopic procedures of OTC[42,54-56] are considered a reasonable safe procedure[48,57], although the benefits need to be balanced against the potential risk of complications, such as bleeding and anaesthetic risks that may occur. Women with transplanted tissue have not been shown to be at increased risk of a relapse[43,58], although it is generally accepted sensible to be cautious with cancers with a high risk of ovarian involvement[59,60]. Promising steps have also been reported regarding in vitro maturation of primordial follicles as an alternative to reimplantation of the ovarian tissue, to circumvent the risk of recrudescence of the original haematogenous malignancy[61].

JOINING CLINICAL AND SCIENTIFIC POWER IN THE NETHERLANDS

In the Netherlands, paediatric oncologic care has recently been centralized at the Prinses Máxima Center for paediatric oncology in Utrecht. In the context of reproductive health in children treated for cancer, this centralization taps into new potential to evaluate the effects of fertility counselling and monitoring the safety of the OTC procedure in a large, controlled clinical setting. In addition, the influence of a cancer diagnosis, cancer treatment and ovarian tissue harvest on the gonadal function can be monitored and longitudinal data can be collected prospectively, a crucial step in the advancement of knowledge and understanding of gonadal function markers, as discussed in the second paragraph of this chapter.

GENETIC DETERMINANTS OF OVARIAN FUNCTION IMPAIRMENT

Determinants of ovarian function impairment include baseline patient characteristics, type of treatment and life-style factors. Groups with low, moderate or high risk of gonadal function impairment after cancer can be identified[62,63], but variation in the extent of gonadotoxicity remains in these groups. In the second part of this thesis, we consider genetic determinants as another factor of ovarian function after childhood cancer treatment[64]. We show that chemotherapy-induced gonadal impairment in female CCS was significantly modified by the *BRSK1* gene. Female CCS who carry the G allele of rs11668344 and received high doses of alkylating agents, were at an increased risk of a low AMH level. To further investigate the modifying effect of genetic variation on the impact of chemotherapy on gonadal impairment, more research is needed, including large childhood cancer survivor cohorts and independent replication cohorts. The design of such a large international cohort, PanCareLIFE, is described[64]. Eventually, this information can help to improve individualized counselling on both fertility preservation prior to cancer treatment and counselling after cancer treatment and may aid future individualized treatment strategies.

COLLABORATION IN SCIENCE

Medical scientific research has historically been based on competition. In the field of genetics, competition typically results in underpowered studies with a high chance on false positive results and reports with little value for the scientific community. The large international collaboration within PanCareLIFE has resulted in the largest European cohort of childhood cancer survivors with genetic data and data on gonadal impairment. Within this endeavour, we have collaborated with research groups from the St. Jude Lifetime Cohort Study, building transatlantic research bridges and improving scientific knowledge

with combined forces. Major recommendations can be drawn from genetic studies in large international collaborations[65]. Firstly, false-positive results will increasingly occur where multiple independent tests are carried out[66]. Failure to correct for multiple testing results in findings that may look 'interesting' and easy to publish, but are worthless to the scientific community in the long run. Therefore, correction for multiple testing should become common standard in all research fields. Another measure against false-positive findings is replication of results in an independent cohort prior to publication, increasing the likelihood of reporting an actual association. Finally, all research data is valuable and scarce. Combining efforts and forming consortia such as within PanCareLIFE can improve power tremendously, meanwhile building bridges between different research groups that may enable knowledge exchange as a valuable spin-off effect. Again, this requires not competition, but trust and collaboration.

Unfortunately, barriers for collaboration can include linguistic, cultural or modus operandi differences[67,68], and concerns about ownership of outputs[68]. Another drawback of collaboration may be the dilution of the definition of authorship. Projects in physics can have hundreds of members, all of whom are listed as authors as a mark of membership of the team – without requirements of writing or revising the paper[69]. Papers in medical science tend to follow suit, with increasing long author lists and shared authorships. According to the International Committee of Medical Journal Editors, authors should meet all four Vancouver criteria for authorship (playing a part in designing or conducting experiments or processing results, help to write or revise the manuscript; approve the published version; take responsibility for the article's contents[70]), a requirement that can hardly be expected from such large author lists. The large gap between 'project membership' and the Vancouver Criteria, while authorships are so highly rewarded in the current scientific field, calls for new definitions and standards of authorship, and new ways of academic achievement evaluation.

