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Impact of androgen action on reproduction and cardiovascular disease in male cancer survivors

Abstract

Childhood and testicular cancer have excellent survival rates, for testicular cancer approaching 98 % and for childhood cancer exceeding 80%. For the many survivors, the post-cancer quality of life is therefore a main issue. The treatments and their side effects are varying and one cannot exclude that genetically determined inter-individual differences in sensitivity to adverse effects of cancer therapy may, in part, have impact on the late effects. Studies have reported increased cardiovascular morbidity, subfertility and risk of hypogonadism in the male cancer survivors. The aim with thesis was to investigate the prevalence of cardiovascular risk factors and their association to hypogonadism and type of treatment and to increase the knowledge regarding the impact of androgen receptor polymorphism on cancer treatment related impairment of reproductive function and occurrence of risk factors of cardiovascular disease.

In paper I impact of treatment and androgen receptor polymorphism on sperm concentration recovery was investigated in 130 testicular cancer survivors. In testicular cancer patients given 3 or 4 cycles of chemotherapy, sperm number one year post-treatment was associated to androgen-receptor CAG repeat lenght. The lowest sperm number was detected in patients with CAG 22-23, pointing at a impact of androgen receptor function on recovery of sperm production.

In paper II the risk of cardiovascular late-effects and impact of treatment and testosterone deficiency was investigated in 92 testicular cancer survivors and the same number of age-matched controls. Hypogonadal patients had the most elevated risk of cardiovascular risk-factors compared to controls and eugonadal patients.

In paper III, the similair study set up as paper II was applied for 125 childhood cancer survivors and age-matched controls. In line with the results from paper II, hypogonadism, but even radiotherapy to CNS, were most strongly associated to cardiovascular risk factors.

In paper IV it was explored whether polymorphisms in the androgen receptor gene modified the association between hypogonadism and risk factors of cardiovascular disease, found in papers II and III. Statistically significant interactions were found for hypogonadism and GGN/ CAG repeat lengths in relation to levels of cholesterol and glucose as well as prevalence of the metabolic syndrome further pointing at impact of androgens on cardiovascular risk factors in young male cancer survivors.

Key words Testicular and childhood cancer, testosterone, androgen receptor, cardiovascular diseaes, CAG, GGN repeat, sperm production

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Impact of androgen action on reproduction and cardiovascular disease in male cancer survivors

Karolina Bogefors

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To my family

Content

List of papers

This thesis is based on the following original publications and manuscripts.

I. **Bogefors, K**, Giwercman YL, Eberhard J, Ståhl O, Cavallin- Ståhl E, Cohn-Cedermark G, Arver S, Giwercman A Androgen receptor gene CAG and GGN repeat lengths as predictors of recovery of spermatogenesis following testicular germ cell cancer treatment. Asian Journal of Andrology, (2017), doi: 10.4103/1008-682X.191126

II. **Bogefors K**, Isaksson S, Leijonhufvud I, Bobjer J, Link K, Kitlinski M, Giwercman A, Hypogonadism in testicular cancer patients is associated with risk factors of cardiovascular disease and the metabolic syndrome, Andrology, (2017) Doi: 10.1111/andr.12354

III. **Bogefors K**, Isaksson S, Leijonhufvud I, Bobjer J, Link K, Kitlinski M, Giwercman A, Increased prevalence of cardiovascular risk factors and metabolic syndrome in hypogonadal childhood cancer survivors. Manuscript. Submitted.

IV. **Bogefors K**, Isaksson S, Leijonhufvud I, Giwercman A Androgen receptor CAG and GGN repeat lengths as modulators of association between hypogonadism and metabolic and cardiovascular parameters in young male cancer survivors. Manuscript. Submitted.

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Abbreviations

ACT	Adjuvant chemotherapy
AFP	Alpha-fetoprotein
AIS	Androgen insensitivity syndrome
AR	Androgen receptor
ART	Adjuvant radiotherapy
ß -HCG	beta-humanochoriongonadotrophin
BEP	Bleomycine, etoposide, cisplatin
BMI	Body mass index
CCS	Childhood cancer survivors
CI	Confidence interval
CV	Coefficient of variation
CVD	Cardiovascular disease
CT	Cancer Therapy
DHT	Dihydrotestosterone
DM	Diabetes Mellitus
DNA	Deoxyribonucleic acid
E2	Estorogen receptor
FSH	Follicle-stimulating hormone
GCNIS	Germ Cell Neoplasia *In Situ*
GnRH	Gonadotrophin-releasing hormone
Gy	Gray
hCG	Human chorionic gonadtrophin
HCT	High dose chemotherapy, 3-4 cycles of chemotherapy
HDL	High density lipoprotein
HL	Hodgkin Lymphoma
HOMAir	Homeostatic model assessment
HPG	Hypothalamic-pituitary-gondal

ICSI	Intra cytoplasmicsperm injection
IVF	*In vitro* fertilization
LDH	Lactate-dehydrogenase
LDL	Low density lipoprotein
LH	Luteinizing hormone
MetS	The Metabolic Syndrome
Mk+	Elevation of tumormarkers (AFP and/ or ß-HCG)
OR	Odds ratio
PEI	Cisplatin, etoposide, ifosfamide
RPNLD	Retroperitoneal lymph node dissection
RT	Radiotherapy
SCT	Standard dose chemotherapy, 1-2 cycles of chemotherapy
SD	Standard deviation
SHBG	Sex-hormone binding globulin
SO	Surgery only
SWENOTECA	Swedish-Norweigan testicular cancer project
T	Testosterone
TC	Testicular cancer
TCS	Testicular cancer survivors
TDS	Testicular dysgenesis syndrome
TRT	Testosterone replacement therapy
VHCT	Very high dose chemotherapy>4 cycles platinum-based chemotherapy +/- Ifosfamide +/- radiotherapy
WHO	World Health Organization

Populärvetenskaplig sammanfattning

Testikelcancer drabbar årligen ca 300 män i åldern 18-35 år och är den vanligaste cancerformen i denna åldersgrupp. Barncancer drabbar ca 300- 350 barn i Sverige varje år. Idag är överlevnadssiffrorna för testikelcancer 98% och för barncancer överstigande 80%. Förbättringar i behandling och uppföljning av patienterna har gjort detta möjligt. I Sverige finns det idag över 10 000 unga canceröverlevare och gruppen ökar ständigt i antal.

Studier har dock visat att de är sjukare än sina jämnåriga, varav 30 % av barncanceröverlevarna har mycket allvarliga biverkningar efter sjukdom och behandling. Vissa får debut av biverkningar direkt efter behandling, medan andra utvecklar dessa senare i livet. De vanligast förekommande är hjärtkärlsjukdomar, hormonstörningar, trötthet, nedstämdhet samt oförmåga att skaffa barn.

För hela gruppen gäller att de dör i förtid. Efter 5 år är det sekundär cancer och hjärtkärlsjukdomar som står för den största dödligheten.

Studier på senare år har visat att många av de manliga canceröverlevarna har brist på det manliga könshormonet testosteron. Testosteronet behövs hos mannen för att upprätthålla flertalet processer i kroppen innefattande hår- och muskelväxt, spermiebildning, samt optimal hjärt- och kärlfunktion. Dessutom motverkar testosteron övervikt, nedstämdhet och nedsatt sexuell lust.

Receptorn dit testosteronet binder för att få önskvärd effekt, androgenreceptorn, finns i de flesta vävnader i kroppen. Det indikerar att den har en mångsidig verkan. Receptorn är en del av mannens arvsmassa och individuella skillnader påverkar hur väl receptorn fungerar. Dessa benämns polymorfier och dess variation kan få olika konsekvenser, till exempel ökad risk för vissa cancerformer och förhöjda blodfetter.

Med denna studie ville vi undersöka om unga manliga canceröverlevare, jämfört med friska kontrollpatienter, har en ökad risk för testosteronbrist och om det påverkades av vilken behandling de fått.

Vi ville också veta om testosteronbristen kan kopplas till tidiga tecken på hjärtkärlsjukdom i form av förhöjda insulin och glukosnivåer, samt stegrat kolesterol, förhöjt blodtryck och en ökad förekomst av metabolt syndrom (som är ett samlingsbegrepp för flera samtidigt förekommande riskfaktorer).

Testosteronbrist kan behandlas med testosteronersättning vilket kanske kan ge en möjlighet att förhindra sjuklighet och för tidig död. Resultaten av dessa studier finns beskrivna i artikel II och III och bekräftar vår teori att canceröverlevare med testosteronbrist har ökad förekomst av flera riskfaktorer i jämförelse med kontrollpatienterna. Vid testosteronbrist ökar också risken för att drabbas av det metabola syndromet jämfört med kontrollerna.

I studie 1 var målet att undersöka om androgenreceptorns polymorfier påverkar hur snabbt man återhämtar sin förmåga att skaffa barn, genom att titta på hur snabbt spermiefunktionen återhämtas. Där visar vi att testikelcanceröverlevande män med den normalt mest effektiva polymorfin är de som återhämtar sin spermieproduktion långsammast.

I studie 4 undersökte vi om androgenreceptorns polymorfier också påverkade risken att drabbas av riskfaktorer för hjärtkärlsjukdomar och metabolt syndrom och hur stor betydelse testosteronbrist har i detta sammanhang. Man kunde i denna studie se att polymorfierna har betydelse för risken att drabbas av förhöjda kolesterolvärden och metabolt syndrom, även när man har testosteronbrist.

Av detta drar vi slutsatsen att testosteronbrist är en riskfaktor för att drabbas av vissa kardiovaskulära riskfaktorer efter cancer och att genetiska varianter av androgenreceptorn ytterligare styr detta, samt att de genetiska varianterna av androgenreceptorn också påverkar hur snabbt man återhämtar sin spermiefunktion.

Denna kunskap hoppas jag kunna leda till förbättrad klinisk uppföljning av unga manliga canceröverlevare, så att man tidigare upptäcker risk-patienter. Provtagning avseende riskfaktorer för hjärtkärlsjukdom och testosteronvärden bör ingå i denna uppföljning. Man skall vid behov initiera förebyggande åtgärder såsom viktminskning och blodfettsminskande mediciner. I vissa situationer bör insättning av testosteronbehandling övervägas. Kunskapen kring de genetiska skillnaderna i androgenreceptorn och dess påverkan på spermieåterhämtningen kan tänkas bidra till att förutse vilka män som har störst risk att påverkas i sin fertilitet. Detta kan få betydelse för pojkar med cancer före puberteten då man inte kan frysa ner spermier för senare provrörsbefruktning, och där andra fertilitetsbevarande åtgärder får övervägas.

Preface

Cure, but to what price?

Being a resident in the Oncology department, I met Anders, who was a survivor of childhood cancer. As a ten-year old he was diagnosed with a malignant brain tumour. The treatment included surgery and radiotherapy and lasted for several years. *"I don´t remember much, but if anything I missed my friends and playing football. The treatments were nothing compared to the disaster that afterwards became my life!"*

The late-term effects with blindness, endocrine dysfunction, seizures, hypertension, and later effects on fertility, changed his life completely.

-I survived, but I am not sure that it was a price worth paying.

The story of testicular and childhood cancer is from a statistical point of view a successful one. Earlier they were highly lethal diseases, from which only few of the young patients survived.

With research and improvements in treatments, the vast majority today become long-term survivors. In Sweden today, one in every five hundered is a young cancer survivor.

Among childhood cancer survivors, three out of four have late-term complications after disease and treatment. One third suffer from serious complications. The late-term mortality is increased and premature death due to cardio-vascular disease (CVD) is common.

In previous studies on healthy men, low testosterone has been linked to an increased risk of CVD. Furthermore, it is indicated that male cancer survivors more often suffer from low testosterone, hypogonadism.

Testosterone and its receptor, the androgen receptor, are crucial for the maintenance of male characteristics and several physiological phenomena. Genetic variants of the receptor, polymorphisms, are known to influence the outcome of the receptor.

With this project the aim was therefore to investigate the associations between low testosterone and risk factors of cardiovascular disease, sperm concentration, recovery and polymorphisms in the androgen receptor gene in male cancer survivors. The completion of this study, including 250 patients and 250 controls have been challenging, but above all, I had the blessing to meet all the cancer survivors, who, in different ways struggle to proceed with the rest of their lives. Their courage and their willing to live a normal life despite suffering from late complications is the driving force of this study.

Anders is just one of many in this growing subgroup in society, with severe sequelae and with a long expected lifetime. More attention should be given the ability to live a normal life with a minimum of complications. With this study my aim was to contribute to the knowledge of late-term morbidity that could lead to improvements in life quality. Hopefully, the future treatment regimens of young cancer patients will give "Cure at a reasonable price", but until then we need to handle the late-term complications.

Part I

Male Survivors of testicular and childhood cancer

Testicular Cancer

Incidence, prevalence and mortality
Testicular cancer (TC) is the most frequent malignancy in young men aged 18-40 years. Yet it is a rare disorder accounting for 1 % of all male cancers (1-3).

The annual incidence has increased by 2.3 % during the last decade with 300 new cases in Sweden every year (4-6). A fivefold rise is detected during the last four decades in developed countries, but with large variation in incidence between Nordic countries. The highest incidence is found in Denmark and Norway, 10.1 and 12.9/ 100000, compared to 7.8/ 100 000 in Sweden. For least developed countries the incidence is considerably lower. The lifetime risk in African countries is 1/1160 compared to 1/235 in Sweden(7,8) (9) (10).

Survival rates have improved remarkably in Western countries (Figure 1). This is mainly due to the introduction of a cisplatin-based chemotherapy, but progress in radiotherapy, improved diagnostics and specialized collaboration groups have contributed. Survival rates have increased from approximately 40 % in the 1960´s to exceed 97 % for the whole group, and 99 % for patients with localized disease (11).

Aetiology
Germ-cell tumours originate from primordial germ cells, the pre-stage of spermatozoa and account for 95 % of all testicular cancer. They constitute two groups; seminomas and non-seminomas. The remaining 5 % are other malignancies; e.g. lymphoma, sarcoma and mesothelioma.

TC occurs in early adult life and it has been suggested that the carcinogenic process starts already in the fetal stage. The primordial germ cell arising during embryogenesis will turn into a gonocyte prior entering mitotic arrest and mature into pre-spermatogonia. Further development will occur at onset of puberty as testosterone levels rise. It is proposed that gonocytes fail to differentiate and instead become cancer precursor cells, indicated by the fact that GCNIS (germ cell

neoplasia *in situ*) cells express markers similar with those expressed by embryonic stem cells and gonocytes (12), (13).
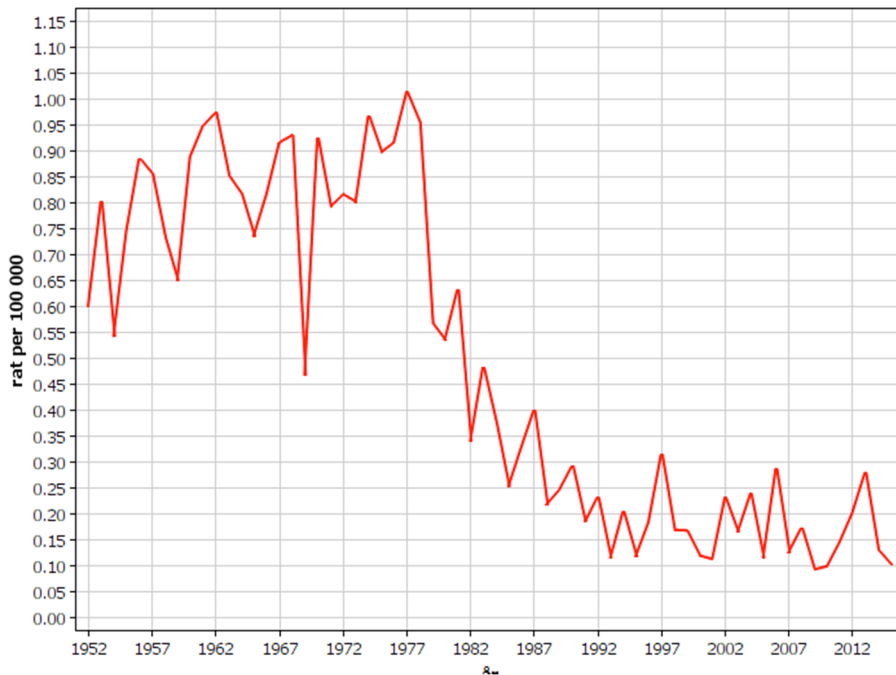
Figure 1.
Testis cancer mortality Sweden 1952-2012 (Nordcan).

Risk factors for TC
The potential background of TC is fetal intrauterine exposure to abnormal levels of sex hormones, initiated by maternal lifestyle (14,15), (maternal smoking, obesity and child-bearing), and specific environmental subjects; (organochloride pesticides, endocrine disruptors.

Some studies have indicated a link between TC and cryptorchidism, (non-descending testis (16). With cryptorchidsm the risk for seminoma is four times enhanced (15,17-19),(20),(21). Also subfertility (22),(23,24), genital malformations, hypospadias (25), (26) and hernias, semen quality decline (24) (27) are associated risk factors. Based on these observations, Skakkebekk formulated the testicular dysgenesis syndrome (TDS) in 2001, where reduced spermatogenesis, cryptorchidism and TC possibly share common ethological features with a genetic impact, including developmental diorders of the testis (28).

Further evidence for a genetic involvement may be represented by the increased risk for TC if a brother or father has been diagnosed with TC (29). Moreover, first-

18

generation immigrants have a similar risk as country of origin, whereas second-generation immigrants have a risk of TC in accordance with natives of the immigrated country (30). Studies on androgen receptor polymorphism have implied an association to TC, further pointing at a genetic susceptibility (31), (32). However, repeated studies of whole genome sequencing and TC, have not reached consensus supporting an existence of a high-penetrating gene (33). Studies on specific families with a minimum of two blood relatives with disease denote that multiple common alleles could be involved (34,35).

