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].

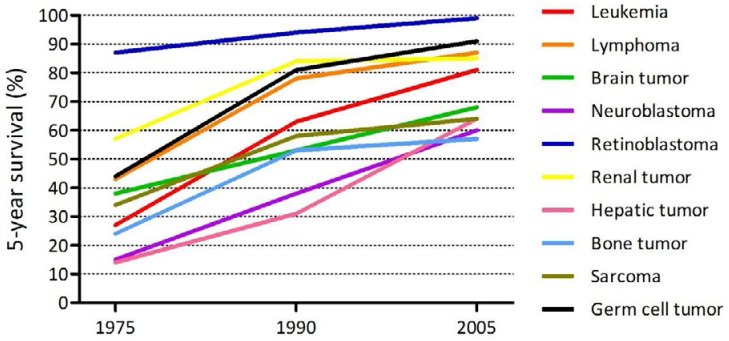

Figure 1. Survival rates for Dutch childhood cancer survivors diagnosed before the age of 18 years. Dutch Childhood Oncology Group (DCOG) national registration 2017.

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

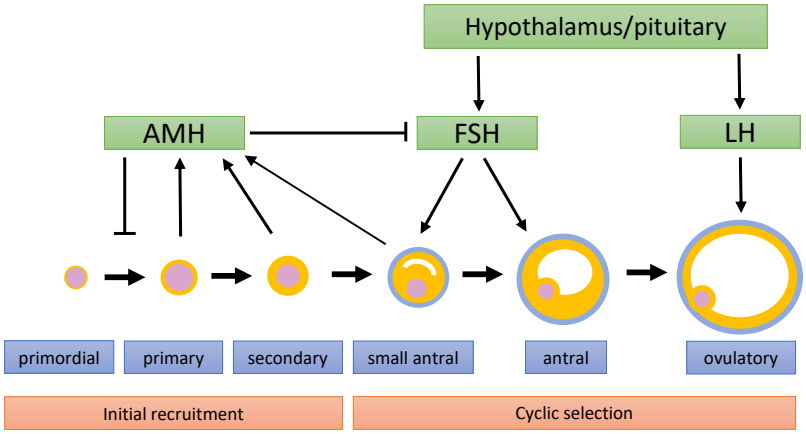

Figure 2. Simplified depiction of initial recruitment and cycle recruitment during folliculogenesis. AMH, anti-Müllerian hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone.

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

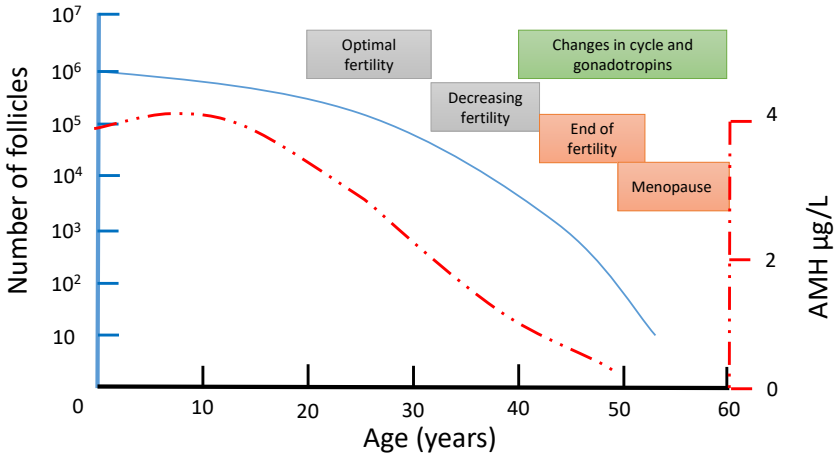

Figure 3. Decline in primordial follicle pool (line) and gradual decline of AMH levels (dotted line). Reproductive events that can be used for ovarian function assessment are indicated on the timeline where they usually occur. AMH = anti-Müllerian hormone.

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

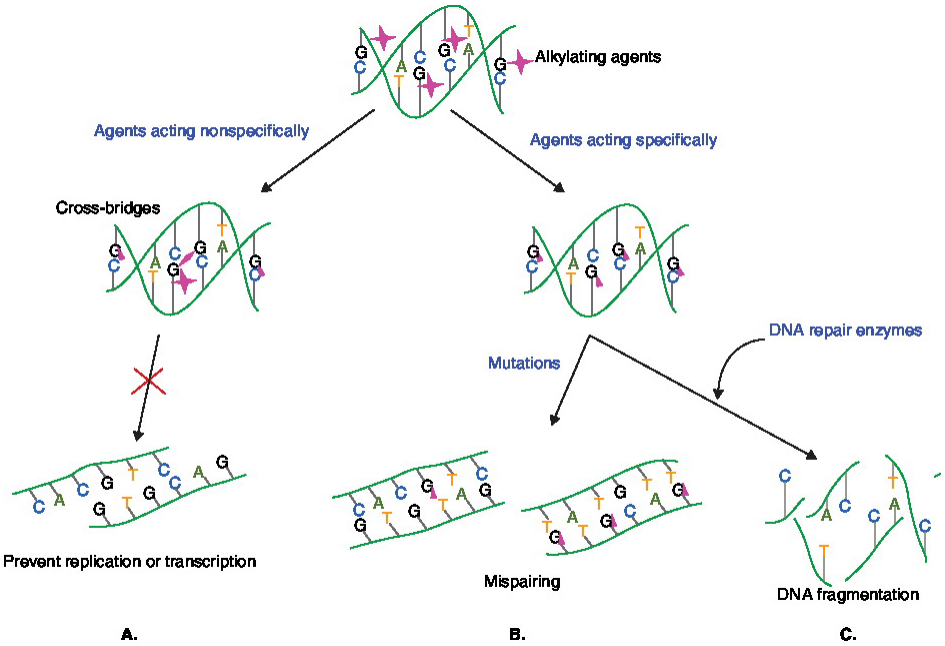

Figure 4. Mechanism of action of alkylating agents. A. Formation of cross-bridges, bonds between atoms in the DNA results in inhibition of replication or transcription. B. Alkylated G bases may erroneously pair with Ts. If this altered pairing is not correct it may lead to a permanent mutation. C. DNA 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