PERINATAL MANAGEMENT AND COMPLICATIONS

Perinatal risks such as premature birth and postpartum haemorrhage are higher in CAYA cancer survivors compared to control groups, and risks seem to increase in survivors who have been treated with abdominal radiotherapy. In a large population-based analysis, we have evaluated the risks of cancer survivors diagnosed before their forties, and show that they are at increased risk of premature delivery and postpartum haemorrhage, but not of giving birth to children small for gestational age or with congenital abnormalities[27]. It was also shown that the risk of an operative delivery and postpartum haemorrhage diminished in the more recent cohorts compared to older ones, resulting in equal risks for those diagnosed in the most recent cohort. The reduced impact of a cancer diagnosis on the risk of

an intervention during delivery may be a result of better targeted treatment strategies, and of a reduction of therapeutic exposures known to be associated with organ toxicity, e.g. radiotherapy in Hodgkin lymphoma[71]. This observation is also in line with decreased late mortality among survivors of childhood cancer as a result of reduced radiotherapy and chemotherapy exposure[72]. It is reassuring that the impact of a cancer diagnosis on postpartum haemorrhage and mode of delivery has been greatly reduced in most recently diagnosed cohorts of survivors, although heightened alertness and careful management in cancer survivors remains appropriate. Evidence-based clinical guidelines may facilitate this careful management by identifying the specific perinatal risks and risk groups.

Perinatal risks in CAYA survivors are generally noted in published clinical practice guidelines by North American and European groups[73-75], but without comprehensive assessment of the risk features of women who may benefit from high-risk obstetrical follow-up. In collaboration with the International Late Effects of Childhood Cancer Guideline Harmonization Group (IGHG)[76], we formulated recommendations for consistent and evidence based clinically effective counselling and care, with regard to obstetrical and perinatal risks for female childhood cancer survivors. Based on the IGHG cardiomyopathy guideline, cardiomyopathy surveillance is reasonable prior to pregnancy or in the first trimester for all female survivors treated with anthracyclines and/or chest radiation. We further recommend that healthcare providers counsel female CAYA cancer survivors treated with radiation to fields including the uterus on the increased risks of miscarriage, premature birth and low birth weight, and reassure survivors that there is no indication of an increased risk of congenital abnormalities. Healthcare providers should be aware of the risk of premature birth and low birth weight during the entire course of pregnancy in CAYA cancer survivors treated with radiation to fields including the uterus (Figure 1). These recommendations will need to be translated and imbedded into national protocols. In the Netherlands, these findings need to be addressed in the continuing dialogue between midwifes and obstetricians concerning medical indications and place of birth for specific risk groups.

CONCLUSIONS

This thesis presents new insights in trends of gonadal function markers. In particular, it shows that gonadal function is already compromised at diagnosis, but also indicates that the ovary has the capacity to recover shortly after cessation of treatment and shows no accelerated decline in the subsequent years as compared to healthy peers. It also shows that although follicle numbers are reduced, the remaining follicles are healthy and perfectly capable to produce vital and largely uncomplicated pregnancies. The latter indicates that ovarian reserve markers generally measured in ageing women in whom quantity as well as quality of follicles are compromised are to be interpreted with caution in CCS. Future