Diagnosis of testicular cancer
Most patients with TC present with a unilateral painless lump and only 20 % have scrotal pain (35). One fourth of patients will suffer symptoms from the site of metastasis; respiratory symptoms if lung metastasis, back pain in patients with retroperitoneal metastasis (the most frequent location of metastasis) (36). The most common site of haematogenous dissemination is the lungs, and other locations, e.g. CNS and skeleton, are more rarely occurring.

Clinical examination, ultrasound of testis, CT-scans and blood sampling for tumor biomarkers (alpha-fetoprotein (AFP); ß-humanochoriongonadotrohin,ß-HCG, lactate dehydrogenase LD are used for proper staging and diagnosis. Among non-seminoma patients, 70 % have elevated AFP and/ or ß -HCG at diagnosis (37,38). LD is a reliable marker of tissue injury and reflects tumour progression and turnover, yet it is a less specific biomarker than AFP and ß -HCG. New biomarkers include PLAP (placental-like alkaline phosphatase), elevated in 60-70% of seminoma patients (39) and circulating micro-RNA (40,41) indicating testicular cancer with 98 % sensitivity.

Treatment and prognosis

A half century ago, testicular cancer was treated primarily by surgery. The survival rates were below 40 %. The introduction of combinational chemotherapy of metatstatic testicular cancer in 1960 resulted in a 30 % response rate (42). When vinblastine and bleomycin were introduced as primary treatment in 1975, 39 % reached complete response (43). Cisplatin was introduced in the 1970´s and in 1977 Einhorn and Donhue reported a 100 % response rate and 74 % complete response of cisplatin in combination with vinblastine and bleomycin (CVB) when surgical excision was performed of residual disease post-chemotherapy (44-46).

In 1983 BEP (Bleomycin, Etoposide and Cisplatin) was introduced, exchanging vinblastine for etoposide due to less toxicity (47). CVB and BEP were compared in a randomized study by (48)where the preeminence of BEP was concluded with

improved efficacy and decreased toxicity. BEP has ever since been first choice of treatment.

Staging and Treatment according to Swenoteca (Swedish Norweigan testicular cancer)
In Sweden, The Royal Marsden Hospital system (Table 1) is applied for screening, (49). After orchiectomy, CT-scans and abnormal tumor-markers are used for detection of metastasis. Due to the risk of Germ Cell Neoplasia *in situ* (GCNIS) in the contra-lateral testicle, present in 2-9 % of patients (50,51), a biopsy is often accomplished. In case of GCNIS, RT of 16 Gy in 8 fractions is recommended (52).

Table 1.
The Royal Marsden Hospital system

Clinical stage	Criteria
CSI	No evidence of metastases
CS MK+	Tumour markers AFP/ beta-HCG persistently elevated (not declining according to half-life), no macroscopic metastases
CS II	Metastatic disease restricted to abdominal nodes. A diameter < 2 cm, B 2-5 cm, C: >5-10 cm. D >10 cm
CS III	Supradiafragmatic node involvement. Abdominal lymph-nodes 0 metastases, no metastases; A-D according to CS II
CS IV	Lung substage: L1 <3 metastases, no metastases >2 cm, L2>3-<2o metastases, no metastases >2 cm; L3; < 20 metastases, > 2cm; L4 >20 metastases. For abdominal lymph-nodes: 0 no metastases; A-D according to CS 2.H+ Liver metastases, Br+ Brain metastases, Bo+ Bone metastases.

Further treatment is dependent on the histology, risk factors and disease stage (53). In Sweden and Norway treatment is given according to Swenoteca recommendations. This collaboration group of Swedish and Norwegian testicular cancer specialists was founded in 1981, providing mutual cancer care programs for testicular cancer (54,55). The IGCCCG (56) is used for staging and patients are divided into good, intermediate and poor prognosis groups.

Table 2.
IGCCCG prognostic system

Prognosis	Seminoma	Non seminoma
Good prognosis	Any primary site, normal AFP, any B-HCG any LD, no pulmonary- visceral metastasis	Testicular or retroperitoneal primary site, no non-pulmonary visceral metastasis. AFP <1000 Ug/ L and B-HCG <5000 IU/ L and LD< 1.5x N
Intermediate prognosis	Any primary site, normal AFP any B-HCG or LD. No pulmonary- visceral metastasis	Testicular or retroperiteoneal primary site no pulmonary visceral metastasis, AFP; 1000 -10000 ug/ L or B-HCG 5000-50 000IU/L or LD 1.5-10 X N
Poor prognosis	None	Mediastinal primary tumour or non- pulmonary visceral metastasis, AFP>10000 ug/ L or B-HCG >50000 IU/ L or LD>10x N.

N=upper normal limit of LD

Treatment

TC patients constitute to 60 % of Seminoma patients, aged 35-40 years, (median 34 years) (57). Non-seminoma appears earlier in life, the peak incidence being 25-35 years, (median 27 years). For seminoma patients, 80 % are in stage I (no evidence of metastases) or IIA (metastases to abdominal lymph nodes <2 cm), while the non-seminoma patients have metastases in 45-50 % of cases (55). For non-seminoma, the histology include several subtypes (Yolk sac tumour, embryonal carcinoma, choriocarcinoma and teratoma).

In clinical stage I (CSI) survival rates are close to 100%. Tumour size > 4cm, and invasion of rete testis are the most important risk factors for relapse in seminoma patients (58), (59). For non-seminoma patients, the strongest predictive factor for relapse is invasion of tumor cells in the blood or lymph nodes (VASC+/ -). Due to 30 % risk of subclinical metastases in CSI disease, a restaging is performed after 6-8 weeks. In case of abnormal tumor-biomarkers, the staging is repeated until normal or increased levels are reached.

Treatment is dependent on histology, presence of risk factors and stage of disease. A risk-adapted strategy for CSI is applied. For seminoma patients no or one risk factor is considered low risk and patients with high risk have more than 2 risk factors, invasion of rete testis or >4 cm tumour size (table 3) and non-seminoma patients (table 4). Strategies vary between hospitals and some use surveillance for all patients and others a risk adapted approach. Studies have however showed that treatment after relapse is as good as treatment up front (60).

Table 3.
Seminoma treatment. According to Swenoteca treatment guidelines.

Stage of disease	Treatment	Prognosis
CSI, low risk	Surveillance	No risk or 1 risk factor, 100%
CSI<40 years	1 dose Carboplatin[c]	Two or more risk factors or rete testis invasion100%
CSI>40 years	RT 20 Gy, 10 fractions[b] para-aortic and ipsi- lateral lymphnodes	Relapse 1-2 %
CS IIA	RT para-aortic and ipsi-lateral lymphnodes, 30 Gy[a]	100 %
CS IIB-good prognosis, >40 years	RT para-aortic and ipsi-lateral lymphnodes, 36 Gy[a]	100 %
CS IIB-good prognosis, <40 years	3 BEP or 4 EP	100%
CS IIB- intermediate prognosis(0.4%)	4 BEP or 4 VIP	50-75 %

a= P Albers, 2015.**b=** Fosså, 99 Jones 05,**c=** Oliver- 05) EP= etoposide, 100 mg/m^2 days 1-5 and carboplatin, BEP= Bleomycine 30 000 IU days 1,5 and 15, etoposide; 100 mg/m^2 days 1-5 and cisplatinum 20 mg/m^2 days 1-5, VIP=etoposide 100mg/m^2, cisplatin 20 mg/m^2 days 1-5, ifosfamide 1200 mg/m^2 , mesna 240/420 mg) given every third week.

Table 4
Non- seminoma treatments according to Swenoteca.

Stage of disease	Treatment	
CSI VASC-	Surveillance/ 1 BEP 10 % relapse rate if no treatment	
CSI-VASC+	Adjuvant 1 BEP: 50 % relapse rate if no treatment	
CSI (if other treatments non-optional)	RPLND	
CSII[a]	3 BEP	
CSII Poor prognosis	Intensified treatment- adding (Paclitaxel, Oxaliplatin, Ifosfamide)[b]	

a= Olofsson 2011, **b=** Fiazzi 2002

Treatment for TC has practically been the same during the last decades reaching excellent survival. The survivors of TC are constantly increasing in number and are now more than 8000 in Sweden (61).

Childhood cancer

Incidence, prevalence and mortality
Childhood cancer prevalence and incidence have been constant during the last decades, with 300-350 new cases every year or 17 children for every 100 000 inhabitants. Until the introduction of radiotherapy in the 1890, surgery was the only treatment option. The survival was first improving when chemotherapy was introduced in 1950.

The incidence depends on age, sex and ethnicity. The incidence is highest during early childhood, decreasing between 5 and nine years of age and then rising again in the ages between 15-19 years of age (62).

The survival rates have improved constantly, now exceeding 80 % for the whole group of childhood cancer patients. For some subtypes, exemplified by acute lymphoblastic lymphoma (ALL), the survival is close to one hundred percent. For other tumour types the progress in survival rates has been less favorable. CNS tumours have plateaued at approximately 50 %. New treatment strategies are needed to improve survival for these diagnoses.

The group of childhood cancer survivors is increasing; among young adults in USA 1/640 is a CCS.
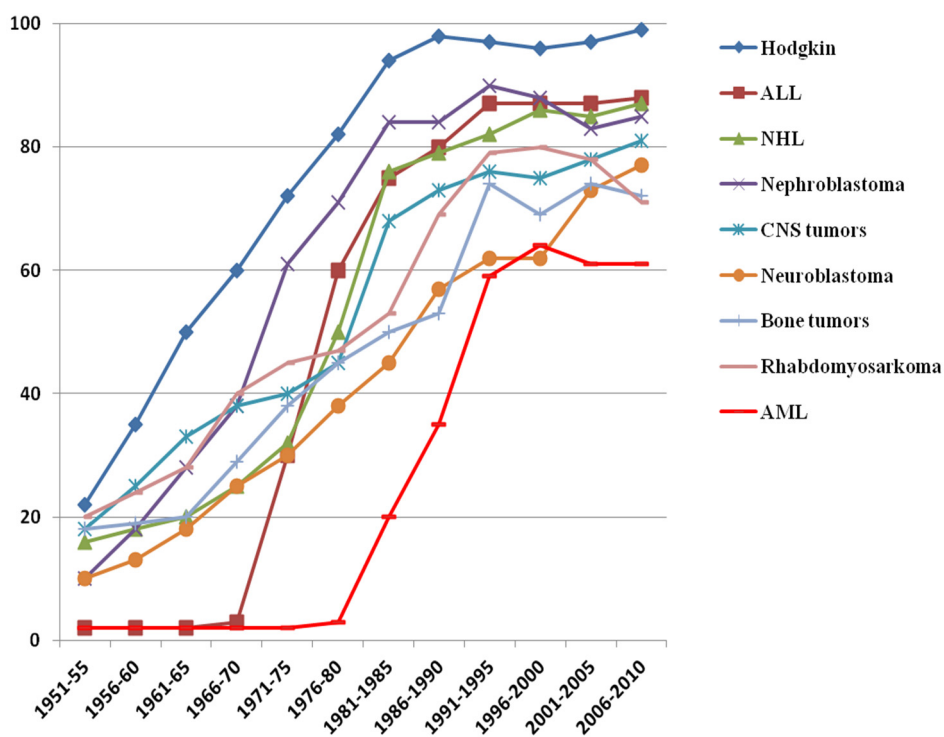

Figure 2.
Survival in childhood cancer in Sweden 1950-2010. (Childhood Cancer Incidence and Survival in Sweden 1984-2010, Report 2013 from the Swedish Childhood Cancer Registry. Editors: G Gustafsson, P Kogner and M Heyman). With permisson.

Aetiology

Childhood cancer is not one disease entity, but a fusion of all malignancies in children up to 18 years of age with different histology, origin, treatment, outcome and side effects.

Treatment and prognosis
The most common subdiagnoses of childhood cancers are; lymphoma and leukemia: 30%, CNS tumors 28% and other solid tumors 42%.

Acute lymphatic lymphoma (ALL; 35 %) has a peak incidence in ages 2-4 years and is more frequently occuring in boys. Primary symptoms constitute fever, fatigue, bone and joint pain. The treatments include vincristine, asparaginase, antimetabolites, anthracyclines and corticosteroids. For patients with leukaemia infiltrating the central nervous system, craniospinal radiation (18–24 Gy) was earlier standard part of treatment, but nowadays due to risk of side effects, selected for high-risk patients. If case of tumor-infiltration of testis, local radiotherapy (RT) is administered. The survival rates have improved from approximately 2% in the 1950s to exceeding 98% today (Gustafsson et al Report 2013 from the Swedish Childhood Cancer Registry. (61)

Acute myeloma patients (AML) constitue a small part of all CCS, thus with a less favorable prognosis. Initially they are treated with less toxic chemotherapy regimens, but due to high relapse risk, high dose chemotherapy regimens and bone marrow transplantation is often added. (63,64)

Hodgkin lymphoma (HL) is the most common malignancy among older children and adolescents. The stage of disease and clinical manifestations will decide the treatment. Chemotherapy, including an alkylating agent e.g. MOPP (mustine, oncovin, procarbazine, prednisolone) increases risk of side effects why ABVD (adramycin, bleomycin, vinblastine and dacarbazin) will be chosen if possible (65). Mantle irradiation was earlier part of treatment regimen, but due to elevated pulmonary diseases and secondary cancer incidence post-treatment, it is no longer given. The late effects from previous mantle irradiation is however still a present challenge. Many protocols include surgery and radiotherapy as supplementary treatment post chemotherapy. Occasionally radiotherapy to lymph nodes below the diaphragm is added.

CNS tumours: The survival rates vary from 50-85% depending on the histology of the tumor. Craniopharyngeoma and gliomas are the most frequent diagnosis. Most of them will be operated, for some RT is added.

Neuroblastoma and other embryonal tumors (Wilm´s tumour, retinoblastoma) have a downward sloping incidence to 5 years of age. Treatment ranges from surgery to

chemotherapy, with or without focal irradiation, including platinum chemotherapy, in severe cases, with addition of autologous bone marrow transplantation.

Sarcoma tumors peak in puberty and early adolescence. Ewing sarcoma is treated with chemotherapy and sometimes with the addition of RT, of the pelvic, head, spine depending on site of tumor. Rhabdomyosarcoma treatment differs according to age and stage of disease but involves surgery, chemotherapy with alkylating agents, irradiation in high doses to CNS if tumor infiltrates the nasal cavity, or to other primary tumor beds.

For any subtype, in severe cases, bone marrow transplantation (BMT) is added. BMT is either autologous (patient´s own cells) or allogeneic (donor cells) transplantation of stem cells from the bone marrow or the peripheral blood. The included CT agents used for pretreatment of BMT are Busulphan, Melphalan, Carmustine and prednisolone.

Some CCS are associated with an increased risk of late-effects on reproduction are listed in table 5.

Table 5.
Childhood cancer diagnoses related to risk of late-effects on reproduction. Adapted from Panel 2 by Wallace et al. (66)

Risk	Diagnose
Low risk	ALL (no RT to CNS or testis) Wilms tumour (RT abdomen) GCCT (no RT) Retinoblastoma CNS tumours- only surgery
Intermediate risk	AML Hepatoblastoma Osteosarcoma Ewing sarcoma CNS tumor RT > 24 Gy Hogdkin and non Hodgkin lymphoma
High risk	CNS RT doses > 30 Gy Patients with alkylating therapy TBI Pelvic or testicular RT Metastatic Ewing and soft- tissue sarcoma, CT conditiong for BMI

Part II

The male reproductive system

Testis

The testes constitute two compartments with different functions. The semineferous tubules with Sertoli and germ cells are responsible for sperm production, and the intestinal space with Leydig cells which account for 95 % of testosterone production (Nieschlag, Andrology; male reproductive health and dysfunction 2nd edition 2001, 454p).

The hypothalamic pituitary gonadal axis HPG

The synthesis of testosterone and spermatogenesis is regulated by gonadotropins, lutheinizing hormone (LH) and follicle- stimulating hormone (FSH) secreted from the anterior pituitary gland. (figure 3) The gonadotropins are stimulated by the pulsatile secretion of gonadotropin-releasing hormone (GnRH) every 60-90 minutes from hypothalamus and further stimulated by kisspeptin by it´s binding to the GPR54-receptor on the surface of the GnRH-neurons (67), (68).
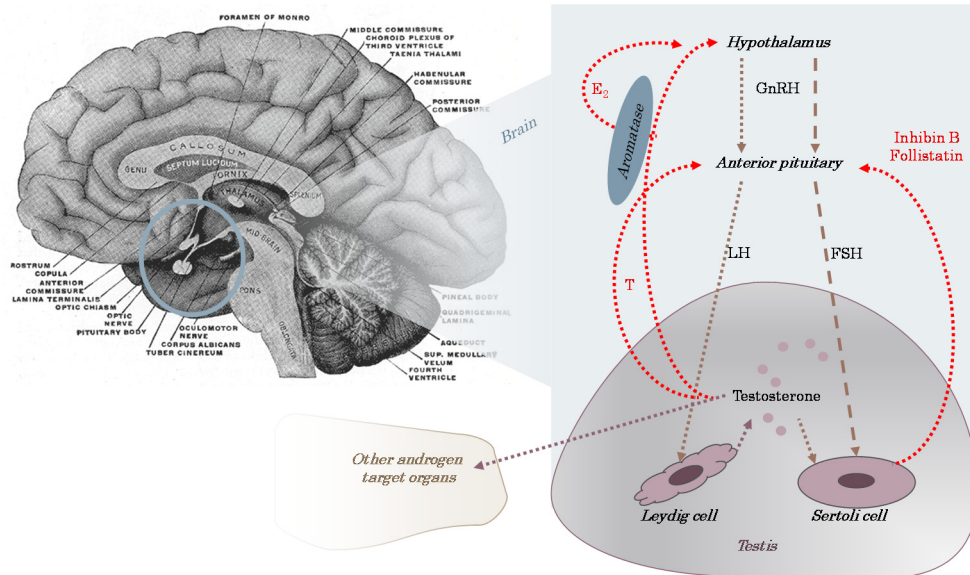
Figure 3.
HPG-axis The regulatory pathways of the hypothalamic pituitary gondal axis. Red dotted lines indicate negative feedback. GnRH=gonadotropin releasing hormone; Lh=luteinizing hormone FSH: follicule stimulating hormone; E2=oestradiol. Picture of brain by Henry Gray, Wikimedia commons. Illustration by Magdalena Bentar-Holgersson.

By negative feedback mechanisms, sex steroids (testosterone and oestradiol: E2) further affect the stimulation of kisspeptin to regulate HPG. LH stimulate steroid genesis in the Leydig cells whereas FSH promotes spermatogenesis. Inhibin B, a peptide produced by Sertoli cells, is dependent on the presence of primary spermatocytes and is under the control of FSH (69). Inhibin B is also involved by negative feedback mechanisms in the regulation of FSH secretion (69).

Control of spermatogenesis
The number of Sertoli cells increases the first 4 months of life due to a rise in gonadotropins and testosterone two weeks after birth, lasting for 6-8 months (70,71). The final number of Sertoli cells is reached at puberty upon start of spermatogenesis (72) determining the number of germ cells that can be supported, (72,73) and thus the capacity of spermatogenesis.

Androgen levels remain low until puberty onset. Pulsatile LH secretion has been observed prior to puberty (74) and spermatogenic bursts (75). The testosterone rise will, along with FSH, stimulate the start of the highly androgen-regulated process of spermatogenesis, which is dependent on high intra-testicular levels of testosterone (76). The importance of testosterone for spermatogenesis was demonstrated in Sertoli-cell specific androgen-receptor deficient mice, leading to spermatogenetic arrest. (77). The germ cells are dependent on the Sertoli cells in

expressing receptors for FSH and T (78) and supporting the maturation of pre-spermatogonia.

Spermatogenesis occurs in the semineferous tubulus, in close relation to the Sertoli cells. Spermatogonias are considered the testicular stem cells, formed as gonocytes differentiate (79). Spermatogonia typ A dark represent the stem cell pool and spermatogonia type A pale differentiates to spermatogonia typ B that initiates DNA synthesis resulting in tetraploidic primary spermatocytes. The primary spermatocytes undergo a first meiotic division, resulting in two secondary spermatocytes entering the second meiotic division, giving rise to four haploid spermatids. The following steps leading to a mature sperm last for 64 days, and the transport to the epididymis, an additional 14 days. (80) (81), (82).

The germ cells are among the most rapidly dividing cells, making them susceptible for oncological treatment. The spermatogenesis is ongoing from puberty and throughout life, with hundreds of millions of sperms produced daily (83).

Androgens

Testosterone is produced from cholesterol (figure) in a multiple step enzymatic process, and eventually converted to testosterone in the Leydig cells in the testis (95 %) or in the adrenal cortex (5%). Testosterone will diffuse into the blood stream from the intestinal compartment and reach distant target AR or act locally in Sertoli cells.
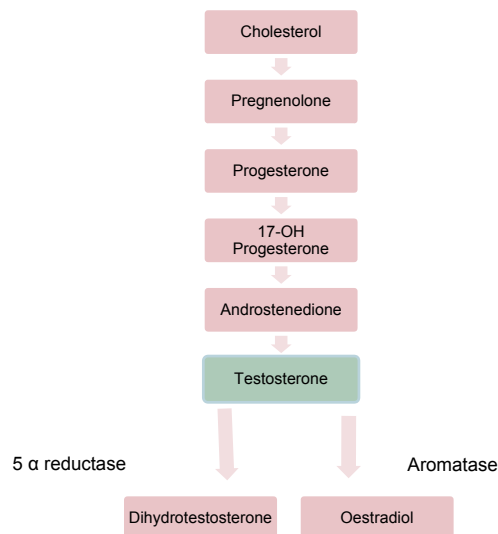

Figure 4
Steroidogenesis

The concentration of testosterone is a hundred times higher in the testicle as compared to plasma. In the plasma 98 % is bound; 40 % with high affinity to SHBG and therefore not considered bioavailable, and 58 % with lower binding affinity to albumin. The albumin bound testosterone is accessible, and together with the 2 % unbound plasma testosterone (84) constitutes the total bioavaliable testosterone.

The circadian variation in testosterone levels is due to diurnal variation in GnRH and perhaps initiated by sleep (85,86). The testosterone level decreases with food intake, due to effect of GLP-1(86) and a fasting morning sampling of testosterone is preferable for determination of testosterone concentrations (87). A normal age decline is proposed, by 1.6-2% every year from 50 years of age (88), which in part (89,90) is explained by an age related SHBG increase (91), (92). Smoking and sexual activity gives transient increased testosterone levels.

Testosterone is crucial for, besides spermatogenesis, the formation of male internal genitalia, muscle growth, elongation of the larynx, normal sexual function including erectile function and libido. The androgen action is however not exclusively performed by testosterone. The metabolite dihydrotestosterone (DHT), spliced by α-reductase, exerts important androgen action by higher androgen receptor affinity and more potent response in selective tissues. DHT is crucial for normal sex differentiation in foetal life, and in puberty for developing male phenotype compromising hair follicle and skin growth, formation of external male genitalia and development and action of the prostate gland (93).

Moreover, several other non-reproductive functions are dependent on androgen action, i.e. bone mineralization (94) and cognitive function (95). Androgen receptor genes are present in the cardiac myocytes (96) modulating the cardiac response to stress. This explains the immediate increase in cardiac contractility capacity of myocytes, and thus the benefit of testosterone for physical performance improvement in athletes (97).

Androgens exert effects through genomic (Described in next chapter) and non-genomic pathways. Knowledge of the latter is scarse, but an example of the indirect effect of androgens is the testosterone-mediated improvement of coronary flow (98) due to vasodilatory effects of testosterone, mediated by calcium channels. Effects of androgen deficiency, with focus on cardio-metabolic parameters, are emphazised in the next section.

Testosterone deficiency / Hypogonadism

For diagnosis of testosterone deficiency (hypogonadism) in a clinical setting, decreased levels of testosterone in two repeated blood samples is required in addition to presence of clinical features (99). According to the Endocrine Society, hypogonadism is a clinical syndrome characterized by androgen deficiency and impaired sperm production due to disruption in the HPG axis (99). International Society for the Study of the Aging Male (ISSAM) on the other hand, describes the syndrome with decreased levels of testosterone and presence of characteristic symptoms, but does not include the impaired spermatogenesis (100), in line with the present criteria in Sweden.

The prevalence of hypogonadism in the general population is uncertain. However, from different observations the estimated variation is 6 to 35 % of male population (101). Normal levels of testosterone are considered 10.4-34.7 nm/L, with likelihood of symptoms with levels below 10.4 nm/L (99,102,103).

The symptoms of testosterone deficiency are non-specific and highly individual. Loss of muscle mass, increased body fat, fatigue, decreased libido, erectile dysfunction and depression are often described, but may be more vague.

Primary gonadal failure may cause primary hypogonadism, characterized by high LH and low testosterone levels in plasma. Possible initiators are genetic variants such as Klinefelters (47 XXY), cryptorchidism, chemotherapy, radiotherapy, trauma, mump orchitis and orchiectomy (104).

If the disruption is on the hypothalamic level, secondary hypogonadism (105) with low levels of LH/ FSH and decreased testosterone may occur. Pituitary neoplasm, hyperprolactinoma, hemocromatoses, infiltrative disorders, anabolic steroids, genetic disorders of GnRH–secretion, idiopatic hypogonadotropic hypogonadism (Kallmans syndrome) and radio/ chemotherapy are possible explanations (104). In addition, an impaired sensitivity to androgens, e.g by androgen receptor polymorphism may result in hypogonadism (106,107).

Androgen insensitivity syndrome (AIS) is due to AR mutations characterized by hypogonadal symptoms, ranging from complete AIS without virilisation, to less evident symptoms despite testosterone and LH levels in normal to marginally elevated range (108,109).

Previous studies indicate an association of hypogonadism with risk factors of cardiovascular disease and the metabolic syndrome (110-113). Moreover, it was argued that cancer survivors have higher prevalence of hypogonadism (114). The knowledge of a link between hypogonadism and the development of late-term effects in male cancer survivors was scarce at study start.

The androgen receptor

Testosterone and dihydrotestosterone target the same receptor, the androgen receptor (AR). The AR is an intra-cellular and nuclear receptor, known to recognize small hydrophobic ligands such as endogenous hormones, vitamins and endocrine disruptors. It is activated by the binding of DHT or testosterone in the cytoplasm, intiating a process in which a heat shock protein is spliced from the AR that dimerizes and phosphorylates into an active transcriptional regulatory-complex, that binds to the androgen response elements (ARE) in the DNA. By this binding, up- or downregulation of androgen dependent genes will occur in the formation of androgen response proteins. (Figure 5).

Figure 5
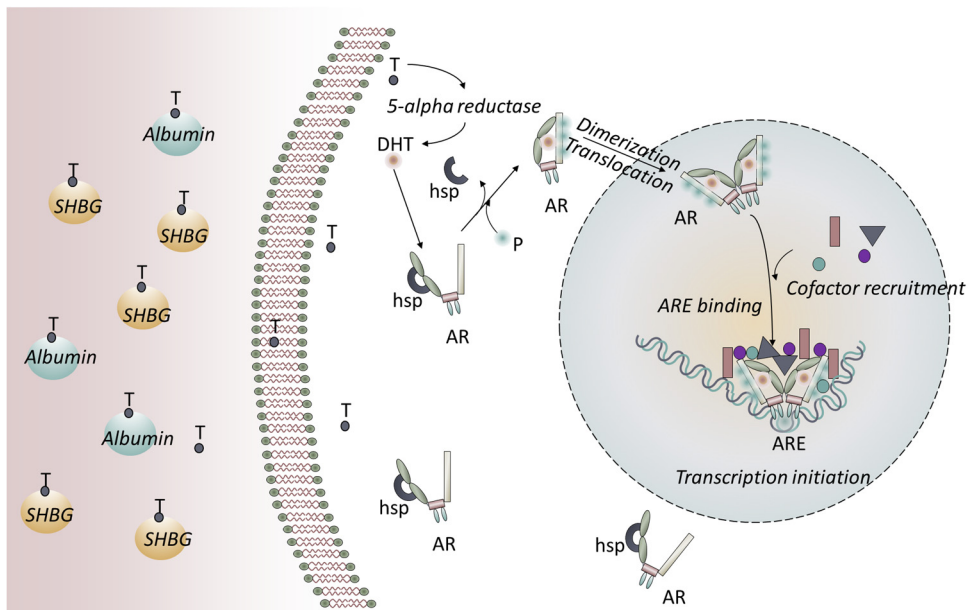Androgen activation of androgen receptor.Adjusted picture:from Magdalena Bentmar.Holgersson.

The AR gene is located on the X-chromosome. (Figure 6), thus only one copy exist in males, inherited from the mother. As for other steroid hormone receptors, it constitutes different functional units; the trans-activating domain in exon 1, (115,116), a DNA- binding part in exon 2 and 3, and a hormone- binding unit in exon 4-8.
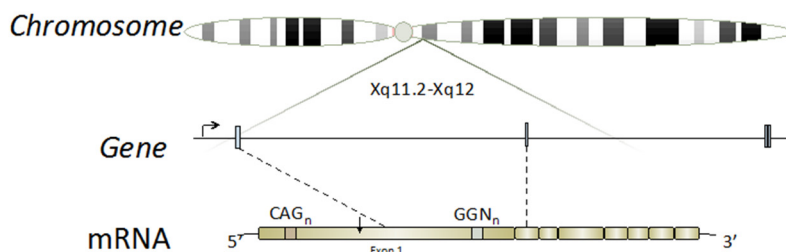
Figure 6
AR gene. Adjusted from a picture by Magdalena Bentmar- Holgersson.

The transactivating domain contains two important polymorphic trinucleotide repeats of polyglutamine and polyglycine tracts, encoded by varying numbers (n). The (CAG) n stretch, the CAG repeat, is followed by a GAA sequence and the GGN-repeat with the genetic code; (GGT)3GGG(GGT)2(GGC)n.

The number of CAG and GGN repeats varies within populations and is dependent on ethnicity (117), (118). CAG-repeats are normally distributed between 10-30, and for Caucasians (32,119) a median of 22 repeats is estimated (119). AR CAG repeats 22-23 are considered the most efficient, confirmed in, *in vitro* and *in vivo* studies by Nenonen (120,121). African American males present with fewer AR CAG repeats and male of Asian origin constitute longer CAG tracts (117).

GGN alleles are normally distributed from 10-27 (117), but GGN 23-24 is by far the most common, covering 80% of Swedish men (122,123). Numerous studies have pointed at associations between CAG and GGN repeat length and androgen receptor outcome. A strict model explaining this phenomena is not revealed, but it is suggested that CAG-repeat length is crucial for the transcriptional activity in the AR (124), (12). Moreover, a feasible effect on receptor stability and fine-tuning of androgen sensibility is proposed.

Clinical implications
A well-known clinical illustration of expanded CAG repeats length is Kennedy's disease, or spino-bulbar muscle atrophy (SBMA). The patients with 40 or more CAG repeats will acquire neurotoxic adverse effects causing muscle spasms, tremor, increased risk of diabetes mellitus, testosterone deficiency, decreased sperm parameters and testis-atrophy (125). Symptoms are positively related to expanding CAG numbers.

Repeated studies implied that long CAG repeat lengths are linked to decreased sperm concentration and subfertility (126), 200, (12),(127). The infertility risk was

20 % higher in males with a less active AR, CAG <22 or >23. Another study emphasizing the possible influence of AR CAG repeat lengths on fertility, was conducted by Wallerand (128). The AR CAG repeat lengths differed between infertile and fertile couples, with a median CAG-repeat length of 23.9 in infertile men vs. 22.2 in healthy men. Hence, there is an equivocality regarding the impact of CAG repeat on fertility. Several European studies have failed to show an association between AR CAG-repeat length and fertility,(119),(129),(130) while numerous Asian-conducted studies verified such an association (12), (131). The ethnical diversity regarding CAG expansion might explain these contradictions.

Fewer studies have been performed for GGN repeats,. *In vitro* results indicated that the most active receptor, (GGN 23), is less associated with hypospadias and cryptorchidism (132). Results by Brokken et al (133) showed increased levels of inhibin-B, a marker of spermatogenesis for GGN <23. In a study by Castro-Nallar (134) long GGN repeats > 24 was linked to an AR associated to cryptorchidism and spermatogenic failure. Earlier studies pointed at a genetic stability for AR GGN 23-24 and less likelihood for impact on reproduction (123,135).

Previous studies have demonstrated that different CAG repeat lengths may play an important part in the onset of testicular cancer. This is further implied as men of African origin have fewer CAG-repeats, and a lower incidence of testicular cancer, than do Caucasian men. Therefore it is suggested that long CAG-repeat regions could reduce the receptor's transactivation capacity (136).

Infertility

The definition by WHO is failure to conceive during 12 months with unprotected intercourse. It has been proposed that 50 % of cases of infertility are due to the male factor, but in 50 % of cases the main cause of infertility is not determined (137). Male infertility is investigated by testicular volume, semen analysis (ejaculate volume, sperm concentration, motility and morphology assessed from WHO (138) and hormonal evaluation (FSH, LH, SHBG and Inhibin B). Sperm chromatin strand breaks is often evaluated, since a high proportion of strand breaks is linked to infertility (139). It is suggested that primary hypogonadism and testicular diseases are responsible for 30-40 % of male infertility.

Assisted fertility
For children, no established methods for fertility preservation exist beyond cryopreservation of spermatozoa. The limitations are often extensive. To deliver semen samples is demanding for peri- or post pubertal boys, and further intriguing while facing a cancer diagnose. In addition, there is often need for an immediate start of therapy.

Epididymal aspiration and testes harvesting or biopsy during anesthesia might be an option, although not yet a standard procedure (73). Semen samples of adequate quality from adolescents with an immature spermatogenesis and cancer is challenging to achieve (140).

For assisted reproduction as an adult, IVF (*in vitro* fertilization) is the most frequently used method. In the case of low sperm numbers or increased DNA fragmentation index (DFI), intra-cytoplasmic sperm injection (ICSI) is preferable (141), (142).

Late puberty can be treated with oestrogen or testosterone therapy, with careful attention to height and growth, as sex steroids prematurely fuse growth plates, not compromising adult height. Thus, hormone replacement therapy should aim to mimic normal pubertal progression.

Part III

Late-term effects of Oncological treatment

In male childhood and testicular cancer survivors, morbidity and mortality are increased due to secondary cancers and late-term complications. The excess rate of deaths from childhood cancers in standard mortality rate (SMR), is 19.4 (143) and due to cardiac causes, 8.2 (SMR), but many more suffer chronic health deficiency. Among all childhood cancer survivors (CCS), two thirds have at least one side effect and for one third of them, the complications are life-threatening (144) in part, due to therapeutic exposure of surgery, chemo- or radiotherapy. The developing and growing tissues in children and adolescents are particularly sensitive to radiation therapy. The damage is dose dependent (145), and influenced by radiation type, daily fraction, cumulative radiation dose and age at time for treatment. The target organ or tissue constitutes varying resilience to RT and risk of late-term complications. The first symptoms may debute as an immediate response to treatment, or arise decades later (hormone deficiency and metabolic disorders) (146).

Among the high cumulative incidence of chronic health deficiency, risk factors of cardiovascular disease and subfertility are major concerns. Anticipating the potential risk is challenging. Many patients receive treatment combinations, and the response varies extensively possibly due to inter-individual dissimilarities, however not entirely elucidated.

More attention is given the influence of androgen deficiency, genetic susceptibility and polymorphic changes in the androgen receptor (147). There is need for prognostifying factors for family planning and for risk-adapted follow up of these young men with long expected life- time.

Oncological effect on reproduction

The ability to father children is an important quality-of-life (QOL) issue for children with cancer and their parents. Despite vast knowledge of treatment effects with respect to fertility, we lack explanations for the inter-individual differences.