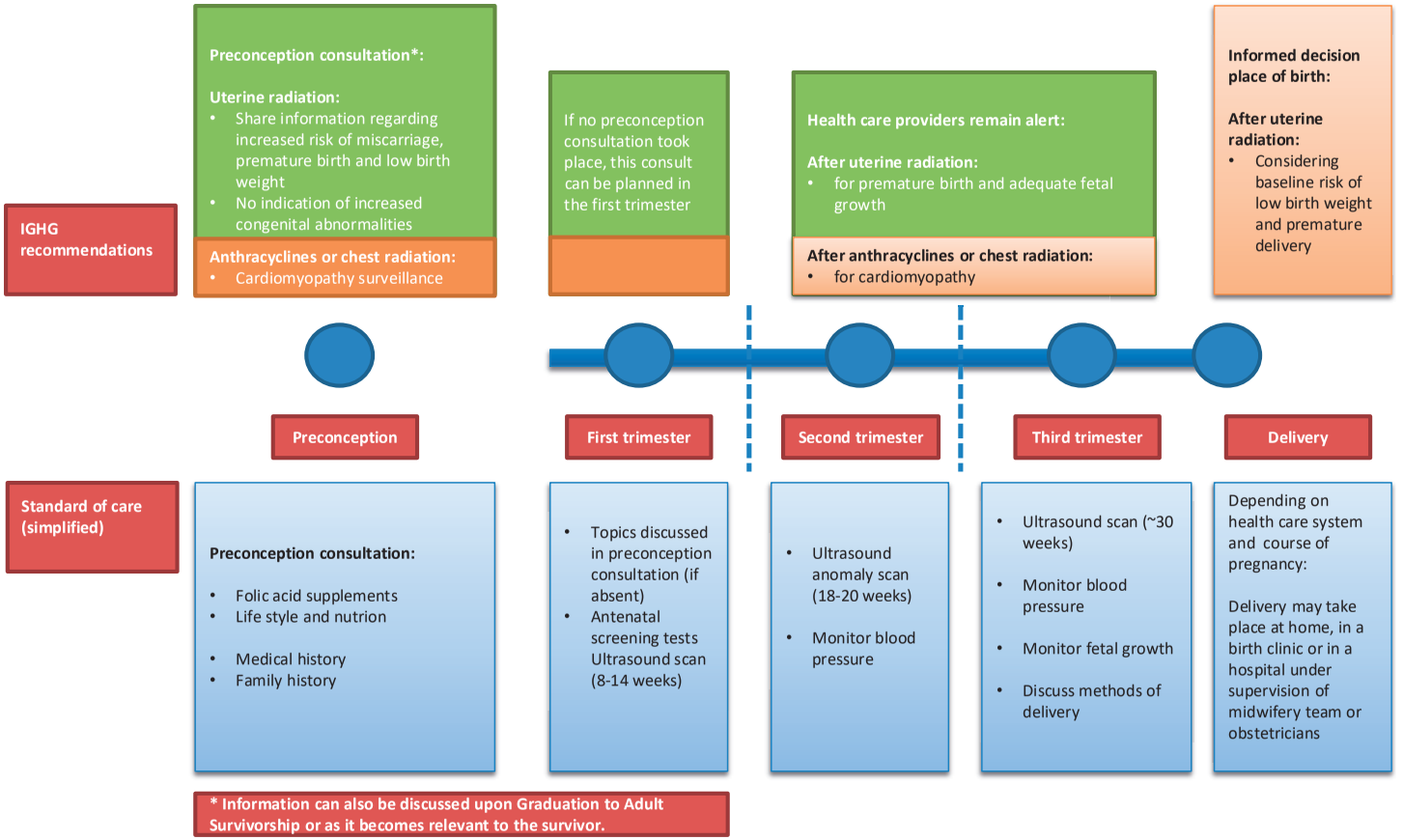

Figure 1. IGHG recommendations for preconception counselling and surveillance during pregnancy

research is needed to determine the best time to evaluate gonadal function damage, and to extrapolate our knowledge of low AMH levels to prediction of fecundity, fertility and age at menopause. The inter-individual variability in gonadotoxicity is for some part influenced by genetic determinants. We have shown that a polymorphism in the *BRSK1* gene is associated with the inter-individual variability of reduced ovarian function as a result of chemotherapy. These findings may be used to develop a prediction model for ovarian function.

Most childhood cancer survivors who become pregnant can expect a normal pregnancy risk. However, we have identified some determinants of high-risk pregnancies. The clinical guideline recommendations offered in this thesis will aid careful and proportional management in cancer survivors. For survivors treated with radiotherapy potentially exposing the uterus, antenatal and postnatal care should be offered in a specialised medical centre to anticipate and deal appropriately with the possible complications.

Future studies should focus on the development of risk prediction models, combining evidence from this thesis and other valuable research. These models could aid health care providers in not only assessing their patients' risk, but also the need for fertility preservation and cycle restoration in order to establish a normal hormonal environment in female CCS. All future research calls for collaboration within research groups, nationally, internationally and globally, to maximize the quality and validity of its results.

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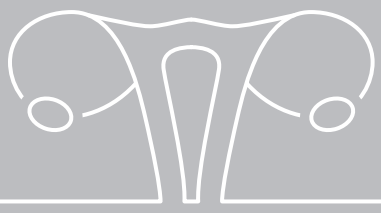


CHAPTER 12

Summary & samenvatting

SUMMARY

Over the past five decades, survival of childhood cancer has improved throughout Europe, with survival rates now approximating 80% as a result of improved treatment strategies. Despite this accomplishment, around 75% of the resulting growing population of long-term childhood cancer survivors (CCS) develops at least one long-term complication as a result of their cancer treatment. Major effects on reproductive health include gonadal function damage and pregnancy complications. The general aim of research described in this thesis was to evaluate the impact of cancer on clinical and genetic aspects of reproductive health.

Gonadal impairment has been demonstrated in girls and young women diagnosed with cancer, prior to therapy. In part I of this thesis, we found that pre-treatment serum levels of inhibin B and testosterone are significantly reduced in boys with newly diagnosed cancer as well. We next showed that gonadal function is further decreased in both boys and girls by childhood cancer treatment. Interestingly, gonadal function markers that were measured directly after end of treatment showed recovery in about half of the children with gonadal impairment when measured again after one year. We concluded that evaluation of gonadal function markers before one year after end of treatment may therefore be unreliable. The next step of the first part of this thesis was to evaluate if the long-term decline of ovarian function, as reflected by a decrease in serum levels of anti-Müllerian hormone (AMH), accelerated over time in female CCS as compared to healthy women of the same age. Although the serum AMH levels were below the P_{50} of normal values at both visits, the median decline of AMH levels in long-term female CCS was not accelerated, and none of the treatment modalities was correlated with a significant acceleration of decline.

The observed gonadal damage among CCS is only partially explained by treatment and baseline patient characteristics. In part II of this thesis we address this inter-individual variability, and hypothesize that genetic variation possibly modifies the association between chemotherapy and reduced ovarian function. We describe the available literature on genetic susceptibility of late toxicity after childhood cancer treatment related to components of gonadal impairment, as well as of metabolic syndrome, bone mineral density, and hearing impairment. We advocate that to advance knowledge related to genetic variation influencing late toxicities among CCS, future studies need adequate power, independent cohorts for replication, harmonization of disease definition and (international) collaboration. We describe the design of the PanCareLIFE study to evaluate the genetic association of chemotherapy-induced gonadal impairment in a large European cohort, and report a modifying effect of a single nucleotide polymorphism of the *BRSK1* gene on alkylating agent-induced ovarian damage in female CCS in three independent cohorts.

The final part of this thesis, part III, addresses obstetric outcome in female cancer survivors. We show that cancer survivors diagnosed before their forties are at increased risk of premature delivery and postpartum haemorrhage, but their children are not at risk of

being small for gestational age or having congenital abnormalities. We also show that the increased risk of an operative delivery and postpartum haemorrhage diminished in the more recent cohorts compared to older ones, resulting in equal risks for those diagnosed in the most recent cohort. In a meta-analysis of the literature we report similar findings, including specifically increased risks of prematurity and low birth weight after treatment with radiotherapy, and increased risk of giving birth to offspring being small for gestational age only after high uterine radiotherapy dosage. The translation of these observed increased risks into clinical practice is not uniform across different national guidelines. The International Late Effects of Childhood Cancer Guideline Harmonization Group has been initiated to harmonize clinical practice guidelines for childhood cancer survivors. We present recommendations for counseling and surveillance of obstetric risks for female survivors of childhood, adolescent, and young adult cancer resulting from our worldwide collaborative effort to harmonize these recommendations.