Spermatogenesis is affected in male cancer survivors due to effect on rapidly dividing germ cells with elevated risk of oligoo- and azoospermia, transiently or permanently (148) (147). In studies on young patients with Hodgkin lymphoma, 70 % had impaired semen quality (149), before treatment start. Young TC patients had increased risk of infertility prior start of treatment (22,150). Orchiectomy *per se* in TC patients, is associated with elevated risk of azoospermia in 10 % of patients (151) and RPLND caused retrograde ejaculation in majority of patients when the old technique (152),(153) was applied. With the modern, nerve-sparing procedure, this risk is diminished (154).

Effect of treatment on reproduction

Radiotherapy
It is well known that chemo- and radiotherapy have possible mutagenic effects on the semineferous epithelium or on the HPG axis (155), with effects on spermatogenesis.

Spermatogonias are more sensitive to irradiation than spermatocytes and spermatids (156) and will be damaged at low doses (< 0.1 Gy). Spermatocytes and spermatids are more robust to RT but are affected when slightly higher doses are given (157). Testicular irradiation doses of 1–3 Gy normally cause reversible azoospermia; at doses above 5–6 Gy, the effects are usually irreversible. This is based on results from a study on healthy prisoners in USA 1974, when RT to the testes in doses of 0.8 Gy caused transient azoospermia. Doses of 4-6 Gy gave elevated risk of permanent azoospermia (158). This was later confirmed by *in vitro* studies (159), comparing acute irradiation effects in juvenile primate testis at low doses, <0,1 Gy to 4 Gy. In line with results from Rowley´s *in vivo* studies, it was concluded that 4 Gy caused an acute depletion of non-differentiating spermatogonias.

RT to treat Germ Cell Cancer In Situ (GCNIS) is given in doses of 14-20 Gy causing permanent azoospermia (160). This is in line with results of RT to testis (in case of relapse of leukemia or as TBI before stem-cell transplantation) causing oligoo- or azoospermia (161),(162),(163).

Secondary hypogonadism due to RT to CNS in craniospinal doses of ≥ 24 Gy will cause gonadotrophin deficiency with subsequent spermatogenesis impairment (164).

Adjuvant RT to abdominal lymph nodes below the diaphragm, most often to lymphoma patients, might impair sperm production transiently by scattered irradiation to the remaining testicle.

Chemotherapy:

The rapidly proliferating cells, spermatogonias, are more sensitive to chemotherapy (CT) than the later stages of spermatogenesis (161), (165). It is well known that cisplatin and alkylating therapy are highly gonadotoxic, acting by the formation of covalent bonds and DNA breaks. The excellent effect of chemotherapy could be due to the effect on spermatogonial stem cells (166), (167). The childhood cancer chemotherapy regimens with potential risk of azoospermia in adulthood are shown in table 6.

Table 6
Chemotherapy drugs with cumulative thresholds with increased risk of impaired spermproduction (according to DeVita *et al, Cancer Principles and Practise of Oncology, 7th edition*).

Chemotherapy	Dos	Action
Carmustine	(1 g/ m^2)	Alkylating
Lomustine	(500 mg/ m^2).	Alkylating
Chloambucil	(1.4g/ m^2),	Alkylating
Cyclophosphamide	(19g/ m^2),	Alkylating
Cisplatin	(500 mg/ m^2),	Alkylating
Melphalan	$(140 \text{ mg/ m}^2$	Alkylating
Procarbazine	(4g/m^2).	Alkylating

Comparing MOPP (mustine, oncovin, procarbazine, prednisolone) and ABVD (adriamycin, bleomycin, vinblastine and dacarbazin), both used in treatment of Hodgkin lymphoma, gave temporary azoospermia in 97% of MOPP compared to 33% in ABVD patients (65).

Repeated studies have demonstrated impaired semen quality in chemotherapy treated TC patients compared to only orchiectomised patients (168) and 33% suffer from oligoo- and azoospermia (148). In 5% of patients a permanent azoospermia will occur, possibly corresponding to patients with disseminated disease given > 4 platinum based cycles, (169) indicating that sperm regeneration is dose dependent (170). Lampe (171) pointed at 80 % recovery of spermatogenesis 5 years after treatment. This was confirmed by Eberhard who in addition concluded that prolonged azoospermia is dose-dependent, and impaired by testosterone deficiency (147).

A late-onset recovery of spermatogenesis is occasionally seen. In a Danish study in 2005, 38 % of TC patients previously treated with high cisplatin doses (>860 mg/m^2) could father children without assisted fertilization after 10 years, and after 15 years to an even higher extent (48%) (150). This might be explained by a slow differentiation of spermatogonial stem cell pool surviving chemotherapy. Results from other studies, however not including patients receiving more than for cycles of BEP, confirm that a majority will regain their spermatogenesis after therapy (172). In mice this process took 3- 6 months before complete regeneration, which

correspond to duration of spermatogenesis. In humans this process is prolonged in some patients for unknown reasons.

It has been discussed whether pre-pubertal testis is less susceptible to oncological treatment. It was argued that the lower amounts of proliferating cells protected the gondal cells from damage (173). Several studies have, however, showed that gondals of pre-pubertal boys will be harmed by oncological treatment (161), (173), GnRh agonists or antagonists, was suggested to decrease testosterone levels and thereby inhibit possible maturation of spermatogonia. However, the HPG axis is quiet during pre-puberty and therefore, the effect of GnRh agonists uncertain (71). In a study by Berensztein (174), it was implied that some sperm maturation already existed in young boys, 1-6 years of age, with proliferation in 11 % of germ cells.

In predicting fertility post-treatment in post-pubertal adolescents, testis volume is a good marker, well corresponding to Sertoli cell function (162). In addition, repeated studies have concluded that FSH and Inhibin B are good predictors of spermatogenesis in order to estimate fertility potential in cancer survivors. (114,175). Predictors of fertility are however lacking for cancer patients before start of treatment. Due to the highly individual risk of subfertility or prolonged recovery of spermatogenesis, and considering the narrow window of fertility, genetics of the AR polymorphism need to be explored.

AR polymorphism
It is argued that 14% of male infertility has genetic causes (176). The importance of androgens for spermatogenesis is previously described. AR is an absolute requirement for complete spermatogenesis, and further maturation to spermatids is dependent on testosterone. Polymorphisms in the AR have impact on receptor outcome with influence on sperm generation after cancer treatment showed in results from pilot study (147). An inverse relationship between CAG repeat length and sperm concentration recovery after TC was detected, with the most prolonged receptor function for CAG > 23. The subjects in the study constituted only nine patients and conclusions were therefore limited. Results regarding the possible impact of GGN polymorphism on reproduction are scarce. A newly published study by (177), was the first to claim that a GGN expansion of > 24 was associated to impaired sperm concentration, but among young healthy men.

Due to prolonged or abundant sperm production recovery in young cancer survivors, fertility guidance is warranted. In general, young couples tend to postpone childbearing and need enhanced awareness of the increased risk of subfertility and the narrowed time-window (178). Cryopreservation is recommended to all postpubertal males, but additional predictive values for individual risk assessment are required. Due to this, the association between AR polymorphism and sperm production recovery was investigated in larger population of male cancer survivors..

Hypogonadism and late-term effects

A three to fourfold increased risk of hypogonadism in male cancer survivors is implied (179),(114),(101). The prevalence differs between studies, ranging from 6-54% (180), due to age, follow-up and treatment regimens. Repeated studies have detected LH-elevation in a large group of patients over time (168),(181). The severity of testosterone was demonstrated in a study in healthy men, for whom decreased testosterone levels were associated with all-cause death in men aged 21-49 years (182).

Local effect and hypogonadism
There is, for some tumor categories an increased risk of hypogonadism *per se,* due to local tumor infiltration. In TC patients there is a risk of persistant Leydig cell damage (183),(179). A CNS tumour may yield direct effect on the pituitary causing secondary hypogonadism.

Almost all CNS tumors in children will be operated. If close to hypothalamus, the third ventricle or the supra sella area as for craniopharyngeomas, the HPG- axis will be affected with subsequent multiple pituitary hormone failure. Hypogonadorophic hypogonadism will then further compromise delay of pubertal development (184).

A unilateral orchiectomy in TC, enhance the risk of Leydig cell insufficiency (179) and as a result; primary hypogonadism. Testosterone levels will decrease after unilateral orchiectomy but a time-related improvement may occur (185). Therefore, initiation of testosterone replacement therapy (TRT) should be postponed in awaiting normal recovery (151). A bilateral orchiectomy will subsequently lead to complete testosterone insufficiency.

Surgical removal of neuroblastomas can result in primary adrenal insufficiency when adrenal tissue cannot be preserved, but only 5 % of testosterone is produced in the adrenal gland and a decrease in testosterone levels is not expected.

Increased risk of hypogonadism with Radiotherapy
CNS radiation: If doses up to 18 Gy are given (total body irradation) there is no risk of secondary HPG effect. Growth hormone deficiency may occur, but not further discussed within this context. In ALL with CNS-involvement, 18-24 Gy is given with low risk of HPG effect. The risk increases when 36 Gy is given as for medulloblastoma, (186). However, radiation reaching 50 Gy increases the risk of permanent damage of hypothalamus and the HPG axis. In 10 % of Rhabdomyosarcomas the primary site is the head and neck area and doses between 40 and 50 Gy in combination with chemotherapy is part of the regimen. With doses exceeding 60 Gy (glial tumours and craniofaryngiomas), (187) seconadary

hypogonadism will arise. Delayed puberty is a consequence for 11 % of CCS with RT to the hypothalamic/ pituitary area (188).

Irradiation to gonds: Irradiation to gonds may cause primary hypogonadism. Leydig cells are more robust than Sertoli and germ cells (161) and may tolerate higher doses. Nevertheless, testosterone deficiency is increased in doses above 12 Gy to testes or TBI (7.5-15 Gy) (189), but with vast individual threshold disparity. Age at treatment and the risk of testosterone deficiency is not clearly associated but pre-pubertal boys should be under careful surveillance, not to compromise puberty (189).

Irradiation to other targets: (e.g. chest and abdomen) as for Hodgkin lymphoma patients when RT is administred to lymph nodes sub-diaphragmally complementary to chemotherapy (190). Gonds may be included in target, directly or by scattered irradiation with the risk of Leydig cell insufficiency.

Chemotherapy induced hypogonadism
Leydig cells are quite robust to CT, but studies are discordant with respect to threshold risk of testosterone deficiency after CT (189). Cisplatin-based CT was previously associated with testosterone deficiency over time, (181,191,192) but in the latest review by Skinner *et al* (189), a low risk of testosterone deficiency after CT is implied, including cyclophosphamide, busulfan or cyclophosphamide and fludarabine and melphalan, BMT conditioning, procarbazine and mechlorethamine, ifosphamaide and cisplatin.

Cardiovascular risk factors in cancer survivors

Male cancer survivors of both CCS and TCS are at elevated risk of cardiovascular disease (193,194). In the 1980s the first reports on acute CVD following cisplatin-based CT was published (195,196). Meinardi *et al.* (197) confirmed the results and argued a 7.1- times increased risk of cardiovascular events in a small group of CT treated-patients. The study lacked controls, but results were confirmed in a larger study by Huddart *et al.*, (198), in which a two-fold increase was found after RT and combination of CT and RT (199). Van den Belt Dusebout *et al* found a 1.5 times increased risk of cardiovascular disease after BEP in TC survivors. Haugnes *et al* (200) found this risk to be 5.7 times increased compared to patients with only surgery as treatment.

Acute cardiovascular and vascular events has been linked to CT treatment, (194) and Nuver *et al.* implied direct effect on blood vessels. This was confirmed in a study on long-term survivors of TC previously treated with CT (201) with evidence of endothelial injury and dysfunction. Wethal *et al*., (202) argued that this was due

to increased inflammatory markers. Treatment induced adverse effects include immediate damage or indirect disruption through other mechanisms. Cisplatin remains in the blood in an active form up to 20 years post-treatment causing damage throughout (203,204),(205).

Antracyclins is a common CT agent in CC regimens, associated with increased risk of CVD due to effect of free radicals on myocyte cells. The cumulative doses are crucial for risk of congestive heart failure, but the threshold is highly individual. It is indicated that 60 % of antracyclin-associated effects are multi factorial or when doses exceed 600 mg/m^2 (206,207). However, a cumulative dose of < 250 mg/m^2 was the previous cut-off value. In children, even lower cumulative doses, 101-150 mg/ m^2 may increase the cardiovascular risk (208). Unmistakably, thresholds are difficult to determine which should be taken into account at follow-up. Accordingly, following radiation therapy, markers of chronic inflammation and endothelial dysfunction increase (202) and contribute to an accelerated risk of atherosclerosis. In 5 % of Hodgkin lymphoma patients, symptomatic heart disease, including coronary artery disease, valvular disease, arrhythmia and pericardial disease were identified (209,210) following chest radiation/ mantle radiation therapy.

During the last decades, more focus is given cardiovascular risk factors following cancer treatment (211). An early detection and initiation of preventive measures might inhibit overt CVD.

Blood-lipids
Several studies have detected increased blood-lipids in cancer survivors. Elevated triglycerides were found among TC survivors (212) and confirmed in other studies along with total cholesterol and LDL (213). Meinardi *et al*. implied increased CVD to be associated to cisplatinum-based treatment. Features of dyslipidemia are closely related to CVD risk and important to diagnose.

Hypertension
Hypertension is a known risk- factor in TC survivors treated with cisplatin-based CT(197), Huddart observed a twofold elevated prevalence of hypertensive treatment in both RT- and CT-treated patients, but the results were not age adjusted (198). In another observation, a cumulative dose of cisplatin (>400 mg/ m^2) resulted in a 24 % prevalence of hypertension compared to 10 % in patients with doses below 400 mg/m^2 (211). The dose-related risk of hypertension was confirmed by Sagstuen *et al*. (214) but at a higher cut-off value for cisplatin (850 mg/ m^2).

Obesity
Obesity is a crucial link in a metabolic and cardiovascular cluster and a risk factor of other cardiovascular-associated dysfunctions (insulin resistance, hypertension and atherosclerosis). The visceral adipose tissue release excessive amounts of free

fatty acids contributing to insulin resistance (144). In TC survivors, cisplatin therapy is correlated to weight gain, but the mechanism not yet elucidated. The association between obesity and cranial CT is however well established (215,216). The risk increases for doses exceeding 24 Gy and for children below 5 years (215,217). Also, RT to CNS tumors including hypothalamus in the radiation field and for high doses (>51 Gy), a postive link to obesity is observed, but the mechanism needs to be elucidated.

Insulin, insulin resistance
Hyperinsulinemia has been identified as a single-factor associated to increased CVD (218). In a study by Bao *et al.*(219), a strong relationship between hyperinsulinemia and the development of CVD risk factors was observed during a eight year follow-up. Steinberger *et al.* (220) concluded that CCS are more insulin-resistant than their siblings prior entering adulthood. Previous studies in adults implied greater insulin resistance in CCS than in healthy controls, as expressed by HOMA-IR or fasting insulin(221). This was confirmed in a subgroup of CS previously given hematopoetic stem cell transplantation and pre-treated with high doses of CT. The risk was increased also for type-2 diabetes and triglycerides (222). Insulin resistance is not considered a disease *per se* in children but prior studies have indicated that low insulin sensitivity is a significant predictor of future increased CV risk (223).

Diabetes
The risk of diabetes mellitus type 2 is partly linked to radiotherapy to abdomen or pelvis with loss of pancreatic β-cell function. In one study, 11.2% of CCS had increased risk if the pancreatic tail was part of the radiation field Accordingly, patients with Wilms tumour who recieved radiotherapy to abdomen were at higher risk of diabetes mellitus type 2 (224).

Total Body Irradiation (TBI) increases the risk of diabetes mellitus typ 2 (225) by 7.2 times, although lower RT doses; 10-18 Gy, are given. However, in TBI, testes are exposed and the risk of testosterone deficiency increased, implying that the risk of DM is related to hypogonadism rather than loss of B-cells. Results showing an elevated prevalence of DM2 by 1.6 times in all CCS compared to the general population supports the theory that other mechanism are involved.

The Metabolic syndrome
The Metabolic syndrome constitutes a cluster of cardio vascular risk factors related to increased mortality, compromising hypertension, dyslipidemia, insulin resistance and abdominal adiposity (226). The definition of MetS is formulated by NCEM-ATP III (227). MetS is a vicious circle where insulin resistance is a cornerstone. The prevalence among cancer survivors expands beyond the prevalence of the general population, ranging from 13- 39 % (228-230). The first study to report increased risk of MetS in ALL patients was published by (231). Nuver *et al.* reported

later a higher risk of MetS in cisplatin treated patients with 36 % prevalence compared to healthy controls. Willemse *et al*, (230) found the prevalence of MetS to be 2.2-fold higher 7.8 years after treatment in TC-survivors treated with CT compared with age-matched healthy subjects.

Observations by De Haas *et al.* in TCS concluded a prevalence of 25 % with MetS and an age-related increase (232). In patients > 40 years, 35% was diagnosed with MetS. Hoffman *et al*. (233) detected increased prevalence of MetS in Ewing sarcoma survivors. In contrast, the risk was increased in males < 40 years of age. Taskinen *et al*. (234) reported a 34% prevalence of MetS in BMT patients, probably due to extensive CT treatment prior to transplantation. The high prevalence in cancer survivors at an earlier age in comparison with normal population and independent of CV risk factors *per se*, might imply that MetS represent the connection between cancer treatment and the long-term CVD risk increased in male cancer survivors. Nevertheless, more knowledge is needed to clarify the mechanism behind.

Increased risk of cardiovascular disease in hypogonadal men

Male gender is considered a risk factor for CVD at an earlier age and with higher CVD mortality than females. A possible link to sex hormones was further raised as women at menopause, approach the similar cardiovascular risk as men (235). An association between testosterone deficiency and increased cardiovascular mortality was detected in multiple studies in older men (236).

Androgen deprived men with prostate cancer, have an increased risk of diabetes mellitus (237). In addition, testosterone had beneficial effects on glycemic status, due to a decrease in adipose tissue (238). In numerous studies later performed, more focus was given androgen deficiency and associations to cardiovascular risk factors. In repeated studies, decreased testosterone has been linked to obesity (239), e.g Klinefelter men, who display higher fat mass and a more central fat distribution, similar with that of the female phenotype. In obese men, excessive adipose tissue increases aromatization and exerts effect on the HPG-axis, and oestrogens act synergistically and conversely to testosterone and have negative feed back on GnRH.