We conclude with a general discussion of the results of this thesis and advocate that more longitudinal data will be needed to extrapolate our findings further. In addition, we stress the necessity of collaboration to ensure well-powered and meaningful research that can be of clinical and individual relevance.

SAMENVATTING

De afgelopen vijftig jaar zijn de overlevingskansen van kinderkanker in heel Europa sterk verbeterd, hetgeen geresulteerd heeft in de huidige 5-jaars overleving van rond de 80% door verbeterde behandelstrategieën. Hoewel dit een geweldige prestatie is, ontwikkelt ongeveer 75% van de groeiende populatie overlevenden van kinderkanker tenminste één langetermijncomplicatie als gevolg van hun kankerbehandeling. Verminderde gonadale functie en zwangerschapscomplicaties zijn voorbeelden van belangrijke effecten op de reproductieve gezondheid. Het doel van het onderzoek in dit proefschrift was om de impact van kanker op klinische en genetische aspecten van reproductieve gezondheid te beschrijven.

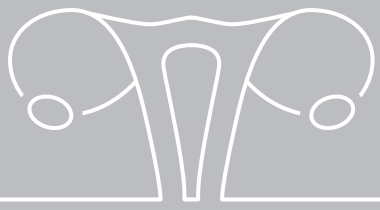
Voorafgaand aan de behandeling voor kinderkanker is de gonadale functie bij meisjes die gediagnosticeerd zijn met kanker al verminderd. In deel I van dit proefschrift hebben we laten zien dat de concentratie inhibine B en testosteron in jongens die gediagnosticeerd zijn met kanker ook al voorafgaand aan de kankerbehandeling verminderd is. De behandeling van kinderkanker zorgt vervolgens voor een verdere daling in gonadale functie bij zowel jongens als meisjes. Interessant genoeg herstelde ongeveer de helft van die zwaar verlaagde gonadale functie markers in het eerste jaar na behandeling. We concluderen dat de interpretatie van gonadale functie markers in het eerste jaar na het einde van de behandeling tot onbetrouwbare conclusies leidt. De volgende stap in het eerste deel van dit proefschrift was om de langetermijndaling van ovariële functie in overlevenden van kinderkanker, gereflecteerd door een daling in anti-Müller hormoon (AMH), te vergelijken met de daling in gezonde vrouwen van dezelfde leeftijd. Hoewel de AMH-concentraties van overlevenden van kinderkanker onder de P_{50} van normaalwaarden was op twee tijdsmomenten (met een tussentijd van ruim 3 jaar), was de mediane daling in AMH-concentratie niet sneller bij vrouwen die kinderkanker hadden overleefd, en geen enkele specifieke therapie was gecorreleerd aan een significante versnelling van die daling.

De geobserveerde verminderde ovariële functie bij overlevenden van kinderkanker kan slechts ten dele worden verklaard door de behandeling en patiëntkarakteristieken. In deel II van dit proefschrift komt daarom de interindividuele variatie aan bod, en de hypothese dat genetische variatie de associatie tussen chemotherapie en een verminderde ovariële functie beïnvloedt. We beschrijven de beschikbare literatuur over genetische susceptibiliteit voor late toxiciteit na kinderkanker behandeling wat betreft gonadale schade, en daarnaast ook van het metabool syndroom, botdichtheid en gehoorverlies. We pleiten voor voldoende statistische power, onafhankelijke cohorten voor replicatie, harmonisatie van definitie en (internationale) samenwerking om kennis van genetische variatie, die van invloed is op late toxiciteiten bij overlevenden van kinderkanker, te verbeteren. We beschrijven het ontwerp van de PanCareLIFE studie om de genetische associatie van chemotherapie geïnduceerde gonadale schade in een groot Europees cohort te onderzoeken, en we doen verslag van

een interactie-effect van een enkel-nucleotide polymorfie op het *BRSK1*-gen op alkylerende middelen geïnduceerde ovariële schade bij vrouwelijke overlevenden van kinderkanker uit drie onafhankelijke cohorten.