Other evidence for an association between testosterone deprivation and cardiovascular risk factors are the results of testosterone replacement therapy (TRT), with beneficial effects in the majority of patients. In a meta-analysis on hypogonadal men, TRT induced decrease of total cholesterol, LDL and HDL (240). In another report, TRT improved the symptoms of angina pectoris in men with coronary heart disease (241). Further, in repeated studies in healthy men, replacement of testosterone reduced fat mass and central obesity (242) (238) (243).

The effect of TRT was confirmed *in vitro* of castrated mice, in which insulin sensitivity were induced but reversed by TRT (244).

In 2004, Laaksonen *et al*. published results on hypogonadal men with increased risk of MetS (245), later confirmed in numerous studies (246),(247). Kaplan *et al*. revealed an inverse relationship between testosterone level and components of MetS.

The results were confirmed to some extent in studies on cancer survivors, De Haas *et al* (232) correlated low testosterone levels to a four-fold increased risk of MetS at a cut-off level of 15 nmol/ L in comparison with the general population. Accordingly, results from Nuver *et al* (228) showed an association for severe MetS and decreased testosterone levels. Haugnes *et al*. revealed a link between low testosterone levels and MetS in TC- survivors, but MetS criteria was not strictly based on NCEP-ATPIII, and thus difficult to compare (229). Willemse *et al*. confirmed an increased risk of MetS in TC survivors, but included patients with anti-hypertensive, statins and anti-diabetics, possibly influencing the results(230).

It can be argued that hypogonadism is a central feature of MetS and that testosterone treatment is of beneficial impact in slowing the progression to CVD.

Some studies have, however pointed to testosterone replacement as risk factor for CVD. This was based on high mortality associated to testosterone therapy in studies of older men (248) with a high burden of CVD and the adverse effects of anabolic steroids (249,250). Few studies are performed on younger healthy men in randomized trials and within larger settings.

Cardiovascular risk factors and AR polymorphism and association to hypogonadism
In searching for inter-individual cardiovascular and hypogonadal risk factors, genetic variation is explored. The knowledge of the modulating effect of the androgen receptor polymorphism on metabolic and cardiovascular effects in male cancer survivors is still scarce. The most common CAG repeat lenghts of the androgen receptor, 22-23, is associated with the most active receptor. Since expanding numbers of CAG repeats are linked to a slower receptor-response, it was assumed to be associated with an elevated risk-profile.

The results from different studies are however diverging regarding most vulnerable AR phenotype and the association to testosterone deficiency and cardiovascular risk factors. Longer CAG repeat length was associated to risk of obesity and MetS in a study by Stanworth (251) and to increased LDL and lower HDL-levels (107). Longer AR was associated to MetS, HDL, HbA1c in Korean men, (252) in line with assumptions that long CAG repeats are less efficient and more vulnerable. Expanded AR CAG tracts were associated with increased hypertension and BMI in a study by Haring *et al*.

In a study on aging men, long AR CAG repeat tracts were associated with higher testosterone levels. However, level of oestrogen was also elevated, which make interpretation difficult. The testosterone increase could be explained by oestrogen increase rather than AR polymorphism (253).

Short AR CAG repeat length responded better to TRT (254) in terms of decreased decreased central fat adiposity and increasing HDL. A large study in men aged 20–79 years, showed that subjects with CAG repeat length lower than 22 had decreased levels of BMI, glycaemia, SBP and hypertension (255,256) also in favor for beneficial effects of CAG tracts.

However in some studies, short AR CAG-repeats were associated to increased prevalence of cardiovascular risk factors, e.g in adolescent boys where low CAG repeat numbers increased the risk for intra-abdominal fat accumulation However, after puberty, the effects disappeared, possibly overruled by a strongly developing hypothalamic-pituitary-gonadal axis.

Hence, the reports are diverging although most studies indicate an increased CV risk with longer AR CAG repeat lengths, thus being less sensitive to testosterone action. The knowledge is scarce and the situation more complex in cancer survivors. The need for a profound understanding of the background to the elevated risk of cardiovascular morbidity in male cancer survivors should include studies on the impact of testosterone deficiency, effect of treatment and AR polymorphism on the risk of cardiovascular disease.

Aims of thesis:

The overall aim of this thesis was to investigate the association between biochemical and genetic indicators of androgen action and post-cancer recovery of spermatogenesis as well as occurrence of cardiovascular risk factors. Specifically, I wanted to:

- Estimate the impact of AR polymorphism on sperm concentration recovery in testicular cancer survivors

- Investigate the association between hypogonadism and type of treatment and risk factors of cardiovascular disease in testicular and childhood cancer survivors compared to controls

- To evaluate the effect of interaction between hypogonadism and AR polymorphism in relation to cardiovascular risk factors

Material and Method

Subjects:

Papers I-IV are based on TCS and male CCS. For paper II and III, age-matched controls were included.

Paper I:
TCS were selected from a larger cohort on reproductive function 2001-2011 (147), all of Caucasian origin and treated for testicular cancer at Lund University hospital, Lund, Sweden or Karolinska University Hospital, Stockholm, Sweden. Inclusion prerequisite was a diagnosis of TGCC and treatment with radiotherapy and/or chemotherapy postorchiectomy. In addition, a baseline semen sample, before initiation of treatment (T0) for adjustment of sperm number at T0, a minimum of one follow- up semen sample and a DNA sample were required.

151 TCS were eligible for the study and DNA was collected from 130 of them and subsequently the number included (Participation rate 86 %).

Patients were given cancer treatment according to SWENOTECA cancer care program (257),(4) 58 patients were diagnosed with non-seminoma and 72 with seminoma. According to treatment, patients were divided in groups for estimation of treatment effect on sperm concentration recovery:

- Adjuvant chemotherapy (ACT): 1-2 cycles of platinum-based CT to patients with clinical stadium I (CSI), n= 54

- Adjuvant radiotherapy (ART): to paraaortic- and ipsilateral iliac lymph nodes, 25.2 Gy in 16 fractions to seminoma patients CSI, n=39

- High dose chemotherapy (HCT): 3-4 cycles of chemotherapy to patients with metastatic disease, n=30

- Very high dose chemotherapy (VHCT): more than 4 cycles of platinum-based chemotherapy +/- Ifosfamide +/- radiotherapy to patients with relapsing disease, n=7

There was no difference in age or stage of disease between included and excluded patients.

Table 7. Characteristics of TCS in treatment groups, paper I.
Sperm count for treatment groups at different time points. Adjuvant chemotherapy (ACT), Standard chemotherapy (SCT), High dose chemotherapy (HCT), Very high dose chemotherapy (VHCT).

Time Points	T0 (x10^6) N=130	T6 (x10^6) N=72	T12 (x10^6) N=81	T24 (x10^6) N=84	T36 (x10^6) N=90	T60 (X10^6) N=78
			Treatment group Mean age (years)			
ACT 31.7	27.7	33.6	28.9	28.8	39.1	30.9
HCT 30.7	23.4	0.2	4.7	18.5	23.4	34.5
ART 36.3	30.6	2.6	18.7	27.9	43.3	43.5
VHCT 29.6	21.4	0/0	0/0	0/0	15.2	18.5

Paper II:

TCS previously included in the study on reproductive function and study I (paper I), treated at Lund university hospital, Lund, between March 1996 and October 2006, constituted 165 eligible TCS aged 18- 65 years. Three TCS were deceased and one had immigrated. 161 were invited and 96 accepted inclusion (60 %), 2 dropped out of the study and 2 were excluded due to diagnose of prostate cancer and prolactinoma, leaving 92 TCS included. One TCS was bilaterally orchiectomised and another TCS treated with RT to the remaining testicle due to GCNIS. Three TCS had extra gondal disease.

Table 8:
Background characteristics Paper II

Parameter	Patients N=92	Controls N=92
Age at inclusion (y) median	40	41
Age at diagnose (y) median	31	Na
Follow up time (y) mean (SD)	9.2	Na
BMI (kg/m2) median (range)	27	26
Hypogonadism n (%)	17(18)	32 (35)
Smoking (%)	8.7	23
Insulin, metformin treatment, n (%)	1 (1)	1 (1)
Lipid loweringTreatment, n (%)	3 (3)	3 (3)
HypertensionTreatment, n (%)	2 (2)	4 (4)
Testosterone Treatment, n (%)	9 (8)	0(0)
MetS	14	9

Na= not applicable

TCS were divided into the following treatment groups

1. SO= surgery only, n= 13,

2. ACT= adjuvant chemotherapy; 1-2 cycles of cisplatin- based CT or carboplatin, n= 28

3. SCT= standard dose chemotherapy in metastatic disease; 3-4 cycles of cisplatin- based CT, n= 22

4. ART= adjuvant radiation therapy to para-aortic and ipsilateral iliac lymph nodes, 25.2 Gy, in 14 fractions to all but one patient who had 24 Gy/16 fractions. n= 23

5. HCT= high dose CT +/- RT to testis, n= 6, 4 = 8 cycles of CT n= 1 got 9 cycles of CT. One patient received RT to CNS, 1 TCS had one cycle of BEP, another was given RT to GCIS and one TCS received RT to para-aortic lymph nodes.

For evaluation of selection bias between included and non-included TCS, the distribution of TCS having 0, 1, 2 or 3/ more children were 12, 37, 47 and 4% and among non- participants; 14, 36, 33 and 17%.

Paper III.
Through the Swedish Cancer Registry we identified male CCS previously diagnosed in Southern Sweden, before 18 years of age, with an extra-cranial malignant neoplasm or any central nervous system (CNS) neoplasm and being at least 18 years of age in December 2009. Further, treatment was completed at least three years earlier.

Among 427 male CCS, 11 men were deceased, 10 could not be located and one patient with testicular cancer participated in study II. Of the 405 eligible men invited by letter, 146 accepted inclusion (36%). Six men dropped out of the study and the following were excluded; one patient with optic glioma (only surveillance), six patients because of non-malignant disease (carcinoid of the appendix) and eight patients with a second malignancy or relapse within 3 years of inclusion, leaving 125 included.

Table 9
Background characteristics, paper III

Parameter	Patients N=125	Controls N=125
Age at inclusion (y) median	33.7	34.4
Age at diagnosis (y) median	9.6	Na
Follow up time (y) mean (SD)	24.3 (7.1)	Na
BMI (kg/m2) median (range)	25.1	24.7
Hypogonadismn (%)	31 (26%)	17 (14%)
Smoking n (%)	10 (9.2%)	15 (12%)
Insulin Treatment, n (%)	0 (0%)	(0.8%)
Metformin/Antidiabetics, n (%)	0 (0%)	1 (0.8%)
Lipid lowering Treatment, n (%)	4 (3.2%)	3 (2.4%)
Hypertension Treatment, n (%)	8 (6.4%)	2 (1.7%)
Testosterone Treatment, n (%)	13 (10.4%)	0 (0%)

Na= not applicable

Cancer treatment categories

The CCS were categorized into therapy groups as described in table 10. In figure 7 the pie chart visualize patients in diagnosis groups.

Table 10
Patient categorization, Paper III

Treatment group	N	Comment
1. Surgery	20	2 CCS treated with unilateral orchiectomy as the only treatment
2. Brain surgery	15	Only surgery
3. CT including alkylating agent, CED[a] < 4000 mg/m^2 Median CED [a]: 4855 mg/m^2	6	No RT given.
4. CT including alkylating agents (CED[a] >4000mg/m^2): Median CED was 9229 mg/m^2.	10	No RT, in 3 CCS surgery other than brain surgery was given
5. CT without alkylating agents	13	No RT given
6. Radiotherapy (RT) to CNS:	12	Included a patient with chondrosarcoma near the sella turcica) no CT, excluding 2 hypophysectomised cases. All cases received brain surgery.
7. RT to CNS +CT	16	Among 2 patients with tumours of the epipharynx, due to infiltration of the scull base and target of irradiation that was partly intracranial
8. RT to testes and CNS:	5	No chemotherapy
9. RT to other organs than item 6-8	5	In n=4 cases combined with surgery other than brain surgery.
10. RT to other organs than item (6-8) + CT	23	Combination of CT and RT

[a]= median Cyclophosphamide Equivalent Dose; CED: (a unit for quantifying alkylating agent exposure independent of study population)
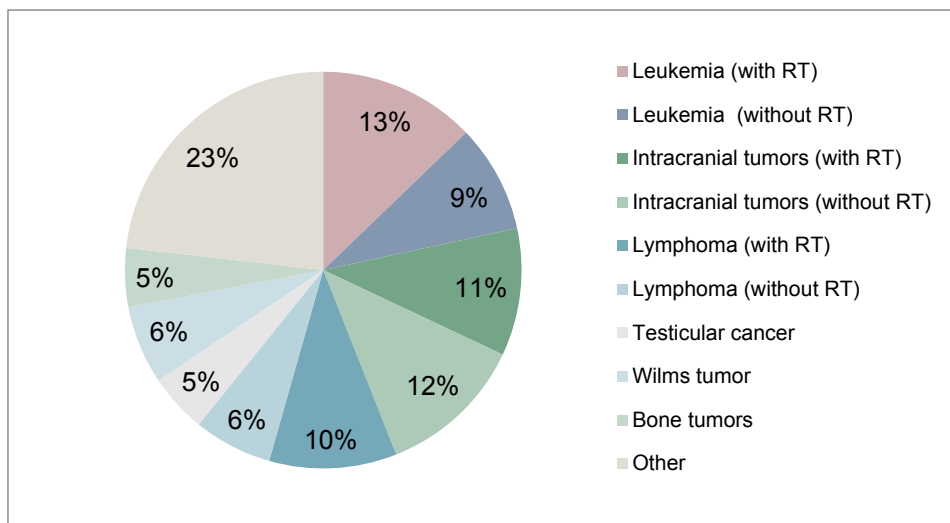
Figure 7
Pie chart for distribution of patients according to diagnosis.

Possible selection bias related to reproductive function was evaluated. Data on number of biological children for participants and non-participants was extracted from the Swedish Multi-Generation Register. The distribution of CCS having 0, 1, 2 or ≥3 children was 52%, 14%, 29% and 5.3% among participants, and 65%, 14%, 17% and 5.1% among non-participants.

Paper IV
The fourth paper is based on CCS and TC previously participating in study II or III, for whom a DNA sample was collected for analyses of androgen receptor CAG and GGN repeat lengths. The inclusion criteria was; for CCS: minimum of 18 years of age, diagnosed with extra-cranial malignant neoplasms or any central nervous system (CNS) neoplasm before 18 years of age, more than 3 years since termination of treatment. For TCS: minimum of 18 years, diagnosed with testicular germ cell cancer and at least 3 years since termination of treatment.

For TCS: unilateral orchiectomy was standard treatment for all but one who had a bilateral orchidectomy.

Of the 125 CCS and 92 TCS included in former studies, 52 CCS and 71 TCS had a DNA sample for analysis of GGN and CAG repeats. A flow chart for the inclusion of patients in study II-IV.(figure 8).

To exclude the risk of selection bias, comparisons between CS with and without DNA (participants/ non participants), were performed, for age, +/- hypogonadism

and for endpoints included in this study. Table 5 for background characteristics and included/ non-included CS.
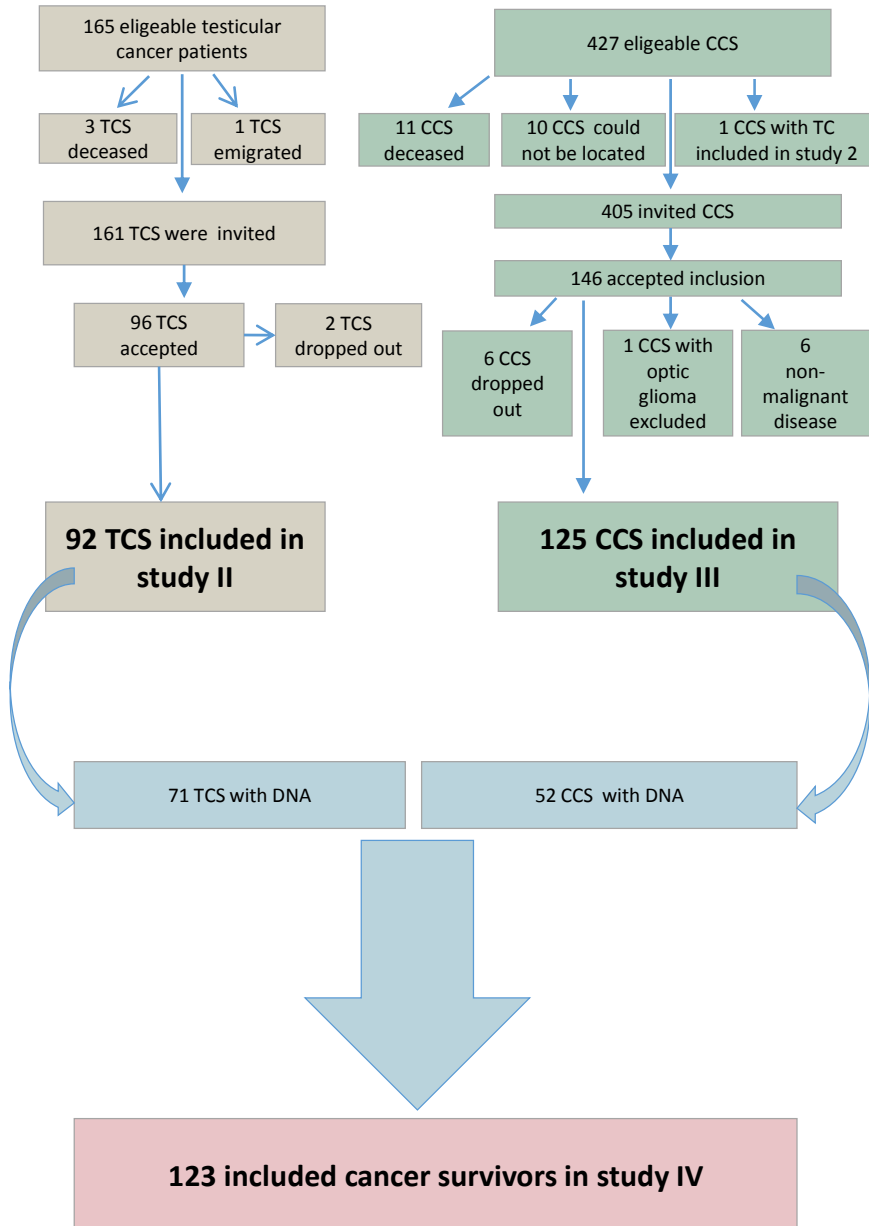

Figure 8.
Demonstrating a flowchart of patient inclusion paper II-IV:

Table 11.
Characteristics of included vs non-included cancer survivors

Parameter Mean values	Included CCS /Non-included CCS	Included TCS/ Non-included TCS
Age at inclusion (y)	34.3/ 35.5	41/ 39.7
Age at diagnosis (y)	9.6 / 11	31/ 31
Waist measure (cm)	91.6 / 91.1	96.4 / 92
HbA1c (mmol/ mol)	34 / 35	36/ 39
HOMA ir	1.4/ 1.7	1.8/ 2
Hypogonadal (%)	21/ 26	31/ 36

Controls

In paper II and III a control, matched by date of birth, living in the nearby area, through the Swedish Population Register for each patient was included. Exclusion criteria for controls were; (i) a previous diagnosis of a tumour in the CNS or malignant disease other than basal cell carcinoma, (ii) Klinefelters syndrome. Of 389 controls invited, 295 did not answer. One dropped out, one was excluded because of RT of a giant cell tumour. Background characteristics for controls in study II and III respectively are visualized in table 8 and 9..

Methods

Study design

Paper I was a cross-sectional study in which semen analysis for estimation of sperm concentration and total sperm number, were performed after orchiectomy (T=0) and compared with 6, 12, 24, 36 and 60 months after completion of therapy (T6, T12, T24, T36, T60). The samples were obtained after 2-7 days of abstinence. Every patient could contribute with one to six samples in addition to T0, due to different time from diagnosis and inclusion in the study. A blood sample for DNA extraction and androgen receptor GGN and CAG repeat lengths was collected.

Paper II was a prospective case-control study with cross-sectional design, were 92 TCS was compared with 92 age-matched controls from the vicinity. A questionnaire was filled out with the study investigator as considers medications and smoking. Weight, height and waist- measure was performed. Blood pressure in arms and legs was measured, the latter for estimation of ankle brachial index (ABI). A fasting blood between 8 and 10 am for the assessment of total testosterone (T), LH, SHBG,

insulin, glucose, HbA1c, cholesterol, TG, LDL and HDL was drawn. HOMAIr for insulin resistance was estimated.

Paper III was of the same design as paper II, but for 125 CCS and 125 controls.

In paper IV was not a case-control study, but otherwise the design was similar as study II and III. We merged the TCS and CCS from study II and III and included them for whom a DNA sample was collected. The total number of patients included was 123. The same DNA categorization for GGN and CAG repeats as for paper I was performed.

Semen analysis (Paper I)

The ejaculates were analysed according to the WHO (2010) (138) manual in which sperm motility, morphology, concentrations and volume were determined. 102/130 semen analyses were either analyzed in the Reproductive Medicine Centre (RMC) Skåne University, Malmö, or for patients recruited in Stockholm, in the Andrology Unit Karolinska Hospital, Solna. Of the remaining 29 samples, 28 semen samples were analysed in the fertility laboratory, Lund University Hospital, Lund, collected at cryopreservation before initiation of treatment. Only one sample was analyzed in Linköping University Hospital, Linköping. All involved laboratories participated in European Society of Human Reproduction and Embryology- Nordic Association for Andrology (ESHRE-NAFA) external quality control program (258).

DNA analysis (Paper I and IV)
The CAG and GGN repeat lengths were analyzed in DNA extracted from peripheral leukocytes. To determine the *GGN*-repeat length, genomic DNA from peripheral leukocytes was extricated and then amplified in a 50 μl PCR containing roughly 10 ng DNA, 0.5 μmol/l of each of the primers: F-CGGTTCTGGGTCACCCTCA, and R-TCACCATGCCGC*CAG*GGTA (Invitrogen), 1.5 mmol/l MgCl$_2$, 200 μmol/l of dATP, dCTP and dTTP each, 100 μmol/l dGTP and 7-deaza-dGTP (Roche) respectively, 45 mmol/l KCl, 10 mmol/l Tris–HCl (pH 8.4 at 70 °C), 0.1% Tween 20 and 0.5 units of Dynazyme DNA polymerase (Finnzymes Oy, Espoo, Finland). Amplification was completed for 35 cycles in an Eppendorf Mastercycler (Eppendorf; Hamburg, Germany). For all cycles, denaturation at 96 °C for 45 s, primer annealing at 61 °C for 45 s and primer extension at 72 °C for 1 min, with an initial denaturation step at 96 °C for 3 min and a final extension step at 72 °C for 5 min.

The *CAG* repeat was amplified in a 50 μl PCR containing around 10 ng DNA, 0.3 μmol/l of the primers: F-TTAGGGCTGGGAAGGGTCTA, and R-TGGGGCCTCTACGATGGGCT, 1.5 mmol/l MgCl$_2$, 200 μmol/l dNTPs, 45 mmol/l KCl, 10 mmol/l Tris–HCl (pH 8.4 at 70 °C) and 0.5 units of Dynazyme

DNA polymerase. As for GGN, the amplification was carried out for 35 cycles. Then followed, denaturation at 96 °C for 1 min, primer annealing at 61 °C for 45 s and primer extension at 72 °C for 2 min, with an initial denaturation step at 96 °C for 3 min and a final extension step at 72 °C for 5 min.

PCR-products were purified, directly sequenced with the reverse primers from the PCRs, precipitated, resuspended, and run externally on an eight-capillary Beckman Coulter CEQ 2000XL (Beckman Coulter; Bromma, Sweden) sequencing gear**.**

Definitions

- **Hypogonadism** was defined as T≤ 10 nmol/ L and or LH ≥ 10 IU/ L (259), or on-going testosterone replacement therapy (TRT). Compensated hypogonadism was defined as, no TRT, T ≥10 nmol/ L and LH ≥10 IU/L.
- **ABI**; ankle-brakial index; the ratio of systolic BP in arms and divided by systolic blood pressure in the legs.
- **Insulin resistance** was estimated using the HOMAir (260) calculated as ((I_f x G_f)/22.5), I= insulin, G= glucose, $_f$= fasting.
- **Total sperm number** was calculated by multiplying the concentration and the semen volume. The latter was determined from weighing the ejaculate.

The criteria for the metabolic syndrome is visualised in table 12, according to NCEP-ATPIII.

Table 12.
NCEP criteria for the metabolic syndrome

Metabolic syndrome (MetS) NCEP[x] , three or more critera for MetS	
Blood pressure, (mmHg)	≥130 mmHg systolic and/ or diastolic < 85 mmHg
Waist measure, (cm)	≥102 cm
High density lipoprotein (HDL)(mmol/ L)	≤ 1.03 mmol/ L
Fasting glucose, (mmol/mol)	≥ 5.6 mmol/ L
Triglycerides (mmol/ L)	≥ 1.7 mmol/ L

[x] The National Cholesterol Education Program (NCEP) Adult Treatment PanelIII (ATPIII); Huang, 2009

Antropoemetric parameters (paper II; II and IV)

Measurements of weight (electronic scale to the nearest 0.1 kg), height (stadiometer - to the nearest 1 mm) and waist circumference were performed (anthropometric tape- to the nearest 1 mm). In a supine lying position brachial blood pressure (Omron Healthcare Ltd Co, Lake Forest, IL, USA), in left and right arm,

respectively, and ankle blood- pressure (continuous wave Doppler) measurements were undertaken and a median of three assessments was used.

Questionnaires (paper II, III and IV)

To obtain best possible accuracy of answers, all study participants filled out a questionnaire regarding smoking habits and medication (anti-hypertensive, anti-diabetic, androgen replacement therapy and/ or lipid modifying treatment) with study investigator.

Biochemical analyses (paper II, III and IV)

All analyses were performed at the Department of Clinical Chemistry, Skåne University Hospital, Malmö, Sweden. A fasting venous blood sample, drawn between 8 and 10 a.m. was used for the assessment of serum levels of total testosterone (TT), sex hormone-binding globulin (SHBG), luteinizing hormone (LH), cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL), triglycerides (TG), glucose, insulin and HbA1c.

Testosterone were assessed by a competitive immunoassay with a luminometric technique (Access: Beckman- Coulter, Chaska, MN, USA; lower detection level: o.o87, CV: 7% at 3 nmol/ LCV 4% at 15 nmol/L.
LH was determined in a two-step immunometric assay with luminometric technique (Access: Beckman- Coulter; lower detection level; 0.10 IU/ L, CV 3% at 5 IU/ L, CV: 2 % at 37 IU/ L).

Sex hormone-binding globulin (SHBG): was analysed with solid-phase two-site chemiluminescent immunometric assay (Immunolite 2000; Siemens healthcare, Camberley, UK; lower detection level: 0.35, CV 3 % at 25 nmol/ L, 3% at 53 nmol/ L.

In May 2012, method of analyses changed to a one- step immunometric sandwich assay with ElectroChemiLuminiscenceImmunoassay (ECLI) detection technique (Cobas; Roche, Basel, Schwitzerland) for SHBG and LH, and to a two- step competition assay with ECLI detection technique for testosterone (Cobas, Roche, Basel, Switzerland). Serum values were reanalysed with the new method from frozen sera in 124 men, in which we had carried out hormonal analyses with previous methods. An internal validation comparing both methods then followed. A linear curve of best fit we obtained factors for mathematical conversion of old values to approximations of the new (261).

Blood lipids; were determined from standard enzymatic methods.

LDL; lower detection level; 0.1mmol/ L, total CV<1% at 2.6 mmol/ L, 1.5% at 3.9 mmol/ L).

Cholesterol; lower detection level 0.1 o.1.2 at 2.6 mmol/ L, 1.0 % at 6.6 mmol/ L

HDL; lower detection level; 0.08, CV:1.3 % at 0.8, CV1.7% at 1.7 mmol/ L.

TG; lower detection level; 0.1, CV: 2.1 % at 1.0, CV 3.3 % at 2.1 mmol/ L

Glucose was assessed with an automated hexokinase method, lower detection level 0.11, CV; 2.1 % at 1.0 mmol/ L, CV: 3.3 % at 2.1 mmol/ L.

Insulin levels in serum were measured with immunometric sandwich assay (Access Ultrasensitive Insulin, Beckman Coulter, 2005 387+67C, Brea, CA, USA). Lower detection level: 0.2, CV 3.2 % at 1.0 mmol/ L, CV: 4.1 at 45.8 mmol/ L).

HbA1c **(IFCC)** were assessed with the VARIANT TURBO Hemoglobin A1c Kit 2.0 program. (VARIANT TURBO; Hercules, CA; USA) using cat ion exchange and gradient elution. Lower detection level; 15 CV1.8 % at 35 mmol/ mol, 1.6 % at 80 mmol/ L).

Statistical considerations:

The statistical methods used in different papers are summarized below with more details. SPSS (IBM SPSS Statistics Chicago, IL; USA) was used and p-value < 0.05 was defined as statistically significant.

Paper I. A unilateral regression model was used for analyses of associations between CAG and GGN numbers and sperm concentration at all six time points (T0, T6, T12, T 24, T36 an T60). All sperm concentrations and total sperm number were adjusted for age, sperm concentrations at T0 and treatment. Nonadjusted analyses were performed if statistically significant associations were found.

The CAG and GGN lengths were primarily trichotomized (<22 CAG, 22-23 CAG and >23 CAG; 23 GGN, 23 GGN, >23 GGN) according to results indicating that GGN 23 (122) and CAG 22-23 (120) are the most active receptors.

The CAG and GGN length tracts linked to the less active AR were then merged and compared with CAG 22-23 and GGN 23 respectively. (CAG 22-23 as reference vs. <22 or >23 CAG), GGN 23 set as reference vs. < 23 GGN and >23 GGN). Sperm concentrations and sperm count were transformed using the natural logarithm (Ln) for normal distribution of residuals. 1 was added to all sperm concentrations/ sperm numbers due to some 0 values (azoospermic cases) before Ln transformation. Group characteristics were expressed as means and standard errors (s.e). Furthermore, back-transformed (95 % confidence intervals of mean differences) were performed.

Paper II and III:

For the comparison between patients and controls, considering mean values of diastolic and systolic blood pressure, ABI, If, Gf, HOMAir, HbA1c, cholesterol, LDL, HDL, TG and waist measure, unilateral regression analyses were performed.

Participants with hypertensive treatment were excluded when calculating means for BP and ABI (ABI was only calculated for TCS and controls). In paper II: n= 2 patients. n= 4 controls, in paper III: n= 8, n= 2 controls.

Patients and controls with lipid lowering treatment were excluded when analysing blood lipids. In paper II; n= 3 patients, n= 3 controls, in paper III: n=4 patients and n=2 controls. When excluding patients from the calculations, corresponding controls was also excluded.

To estimate the impact of hypogonadism on metabolic and cardiovascular risk factors, the same comparisons as above were made for hypogonadal patients compared to eugonadal and controls.

To evaluate the association of given treatment, the above comparisons were made between the five treatment groups; for TCS; (SCT, ACT, ART, HCT and SO) and the ten treatment groups for CCS; (see table for all treatment groups) vs. controls. Statistically significant associations were adjusted for +/- hypogonadism, to evaluate if androgen deficiency is part of the pathogenetic link between treatment type and metabolic and cardiovascular parameters.

OR for MetS were calculated using logistic regression analysis. All TCS/ CCS and hypogonadal patients were compared with controls. Patients were divided in the following hypogonadal subgroups: compensated (LH >10 nmol/ L, T>10 nmol/ L), uncompensated (T<10 nmol/ L, LH/>10nmol/L) or testosterone replacement therapy).

OR for MetS was calculated for the groups with controls and eugonadal TCS, respectively, as reference. All calculations were adjusted for age and smoking.

Paper IV

For each of the two repetitive sequences, CAG and GGN, three categories according to the number of repeats (CAG: <22; 22-23; >23; GGN: <23; 23; >23) were defined.

Since similar associations between hypogonadism and the risk of MetS were found in both TC and CC patients, the two groups were merged.

From study II and III, we identified HbA1c, HOMAir, waist measure, glucose, insulin, triglycerides, cholesterol, HDL and BMI to be associated with hypogonadism in young cancer survivors. For each of those parameters, with and without adjustments for smoking and age, using linear regression, analyses with either CAG or GGN category * +/- hypogonadism as interaction parameter, were

performed. CAG/GGN category and +/- hypogonadism were included as co-variates.

For statistically significant interactions, 2 (+/-hypogonadism) x 3 (CAG/GGN category) tables were made to visualize interaction type between genotype and +/-hypogonadism. Thereafter, for the phenotype (+ or – hypogonadism) for which the 2 x 3 tables indicated modulating effect of genotype, linear regression analyses, comparing the three genotype groups within each type of repeat (CAG or GGN) was performed. These calculations were completed without and with adjustment for age at inclusion and smoking. At last, an interaction analysis for MetS using logistic binary regression was accomplished.

Results

The impact of Androgen Receptor polymorphism on male reproduction- (Paper I)

The AR GGN- and CAG-repeats lengths were normally distributed in the 130 included testicular cancer survivors. For CAG; 15-28 (figure 9.), median=21, and for GGN; 10-29, median GGN= 23 (figure 10).
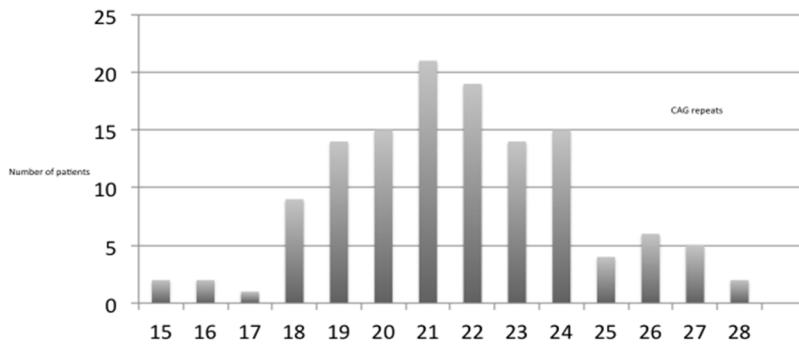
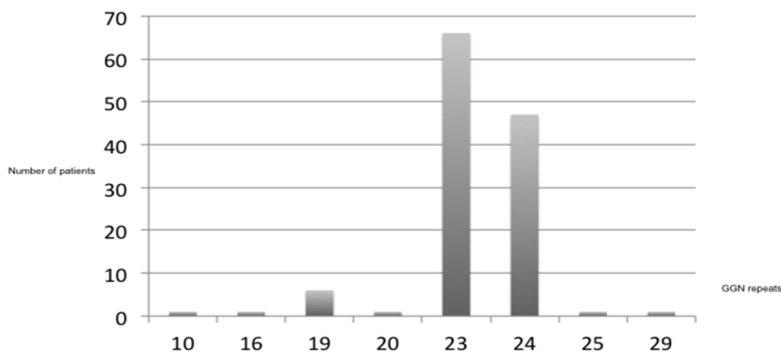**Figure 9.**
CAG repeat lengths in a graph bar

Figure 10.
GGN-repeat lengths in a graph bar

At baseline (T0) before initiation of treatment, there were no statistically significant differences in sperm concentration or total sperm number between the CAG groups (<22 CAG, 22-23 CAG and >23 CAG), (p =0.16 and p =0.14).

After one and 2,5 years, respectively, men with 22-23 CAG exhibited lower sperm concentrations than the other CAG groups, but it was statistically significant only after one year. (95 % CI for ratio; 1.01; 2.65, p= 0.045). The association was robust when we adjusted for age, treatment type and sperm concentration at baseline (95% CI 1.13; 4.9, p= 0.02). (Table 13).