Het laatste deel van dit proefschrift, deel III, beschouwt de obstetrische uitkomsten bij vrouwelijke overlevenden van kanker. We laten zien dat vrouwen die gediagnosticeerd zijn met kanker voor hun 40ste levensjaar een verhoogd risico hebben op een premature bevalling en een fluxus post partum, maar dat hun kinderen niet vaker een te laag geboortegewicht hebben voor de zwangerschapsduur of vaker een congenitale afwijking hebben. We laten ook zien dat de verhoogde kans op een operatieve bevalling en een fluxus post partum in de meest recente cohorten belangrijk is afgenomen ten opzichte van de oudere cohorten, met als resultaat gelijke risico's voor vrouwen die het meest recentelijk kanker hebben overleefd ten opzichte van gezonde vrouwen. In een meta-analyse laten we ook deze obstetrische risico's zien: een verhoogd risico op prematuriteit en een laag geboortegewicht na bestraling, en alleen na hoge dosis bestraling op de baarmoeder bestaat een verhoogd risico op een te laag geboortegewicht voor de zwangerschapsduur. De vertaalslag van deze verhoogde risico's naar de klinische praktijk is verschillend bij nationale protocollen. De International Late Effects of Childhood Cancer Guideline Harmonization Group is opgericht om protocollen voor kinderkankeroverlevenden te harmoniseren. We beschrijven de internationaal tot stand gekomen aanbevelingen voor counseling en surveillance van zwangerschapsrisico's bij overlevenden van kinder- en jeugdkanker.

We eindigen met een algemene beschouwing van de resultaten beschreven in dit proefschrift, en pleiten voor meer longitudinale data om de resultaten te kunnen extrapoleren. We benadrukken bovendien de noodzaak van samenwerking voor voldoende statistische power en zinvol onderzoek dat van klinische en individuele waarde is.



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BIBLIOGRAPHY

In this thesis

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Wigny, K. M.*, W. van Dorp*, **A.L.F. van der Kooi,** Y.B. de Rijke, A.C.M. de Vries, M. Smit, S.M.F. Pluijm, E.L. van den Akker, R. Pieters, J.S.E. Laven and M.M. van den Heuvel-Eibrink (2016). "Gonadal function in boys with newly diagnosed cancer before the start of treatment." Hum Reprod 31(11): 2613-2618.

Other manuscripts

Clemens, E., A.J. Meijer, L. Broer, T. Langer, **A.L.F. van der Kooi**, A. G. Uitterlinden, A. de Vries, C.E. Kuehni, M.L. Garre, T. Kepak, J. Kruseova, J.F. Winther, L.C. Kremer, E. van Dulmen-den Broeder, W. J. Tissing, C. Rechnitzer, L. Kenborg, H. Hasle, D. Grabow, R. Parfitt, H. Binder, B. C. Carleton, J. Byrne, P. Kaatsch, A. Am Zehnhoff-Dinnesen, O. Zolk and M.M. van den Heuvel-Eibrink (2019). "Genetic Determinants of Ototoxicity During and After Childhood Cancer Treatment: Protocol for the PanCareLIFE Study." JMIR Res Protoc 8(3): e11868.

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G. Uitterlinden, M. van den Berg**, A.L.F. van der Kooi**, M. van Dijk, F. van Leeuwen, O. Zolk, D. Zoller and P. Kaatsch PanCare (2018). "PanCareLIFE: The scientific basis for a European project to improve long-term care regarding fertility, ototoxicity and health-related quality of life after cancer occurring among children and adolescents." Eur J Cancer 103: 227-237.