Table 13
The effect of CAG repeat number on total sperm number in relation to follow up.

Time point (month)						
CAG repeats (x10⁶)	0	6	12	24	36	60
CAG 22-23 mean (s.e.)	65(17)	210(64)	41 (21)	67 (22)	76 (29)	130 (44)
CAG <22 mean (s.e.)	76 (13)	48 (42)	64 (16)	80 (15)	89 (20)	120 (32)
CAG >23 mean (s.e)	88 (18)	50 (62)	60 (22)	65 (20)	120(27)	120 (32)
95 % CI of CAG <22 or CAG >23[b]/ CAG 22-23	0.9-2.9	0.5-6.4	1.2-6.6	1.0-5.8	0.6-3.6	0.4-2.7
P	0.1	0.4	0.02	0.1	0.4	1.0

[a]Values adjusted for age, treatment and sperm concentration at time point 6-60 months,[b]Due to back transformation from logarithm, the figures represent 95% CI for the ratio between total sperm number in the CAG groups "<22 and >23" and that in the reference group .

CAG <22 and CAG >23 had higher sperm concentrations than CAG 22-23 and statistically significantly higher in comparison with CAG <22 (p=0.04) but not for CAG>23 (p = 0.18). (Table 13).
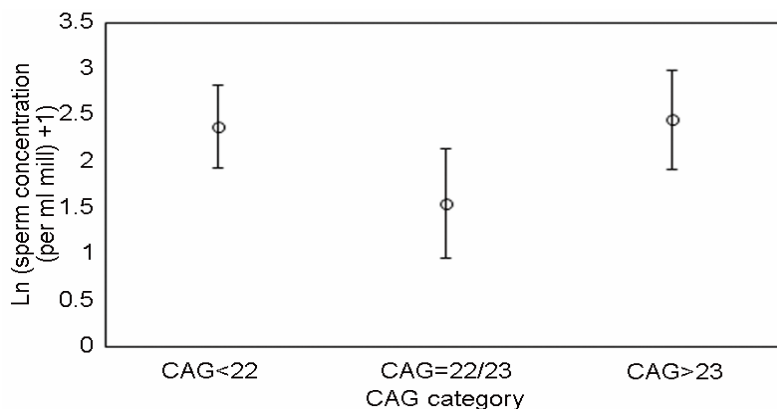

Figure. 11.
Error bar for the androgen receptor CAG repeat number in the trichotomized groups at T_{12} after logarithm-transformation after adding 1 to all concentrations. Ln= logarithm. Adapted from Bogefors et al, Andrology 2017.

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Results from this study indicate that CAG repeat number could influence the sperm concentration recovery after testicular cancer. Significant differences were seen for patients treated with 3-4 cycles of chemotherapy and only 12 months after treatment in line with previous studies (147). The AR receptor with CAG 22-23, considered the most active, had slower recovery than CAG<22 and CAG >23. CAG >23 and CAG repeat<22 had higher sperm concentrations than CAG 22-23, but only CAG<22 differed significantly.

Increased prevalence of cardiovascular risk factors in male cancer survivors (Paper II, III and IV)

- Impact of hypogonadism, treatment type and AR polymorphism

Metabolic parameters and lipids

Insulin and HOMAir
Insulin levels were borderline significantly increased in both TCS and CCS, as compared to controls. In addition, for childhood cancer survivors vs. controls, insulin resistance (HOMAir) was significantly increased (Table 14).

Impact of hypogonadism: In hypogonadal vs. eugonadal TCS, a statistically significantly higher insulin and HOMAir was detected. This was also the case for uncompensated hypogonadal (U-HG) CCS and for those with testosterone replacement therapy (TRT), in comparison with controls. (Table 14.)

With impact of treatment: In TCS, adjuvant chemotherapy (CT) and 3-4 cycles of chemotherapy was associated with statistically significant increased insulin levels, and the latter was robust for adjustments to hypogonadism. Adjuvant radiotherapy in testicular cancer survivors was also linked to significantly elevated HOMAir. Among CCS, radiotherapy to CNS was the single treatment associated to increased insulin and HOMAir. Neither this comparison nor adjuvant radiotherapy in TCS vs. controls, remained statistically significant after adjustment for hypogonadism (Table 15).

Impact of AR polymorphism:

HOMAir was borderline statistically significantly increased in interaction analysis for GGN >23, however linear regression analysis showed no statistically significant differences between GGN categories. (Table17).

Glucose and HbA1c
HbA1c was statistically significantly higher in the whole childhood cancer group compared to controls. (Table.14)

Impact of hypogonadism: Uncompensated hypogonadal CCS and those treated with TRT had increased levels of HbA1c. No significant associations were seen for hypogonadal testicular cancer survivors. (Table 14).

Impact of treatment: In both TCS- and CCS, radiation therapy was the treatment type most prone to be associated to increased HbA1c, and above all, radiotherapy to CNS. However, radiotherapy to testes and CNS and adjuvant radiotherapy in testicular cancer survivors was also significantly increased and robust for adjustment to hypogonadism. **RT** to other organs (than CNS and testes) and to CNS in combination with chemotherapy, increased the levels of glucose and was robust when adjusting for hypogonadism. (Table 15 and 16).

Impact of AR polymorphism:

Interaction analyses for the association between GGN categories +/- hypogonadism found statistically significant associations for glucose being robust when adjusted for smoking and age at inclusion. The highest value was seen in the hypogonadal group with GGN=23. Comparisons between GGN>23 and GGN 23 showed a mean difference of 0. 43 mmol/L (95% CI: 0.023 mmol/L; 0.81 mmol/L) and the mean difference in hypogonadal GGN=23 vs. GGN<23 not being statistically significant. (Table 17)

Table 14. Comparisons of metabolic values, TCS vs. controls, hypogonadal TCS vs. eugonadal patients and CCS vs. controls.

Mean difference (95% CI). EG= eugonadal, HG=hypogonadal. TCS= testicular cancer survivors, CCS= childhood cancer survivors. U-HG= uncompensated hypogonadal, TRT-HG= Testosterone treated hypogonadal,

	HbA1c (mmol/mol)	Insulin (mmol/L)	HOMAir	Glucose (mmol/L)	HDL (mmol/L)	LDL mmol/L	TG mmol/L	Cholesterol (mmol/L)
TCS	1.41 (0.30; 2.5)	1.28 (-2.6; 0.036)	0.22 (0.58; 0.13)	0.042 (-0.19; 0.10)	0.05 (-0.04; 0.14)	0.09 (0.017; 0.36)	0.14 (-0.38; 0.09)	0.08 (-0.19; 0.35)
CCS	1.45 (0.51; 2.38)	1.23 (-0.008; 2.47)	0.32 (0.08; 0.64)	0.08 (-0.05; 0.20)	0.06 (-0.13; 0.04)	0.02 (-0.25; 0.21)	0.66 (-0.98; 1.22)	0.04 (0.23; 0.26)
TCS-HG	1.56 (-0.07; 3.29)	3 (1.21; 5.0)	0.79 (0.23; 1.36)	0.16 (-0.74; 0.34)	0.12 (-0.25; 0.006)	0 (-0.41; 0.41)	0.45 (0.062; 0.84)	0.067 (-0.2; 0.31)
CCS-U-HG	2.1 (0.13; 4.1,)	3.2 (0.84; 5.6)	0.78 (0.18; 1.4)	0.16 (-0.4; 0.1)	0.09 (-0.09; 0.26)	0.05 (-0.56; 0.46)	0.28 (-0.6; 0.08)	0.12 (-0.63; 0.39)
CCS TRT-HG	2.5 (0.07; 4.4)	9.0 (6.3; 11.6,)	2.3 (1.7; 3.0)	0.2 (-0.5, 0.06)	0.25 (0.06; 0.44)	0.3 (-0.67; 2.7)	0.48 (0.05; 0.8).	0.24 (-2.8; 0.93)

Table 15 Comparisons of metabolic values, for TCS in treatment groups vs controls.

Mean difference (95% CI). SO= surgery only, ACT= adjuvant chemotherapy, SCT= standard chemotherapy, HCT= high dose chemotherapy, ART= adjuvant radiotherapy

Treatment	HbA1c (mmol/mol)	Insulin (mmol/L)	HOMAir	Glucose (mmol/L)	HDL (mmol/L)	LDL mmol/L	TG mmol/L	Cholesterol (mmol/L)
SO	0.065 (-2.46; 2.33)	0.59 (-2.25; 3.42)	0.21 (-0.77; 1.19)	0.064 (-0.4; 0.19)	0.004 (-0.16; 0.17)	0.028 (0.55; 0.50)	0.19 (-0.25; 0.63)	0.10 (-43; 0.64)
ART	3.99 (2.07; 5.92)	1.96 (-4.18; 0.25)	0.74 (0.014; 1.46)	0.23 (-0.56; 0.09)	0.09 (-0.05; 0.22)	0.15 (-0.28; 0.58)	0.25 (-0.60; 0.11)	0.09 (8-0.35; 0.53)
ACT	0.25 (-2.0; 1.51)	2.64 (0.64; 4.63)	0.49 (-1.21; 0.22)	0.10 (-0.4; 0.19)	0.10 (-0.03; 0.22)	0.02 (-0.39; 0.43)	0.34 (0.004; 0.67)	0.02 (-0.42; 0.39)
SCT	1.3 (-3.17; 0.57)	0.50 (-2.66; 1.67)	0.06 (-0.67; 0.80)	0.10 (-0.4; 0.19)	0.05 (-0.19; 0.09)	0.12 (-0.31; 0.55)	0.19 (-0.17; 0.54)	0.14 (-0.30; 0.57)
HCT	3.9 (0.37; 7.13)	4.38 (0.59; 8.2)	1.13 (-2.35; 0.08)	0.24 (-0.8; 0.32)	0.20; (-0.36; 0.43)	0.21 (-0.56; 0.97)	0.9 (80.29; 1.5)	0.04 (-0.70; 0.79)

Table 16. Significant interactions for childhood cancer patients in treatment groups vs. controls.
Mean difference (95% CI)

Treatment	Metabolic or antropoemetric values
RT CNS	HbA1c; 2.5mmol/ L (0.05; 4.6), HOMAir; 1.1(0.035; 1.8), Insulin 4.1 mmol/ L (0.6; 7.0)
RT other	Distolic BP; 4.8 mmHg(0.55, 9.0), ABI; 15.4 (0.47; 30.2), Glucose: 0.66 mmol/ L (0.13; 1.19)
RT CNS and testes	HbA1c: 4.7 mmol/ mol(1.5; 7.9)[c]
CT >4000 CED	HbA1c: 2.4 mmol/ mol (0.007; 4.7)
RT CNS and CT	Glucose: 0.37 mmol/ L (0.1, 0.6)
RT other and CT	HbA1c: 1.8 mmol/ mol (0.01; 3.6), TG: 0.5 mmol/ L(0.20; 0.83)

Lipids

Impact hypogonadism: Hypogonadal childhood cancer survivors with TRT had increased levels of HDL and triglycerides. (Table 14).

Impact of treatment: CCS with RT to other organs and chemotherapy, and testicular cancer survivors with both adjuvant and high- dose chemotherapy, had increased triglycerides compared to controls and were robust for adjustment to hypogonadism. (Table 15 and 16).

Impact of AR polymorphism:

Interaction analyses for the association between GGN categories +/- hypogonadism and metabolic parameters were performed and a statistically significant association for cholesterol was found, being robust when adjusted for smoking and age at inclusion. (Table 17). The most elevated values were detected in the hypogonadal groups with GGN>23. Linear regression analysis showed that CS in hypogonadal GGN>23 had statistically significant increased values compared to GGN <23; mean difference (md): 1.4 mmol/L (95% CI 0.3 mmol/L; 2.3 mmol/L) and GGN 23 (md: 0.8 mmol/L, (95% CI; 0.06mmol/L; 1.6 mmol/L). (Table 18).

Table 17.
Interaction analysis for GGN categories x+/- hypogonadism i relation to different metabolic and antropometric end points

Parameter	GGN x hypogonadism Non-adjusted	GGN x hypogonadism Non-adjusted
HbA1c (mmol/ mol)	P= 0.63	P=0.72
HOMAir	**P=0.05**	**P=0.06**
TG, (mmol/ L)	P= 0.76	P=0.80
Glucose (mmol/ L)	**P= 0.014**	**P=0.009**
Insulin, (mmol/ L)	P= 0.51	P= 0.23
BMI, (kg/ m^2)	P=0.23	P=0.23
Waist, (cm)	P=0.43	P=0.51
Cholesterol mmol/ L	**P=0.002**	**P=0.003**
MetS	P=0.93	P=0.99

Table 18.

Parameter	GGN<23 Mean (sd)	GGN 23 Mean (sd)	GGN >23 Mean (sd)
HOMAir +/ - hypogonadism	1.6 (0.5)/ 2.0 (1.1) n=6/5	1.5 (0.9)/ 1.9 (1.4) n=40/16	1.2(0.7)/ 2.7 (1.9) n=32/ 8
Glucose(mmol/ L) +/- hypogonadism	5.6(0.5)/ 9.9 (3.2) n=7/5	5.3(0.4)/ 12.0 (4.9) n=40/19	5.1(0.5)/ 9.9 (3.8) n=35/10
Cholesterol mmol/ L -/+ hypogonadism	5.7 (0.9)/ 4.5 (0.6) n=7/ 6	5.0 (0.8)/ 5.0 (0.9) n=39/17	5.0(1.0)/ 5.9 (1.0) n=36/ 10

Anthropometric parameters and blood pressure

In comparisons between all testicular cancer patients and controls, a higher waist measure was identified. (Table 19)

Impact of hypogonadism: Waist measure was increased in both hypogonadal testicular- and childhood cancer survivors (uncompensated and TRT) compared to controls. In addition, for childhoodcancer survivors with TRT, an increased diastolic BP was detected. (Table 19)

Impact of treatment: CCS with radiotherapy to other organs (not CNS/ testes); had a statistically significantly higher systolic and diastolic BP. None of the associations were however robust when adjusted for hypogonadism. Testicular cancer patients with adjuvant radiation therapy had increased waist measure, but did not remain significant when adjusted for hypogonadism. (Table 20 and 16).

No significant mean differences were found for patients treated with surgery only. (CCS treated with surgery other than brain surgery and brain surgery).

Table 19
Anthropometric values in comparison between TCS and CCS vs. controls and hypogonadal TCS vs. eugonadal TCS and hypogonadal CCS vs. controls. Mean difference (95% CI). Na= not applicable.

| Treatment group	Waist (cm)	DBP (mmHg)	SBP (mmHg)	ABI
TCS	4.06 (1.08; 7.03)	0.48 (-3.1; 2.2)	0.97 (-2.8; -4.7)	0.046 (0.011 0.082)
CCS	2.60 (5.42; 214)	0.03 (-2.3; 2.4)	-4.6 (-8.5; 0.65)	Na
TCS-HG	5.8 (0.57; 11)	2.6 (-6.7; 1.4)	4.93 (-11.3; 1.47)	0.026 (-0.4; 0.09)
CCS-U-HG	8.5 (2.8; 14.2)	1.3 (-6.12; 3.5)	3.1 (-11; 8.9)	Na
CCS TRT-HG	12.1 (5.9; 18.3)	5.8(-10.9; 0.08)	8.5 (-0.63; 17.6)	Na

Table 20.
Anthropometric values in comparison between TCS in treatment groups vs. controls. Mean difference (95% CI).

Treatment group	Waist (cm)	DBP (mmHg)	SBP (mmHg)	ABI
SO	3.37 (-9.3; 2.59)	0.53 (-5.8; 4.7)	4.42 (-3.1; 11.9)	0.09 (0.015; 0.16)
ACT	6.06 (1.58; 10.5)	1.09 (-5.01; 2.83)	0.22 (-5.39; 5.83)	-0.07 (0.12; 0.13)[a]
ART	5.7 (0.88; 10.5)	3.46 (-7.7; 0.8)	2.38 (-8.5; 3.7)	0.006; (-0.06; 0.05)
SCT	0.52 (-5.27; 4.24)	2.08 (-2.08; 6.24)	2.64 (-3.31; 8.59)	0.03 (-0.09; 0.05)
HCT	5.4 (-13.7; 2.93)	0.63 (-6.67; 7.93)	1.37 (-9.07; 11.8)	-0.035 (-0.14; 0.07)

The Metabolic syndrome in testicular cancer survivors

MetS was diagnosed in 9.8 % of controls and in 15.2 % of TCS. However, OR for MetS compared to controls, was not statistically significantly increased (p=0.6).

The amount of hypogonadal TCS with MetS was further increased to 36 % and in comparison with controls, the OR was 4.4 (95 % CI; 1.4; 14 p= 0.01). In comparisons with eugonadal TCS, the difference was even higher; OR 15 (95 % CI; 2.8; 84, p=0.02). For the subgroup of uncompensated hypogonadal TCS, a statistically significant increased OR of MetS vs. controls was found; OR 6.4, 95 % CI 1.7; 25 p= 0. 007). When comparing with eugonadal TCS, the OR was 20 (95 % CI 3.2; 120). In another hypogonadal subgroup, constituting hypogonadal patients with TRT, compared with controls; OR was 13 (95 % CI; 1.3; 120 p=0.03). (Figure 12).
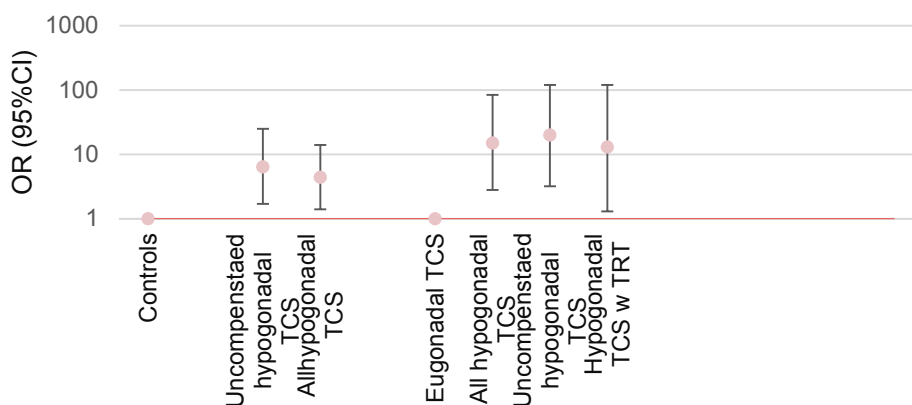
Figure 12.
OR for Metabolic syndrome i testicular cancer survivors compared to controls and eugonadal patients

The Metabolic syndrome in Childhood cancer survivors

Thirteen per cent of childhood cancer survicors and 10% of controls fulfilled criteria for MetS and in comparison, no statistically significantly increased OR for metabolic syndrome was found, OR=1.5 (95 % CI; 0.65; 3.2).

In comparisons between all hypogonadal CCS and controls, 19 % of the hypogonadal patients fulfilled the criteria for MetS and the risk was statistically significantly increased; OR =3.4 (95% CI; 1.2; 9.7). When comparing eugonadal and hypogonadal CCS subgroups and controls, MetS was diagnosed in 17.6 % of uncompensated hypogonadal patients but the risk-increase did not reach level of statistical significance; OR=2.3(95% CI: 0.8; 16.4). Among hypogonadal CCS given TRT, 39 % were diagnosed with MetS compared to controls; OR=5.9 (95% CI: 1.6; 21.8). None of the CCS with compensated hypogonadism fulfilled the MetS criteria. MetS was found in 9.9 % of the eugonadal patients, compared to controls; OR=0.95, (95% CI: 0.39; 2.4).

In the CCS RT CNS group, 33.3% of the patients were diagnosed with MetS. The OR, as compared to controls, was 6.7 (95% CI: 1.6; 28.9). After adjustment for hypogonadism the OR was 4.4 (95% CI: 0.99; 20.5) thus, borderline statistically significant. No other treatment group had increased OR for MetS. (Figure 13).
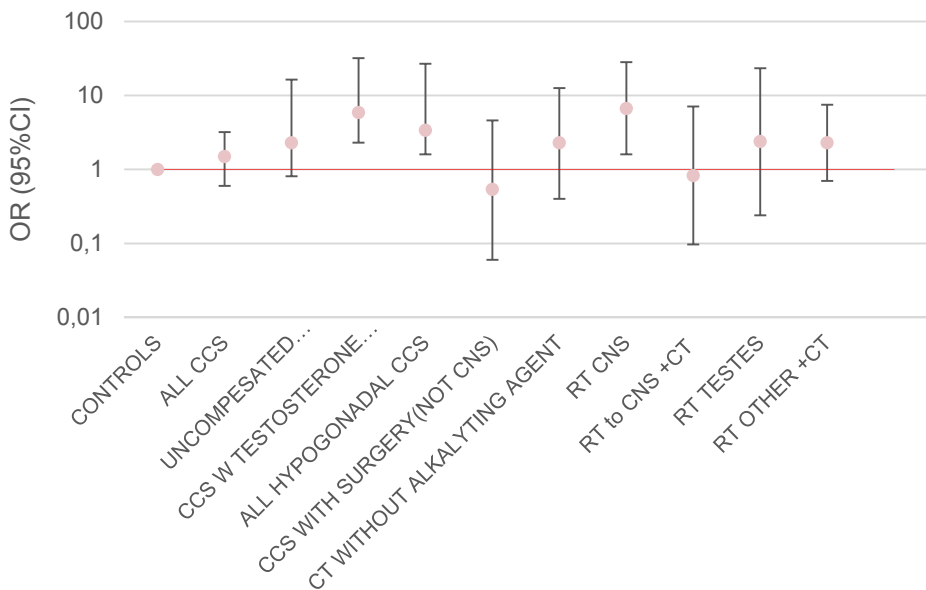

Figure 13
OR for Metabolic syndrome in childhood cancer survivors compared to controls

Effect of AR polymorphism

Logistic regression analyses revealed statistically significant interactions between CAG categories and +/- hypogonadism in relation to MetS (p= 0.018) in interaction analyses. The interaction was robust for adjustment to age at inclusion and smoking (p=0.041). The prevalence of MetS was highest in the hypogondal CS in the CAG 22-23. (Table 21). There was no statistically significant difference in OR for MetS when comparing CAG categories among hypogonadal patients.

Table 21
Cancer survivors with Metabolic syndrome(%) in different CAG categories +/ hypogonadism

	CAG <22	CAG 22-23	CAG >23
MetS+ hypogonadism	36% n=11	40% n=10	25% n=12
MetS- Hypogonadism	4% n=50	18% n=17	5% n=20

Interaction analyses for CAG

Interaction analyses for CAG categories and +/- hypogonadism did not give any statistically significant associations for waist measure, HbA1c, HOMAir, TG, BMI, cholesterol, glucose or insulin. Table 22.

Table 22
Interaction analysis CAG

Parameter	CAG x hypogonadism Non-adjusted	CAG x hypogonadism Adjusted (for age and smoking)
HbA1c (mmol/ mol)	P= 0.35	P=0.67
HOMAir	P=0.31	P=0.3
TG, (mmol/ L)	P= 0.43	P=0.53
Glucose (mmol/ L)	P= 0.63	P=0.81
Insulin, (mmol/ L)	P= 0.33	P= 0.26
BMI, (kg/ m^2)	P=0.71	P=0.53
Waist, (cm)	P=0.47	P=0.24
Cholesterol mmol/ L	P=0.91	P=0.88
MetS	**P=0.018**	**P=0.041**

We analyzed the distribution of CAG (mean = 22, median= 21) and GGN repeat lengths for all 123 included male cancer survivors whereas GGN repeats lengths (mean= 23, median = 23).

Discussion

The results from the studies this thesis is based on, provide a possible understanding for the increased cardiovascular morbidity in male cancer survivors. From three of the studies it can be concluded that testosterone deficiency has great impact on CVD risk factors. It is therefore suggested that testosterone measurements should be part of follow up in cancer survivors for preventive actions as considers CVD. Along with testosterone, insulin resistance, waist measure and levels of insulin and HbA1c should be kept under surveillance, being early signs of the metabolic syndrome and CVD.

In childhood cancer survivors, patients with testosterone replacement therapy had a higher risk than other hypogonadal groups for increased metabolic disturbances. This was unexpected and further challenging to evaluate due to lack of information regarding the dose and interval of the testosterone treatment. In addition, we had no information on pretreatment levels of testosterone. Thus, the interpretation was that these patients suffered the most severe hypogonadism, yet not treated to the full extent. However, previous studies are lacking to prove CVD benefits from exogenous testosterone treatment (262). Also, endpoints frequently focused on overt and more severe CVD further difficult to revoke. Nevertheless, more studies are needed to investigate potential benefits of testosterone replacement therapy on cardiovascular risk factors in large prospective studies for young cancer survivors.

We confirmed an association between AR polymorphism and sperm production recovery from a previous pilot study (147), AR CAG 22-23, considered the most optimal AR phenotype, had significantly lower sperm concentration than short tracts (<22) for both studies. Although the curve for sperm concentration was u-shaped and not negatively linear as for the pilot study, this difference could be explained by the fact that, in the piloted study, only two patients constituted the group >23 CAG repeats.

Statistical significance was obtained for one treatment group (3-4 CT) at one time point in both studies, which is an indication of a genetic association and not a chance finding. However, to be able to use AR CAG- and GGN-repeat lengths as predictive values, larger studies are warranted. Giving that an interaction with hypogonadism could account for the inter-individual disparities, seen in paper IV, hypogonadism is suggested to be included in the model when looking at the effect of AR polymorphism in future studies.

The risk of the metabolic syndrome was higher among hypogonadal CS in the CAG 22-23 group, compared to those with long (<23) and short (<22) CAG repeat lengths although non significant. Interaction analysis for CAG repeat lengths and hypogonadism was significant for MetS, pointing at an important interaction between hypogonadism and genetics. This is in line with the results reported by Skjarpe et al (263) where low CAG repeat lengths in a hypogondal context was associated with the risk of MetS. In contrast, an association for long repeats and risk of MetS in hypogonadal patients was seen in studies by Haring et al (256) and Zitzman et al (107), but studies were performed in healthy men and not in cancer survivors. Thus, the results are not showing a clear pattern as considers the inter-subgroup difference in prevalence of MetS. Nevertheless, it can be argued that low testosterone levels have impact on androgen receptor CAG repeat length for the risk of MetS. In that case one can agree with Hammond postulating that genetic polymorphism influence the symptomatic threshold of testosterone (191,264), Also when testosterone is administered the response can be individual, due to CAG repeat length, showed in a study by Tirabassi et al. (265) for which testosterone administration yield enhanced metabolic improvement in male with hypogonadism and shorter AR CAG repeat length.

The results are not convincingly pointing in one direction regarding the CAG and GGN categories. We anticipated the combination of a less active AR variant and hypogonadism associated with the most pronounced metabolic and cardiovascular risk profile. This assumption was partly established for the GGN lengths in relation to HOMAir and cholesterol levels whereas for glucose the highest values were seen for GGN=23 assumed to be associated with the most active AR (122). Previous studies are partly in agreement with our results, pointing at the increased risk of androgen deficiency related pathologies, associated with an expansion of GGN repeat (132) Being the highest cholesterol and HOMAir values among hypogonadal CS GGN>23 whereas for glucose, the concentration was highest in the GGN=23 group.

Although the results are not conclusive and the study population needs to be expanded in order to increase the statistical power of the study, we found some indications of modulating effect of AR CAG and GGN repeat lengths in relation to adverse effects of hypogonadism. Further exploration of this phenomenon may be an important link in understanding of the increased morbidity and mortality among cancer survivors as well as an identification of high-risk groups. CAG repeat length could allow us to individually tailor testosterone replacement therapy.

Hypogonadism seems to be a significant risk factor for MetS among cancer survivors. For the individual components of MetS the pattern is not as clear. Hypertension was increased for patients in RT other group but no other associations were found, indicating that it could be a chance finding. In line with our results, there is often a later appearance of hypertension secondary to other factors and long-

term effect of endothelial damage of CT or RT (194)or hypogonadism. ABI is a marker to the risk of atherosclerosis, and no significant differences were identified in comparisons with controls. This might reflect the prolonged process of atherosclerosis. Again with low levels of testosterone this process will be accelerated. This is in part explained by favorable influence of testosterone on the uptake of cholesterol and foam cell formation the earliest step in atherosclerosis (266). This is mediated through androgen receptors represented in high numbers in macrophages. From paper IV results indicate that GGN >23 had increased risk of elevated cholesterol and it is therefore plausible that AR polymorphism could modulate this effect. Low testosterone is also associated with increased intima media thickness (267) further empazising this association. The importance of adequate levels of testosterone points at the importance of testosterone surveillance after cancer. This suggests that the pivotal role of polymorphism in modulating androgen effects on cardiovascular risk factors is of a complex nature and implies that its clinical impact, similar to that of androgens, is dependent on exogenous factors.

To which degree inflammation is involved in the pathogenic link between hypogonadism and CVD is not fully explored. It is known that cancer and treatment induces rise in inflammatory agents, increasing risk of insulin resistance. Hyperinsulinemia and increased HOMAir were seen in the cancer survivors and most evident in hypogonadal patients. Insulin per se stimulates the smooth muscle growth and endothelial by increasing growth factors, (268) explaining the strong correlation to MetS and CVD. Increased levels of low-grade inflammation are found in subfertile men with hypogonadism (269). This is confirmed in several other studies, where testosterone deficiency was linked to increased inflammatory markers. Further, it is suggested that immune-regulatory effects of testosterone is a link to CVD and diabetes type II. Other studies indicated an association between increased obesity, (270) visceral adiposity and increased body-fat and leptin (271,272) by CAG polymorphism. This might be explained by the high representation of androgen receptors in adipose tissue, secreting inflammatory mediators. Further, testosterone replacement therapy on the other hand, reduces leptin and adiponectin (273), again pointing at a crucial interaction in the AR-mediated androgen effect (274).

In former studies increased LH-levels have been identified as risk factors for increased mortality (275). In contrast, in this thesis, patients with isolated LH-increase and normal testosterone levels, i.e. compensated hypogonadal patients were few and no significant associations to cardiovascular disease were found. Therefore we cannot recommend LH as predictor for CVD in cancer surveillance.

Summary and conclusions

The overall aims with this thesis was to assess the impact of hypogonadism on cardiovascular risk factors in male cancer survivors and the genetic influence in those patients by androgen receptor polymorphism. Further, it aimed at investigating the association between AR polymorphism and reproduction post treatment in a larger study, previously indicated in a pilot study (147).

Based on the results, the following was concluded:

- Hypogonadism is associated with increased prevalence of several cardiovascular associated risk factors in male cancer survivors.

- Insulin resistance (HOMAir), insulin, HbA1c and waist measure are the risk factors most significantly associated with hypogonadism.

- The metabolic syndrome occurred in 33% of hypogonadal TCS and in 19% of hypogondal CCS and the risk was statistically significantly enhanced compared to controls

- Radiation to CNS in CCS was the treatment most clearly associated with increased risk of MetS.

- The androgen receptor polymorphism had significant impact on glucose, cholesterol and MetS in hypogonadal cancer survivors

- The most efficient androgen receptor, CAG 22-23, had higher prevalence of MetS than <22 and >23.

- AR CAG repeat length of 22-23 had lower sperm concentrations than other CAG groups, indicating a genetic influence on reproduction after cancer treatment

This provides evidence for increased incidence of cardiovascular risk factors in both male TCS and CCS and a strong link to hypogonadism, displaying a big risk factor in these young patients. Insulin, waist measure, HbA1c and HOMAir are the most strongly associated risk factors and further pronounced in hypogonadal patients and in patients with radiation therapy to CNS. The metabolic syndrome is significantly increased in hypogonadal patients of both patient cohorts, approaching 40 % of hypogonadal patients. The results from paper IV provide novel insight on the effect of interaction between hypogonadism and AR polymorphism on cardiovascular risk

factors. We suggest that low testosterone can be used as predictive value for CVD in male cancer survivors and in the future, hopefully AR polymorphism can further predict cardiovascular risk and sperm production recovery, but more studies are needed.

Strenght and limitations of thesis

This thesis has some important strength due to the prospective design of all included studies and due to inclusion of age-matched controls in study II-IV, randomly selected from the normal population. The patient groups were fairly large, giving some power to the statistical analyses.

All blood samples, including a wide range of hormone measurements, were drawn in the morning, fasting, before 10 am, thus eliminating risk of low concentrations caused by diurnal variation of testosterone. All comparisons were performed adjusting for age and smoking, known to have an impact on testosterone levels as well as the risk of cardiovascular disease.

The study investigators met all patients and controls at RMC Malmö and the examination, blood sampling and questionnaries were all done at the same time.

The participation rate was rather high for TC patients, but lower for CCS, probably due to longer follow up (9.2 vs 24 years). There was however no difference in number of children between participants and non-participants, reducing the risk of selection bias. For controls the participation rate was low but when comparing participants and non-participants, no difference was detected for number of children, also decreasing the risk of selection bias. For patients with and without DNA, included in study IV, mean values for age and metabolic endpoints were compared and no differences were detected.

The CCS presented with a wide spectrum of oncological diagnoses and although they were divided in ten treatment groups, these subgroups remained heterogenic.

The number of patients with compensated hypogonadism was too low to draw any conclusions for this category of patients.

Age at diagnose might influence the outcome of late effects, but only age at inclusion was taken into account. However, Skinner concluded that age at diagnose was of less impact on the risk of hypogonadism than previously reported.

The hypogonadism diagnose in study II-IV was based on biochemical analyses only, whereas in the clinical setting, repeated analyses are recommended for making decision regarding androgen replacement therapy. However, earlier studies have shown that single measurements are applicable for identifying high-risk patients (276).

As earlier mentioned, the lack of detailed information on testosterone treatment limits the possibility to draw conclusions on treatment impact. Some of the patient groups are fairly small and there is a risk of statistical type two errors. The abstinence time was not included as a cofactor in paper I due to missing values for 40% of patients.

Future perspectives

The future perspectives are enlightend under discussion and therefore only briefly discussed here.

The results from this thesis indicate the prerequisite for an individualized treatment and follow up due to inter-individual risk of adverse effect of treatment. More predictors are needed to identify high-risk patients in order to offer preventive measures and for individual tailored treatments. Therefore, future studies that could contribute with such information are;

– Studies with longer follow-up of insulin, MetS, HbA1c, HOMAir, waist measure and testosterone after treatment and over time. Patients should be randomized in large multi-centre studies to line out the risk of metabolic disturbances and the association to hypogonadism.

– Testosterone treatment should be given and evaluated in randomized placebo controlled studies as considers risk-benefit regarding cardiovascular risk factors in cancer survivors. Early onset-hypogonadism should be given special attention in follow up surveillance. Previous studies indicate that some individuals are more sensitive to testosterone replacement therapy whereas others require higher doses of testosterone for equal outcome, which should be further analysed.

– Additional studies are required to understand the impact of androgen receptor polymorphism on cardiovascular risk factors and reproduction also when hypogonadism is taken into account. Overall, in a larger study the AR CAG and GGN groups will more accurately reflect the normal population. Subsequently, genetic alteration would therefore be plausible and applied as predictive values.

– With the intention to avoid future late-term side effects of treatment, and with the knowledge that radiotherapy is associated with the highest risk of adverse effects, studies investigating treatment regimens with low doses of radiotherapy or without radiotherapy are warrented.

We could not prove any effect of compensated hypogonadism on the risk of late effects, perhaps due to low number of patients, and too short observation follow-up period for this group. More information regarding long-term risk of compensated hypogonadism in male cancer survivors is needed.

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I am a Medical Oncologist specialized in sarcoma and lymphoma cancers. The focus with this thesis has been to evaluate the impact of testosterone deficiency and genetics on the risk of cardiovascular disease and reproduction in young male cancer survivors.

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