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PHD PORTFOLIO

Name:	Anne Lolkje Femke (Anne-Lotte) van der Kooi
Erasmus MC Department:	Obstetrics and Gynaecology, Division of Reproductive Medicine
Research school:	NIHES MolMed
PhD period:	June 2015 – July 2019
Promotores:	Prof. J.S.E. Laven Prof. M.M. van den Heuvel-Eibrink

General courses	**Year**
BROK (Basiscursus Regelgeving Klinisch Onderzoek)	2016
English Biomedical Writing and Communication	2016
Workshop Indesign CS6, Photoshop and Illustrator	2016
Research Integrity	2017
Specific courses (e.g. Research school, Medical Training)	
Principles of Genetic Epidemiology	2015
Genomics in Molecular Medicine	2015
SNPs and Human Diseases	2015
Repeated Measurements	2016
The Course on R	2016
Markers and Prediction Research	2017
Biostatistical Methods: Basic Principles	2018
Bayesian statistics and JASP	2018
Genome-wide association studies	2018
Principles of research in medicine and epidemiology	2018
Human epigenomics	2018
Survival Analysis	2019
Presentations at (inter)national conferences	
Najaarsvergadering Vereniging voor Fertiliteitsstudie, Antwerp, Belgium (oral presentation)	2015
Refereeravond “What’s new in reproductive medicine” (oral), Erasmus MC, Rotterdam	2015
European Symposium on Late Complications after Childhood Cancer, Copenhagen, Denmark (poster presentation)	2016
71st Annual Meeting of the American Society for Reproductive Medicine, Salt Lake City, USA (oral presentation)	2016
48th Congress of the International Society of Paediatric Oncology, Dublin, Ireland (oral presentation)	2016
15th Annual International Conference on Long-Term Complications of Treatment of Children and Adolescents for Cancer, Atlanta, Georgia, USA (poster presentation)	2017
1st UK Fertility Preservation Annual Meeting, Edinburgh	2017
PanCare meeting, Prague, Czech Republic (oral presentation)	2017
Sophia Research Day, Erasmus MC, Rotterdam (oral presentation)	2018

AAV wetenschapsmiddag, Erasmus MC, Rotterdam (oral presentation) *Award Best Presentation*	2018
Voordracht werkgroep kinder- en adolescenten gynaecologie, Utrecht	2018
Wladimiroff Award Meeting (RCOG), Erasmus MC, Rotterdam (oral presentation)	2018
PanCare meeting, Paris, France (oral presentation)	2018
50th Congress of the International Society of Paediatric Oncology, Kyoto, Japan (poster presentation)	2018
66th Annual Meeting of the Society for Reproductive Investigation, Paris, France (poster presentation)	2019
Wladimiroff Award Meeting (RCOG), Erasmus MC, Rotterdam (oral presentation) *Award Best Presentation*	2019
Invited speaker ESHRE Campus Course "Fertility preservation in children and adolescents: the next frontier", Edinburgh, UK	2019

Seminars, workshops and research meetings

PhD-day Erasmus MC	2015
AAV wetenschapsmiddag	2015
Weekly Quality of Care and Toxicity (QCAT) research meeting	2015 – 2016
Biweekly research meetings Department of Obstetrics and Gynaecology, division of Reproductive Medicine	2015 – 2019
Biweekly multidisciplinary Reproductive Medicine – Endocrinology meeting	2015 – 2019
Sophia Research Day	2016 – 2018
AAV wetenschapsmiddag	2018
Prinses Máxima Centrum (LATER) research meetings	2018 – 2019

Teaching

Coaching (onderdeel BKO)	2016 – 2019
Lecturing/supervising practicals Bachelor Medicine, Faculty Medicine and Health Sciences, Erasmus University MC	2016
Tutoring (onderdeel BKO)	2017
Supervising minorstudents	2017
Supervising Masters' theses	2018 – 2019

Other

Organizing committee PhD-day Erasmus MC	2016 – 2017
Coordinator of the International Guideline Harmonization Group Obstetrics	2016 – 2019
Recipient Koninklijke Nederlandse Akademie van Wetenschappen Ter Meulen Beurs	2017 – 2018
Peer reviewing of articles for scientific journals	2017 – 2019
Collaborator of the Fertiliteitsconsortium Prinses Máxima Centrum and UMC Utrecht	2018 – 2019

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