Life-Threatening Long-Term Treatment-Related Adverse Effects in Testicular Cancer and Hodgkin's Lymphoma Survivors with emphasis on Cardiovascular Disease

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ABBREVIATIONS

ABOD: adriamycin, bleomycin, vincristin, dacarbazine
BEP: Cisplatin, etoposide, bleomycin
CACS: Coronary artery calcium score
CAD: coronary artery disease
ChlVPP: chlorambucil, vinblastin, procarbazine, prednisolon
CI, cardiac index
CVD: cardiovascular disease
EBVP: epirubicin, bleomycin, vinblastin, prednisolon
EF: ejection fraction
FU-1: first follow-up of testicular cancer survivors or Hodgkin's lymphoma survivors
FU-2: second follow-up of testicular cancer or Hodgkin's lymphoma survivors
HL: Hodgkin's lymphoma
HLSs: Hodgkin's lymphoma survivors
hsCRP: high sensitivity CRP
IVSd: interventricular septum thickness
LA: left atrium diameter
LVEDD: left ventricular end diastolic diameter
LVEDV: left ventricular end diastolic volume
LVESD: left ventricular end systolic diameter
LV-FS: left ventricular shortening fraction
LVPW, left ventricular posterior wall
MDCT: multi-detector computed tomography scanner
MOPP: nitrogen mustard, vincristine, procarbazine, prednisone
MVPP: nitrogen mustard, vinblastine, procarbazine, prednisone
NRH: Norwegian Radium Hospital
PAS: pre-cranial atherosclerosis score
Pro-BNP: pro-Brain Natriuretic Peptide
PVB: cisplatin, vinblastin, bleomycin
RPLND: retroperitoneal lymph node dissection
sCD40L: soluble CD40 Ligand
TC: Testicular cancer
TCSs: Testicular cancer survivors
vWF: von Willebrand factor

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I. **Wethal T**, Kjekshus J, Røislien J, Ueland T, Andreassen A.K, Wergeland R, Aukrust P, Fosså S.D. Treatment-related differences in cardiovascular risk factors in long-term survivors of testicular cancer. *Journal of Cancer Survivorship* 2007; 1: 8 – 16.

II. **Wethal T**, Haugnes H.S, Kjekshus J, Ueland T, Aukrust P, Fosså S.D. C–reactive protein; a marker of second cancer and cardiovascular disease in testicular cancer survivors? Submitted

III. **Wethal T**, Lund M-B, Edvardsen T, Fosså S.D, Pripp A.H, Holte H, Kjekshus J, Fosså A. Valvular dysfunction and left ventricular changes in Hodgkin's lymphoma survivors. A longitudinal study. *British Journal of Cancer* 2009; 101: 575 – 581.

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V. **Wethal T**, Nedregaard B, Andersen R, Fosså A, Günther A, Lund M-B, Kvaløy S, Fosså S.D, Kjekshus J. Effect of cholesterol on radiotherapy-induced atherosclerosis and peripheral endothelial dysfunction. Submitted.

1 INTRODUCTION

1.1 Testicular cancer

1.1.1 Epidemiology

Testicular germ cell tumors (TC) accounted for about 2% of all new cases of cancer in Norway in 2007 and is the most common cancer in males below the age of 40 years.[1] In Norway the incidence of TC has increased from 3 to 11 per 100 000 the last 50 years.[2] A similar increase in the incidence of testicular cancer is seen in most European countries.[3] The 5 year relative survival rate registered in Norway in 1998 – 2002 was 97%.[1] As most cases of TC appear before the age of 40, the life expectancy of testicular survivors (TCSs) is 30 to 50 years if not curtailed by treatment-related sequelae. The consequence is that the prevalence of TCSs is increasing. In 2007 there were 5515 men with TC which represent about 3% of all males with a diagnosis of cancer.[1]

Genetic and environmental factors probably starts their influence during early embryonic life and may cause TC through cryptorchidism (incomplete desence of one or both testis during embryological life), hypogonadism and infertility.[3-5]

1.1.2 Histopathology and staging

Approximately half of the patients are diagnosed with seminomas at a mean age of 37 years. Seminomas are highly radio-sensitive and 80 % of the patients are diagnosed with non-metastatic disease.[1, 6] The majority of primary germ cell tumors are homogenous cells with solely seminomal elements.[7]

Non-seminoma accounts for the remaining 50% of TC and consists of several histological subgroups like choriocarcinoma, yolk sac tumor, teratoma.[7] Most cases appear in the third decade.[6] Approximately 50% of the patients have metastases at the time of diagnosis. Non-seminomas are relatively radio-resistant. Malignant germ cell tumors may produce human chorionic gonadotropin (HCG) and/or glycoprotein α-fetoprotein (AFP), tumormarkers which can be demonstrated in serum. Staging is performed according to the Royal Marsden Staging System (Table 1).[8]

Table 1 The Royal Marsden Staging System.

Stage	Description
I	Tumor confined to the testicle. No evidence of metastases.
IM	No radiological evidence of metastases, but positive markers after orchiectomy
II	Involvement of infra-diaphragmal lymphadenopathy.
A	Maximum diameter of metastases < 2 cm
B	Maximum diameter of metastases 2-5 cm
C	Maximum diameter of metastases >5 cm
III	Involvement of supradiaphragmatic lymphadenopathy. A, B and C as for stage II.
IV	Hematological metastases. Involvement of lungs, liver, skeleton and/or brain.

Except for the use of Royal Marsden Staging System, the International Germ Cell Cancer Collobarative Group (IGCCCG) has developed a prognostic classification for metastatic cases based on histology (i.e. seminoma or non-seminoma), extent and localization of metastases and the level of tumor markers.[9]

1.1.3 Treatment of TC

Treatment of TC patients starts with orchiectomy of the tumor bearing testicle. Post-orchiectomy therapy depends on histology (seminoma vs non-seminoma) and the stage of the disease. As the present investigations are restricted to TC patients treated at the NRH from 1980-1994, Table 2 summarizes the standard treatment policies during these 15 years. Most of the patients are included in international trials and the results are published elsewhere.[9-32]

Table 2 Post-orchiectomy policies for TC at NRH, 1980 – 1994.

	Seminoma	Non-seminoma
Stage I	Infradiaphragmatic para-aortic/pelvic or Para-aortic radiotherapy 20-30 Gy	≤1988 RPLND with adjuvant chemotherapy in case of metastases (2-3 cycles) ≥1989 Surveillance or adjuvant chemotherapy (2 cycles)
Stage II (incl. stage 1M)	Limited: Infradiaphragmatic radiotherapy (36 Gy) with or without adjuvant chemotherapy (1 cycle) Extended: Chemotherapy (3-4 cycles)	Limited: RPLND with adjuvant chemotherapy in case of metastases (2-3 cycles) Extended: 3-6 cycles chemotherapy followed by surgery and post-op. adjuvant chemotherapy if residual metastases are present
Stage III-IV	Chemotherapy (4 cycles) occasionally followed by post-chemotherapy surgery or radiotherapy ≤ 36 Gy	As outlined for stage II (Extended)
Post-chemotherapy relapse/progression	Alternative chemotherapy ± surgery / radiotherapy ≤ 45 Gy	

Radiotherapy: Only infra-diaphragmatic radiotherapy was provided as initial treatment, the upper border of the target field being the disk between the 10th and 11th thoracic vertebra. If more than 1/3 of the renal parenchyma was within the target field, lead shields were applied after 20 Gy, restricting the target dose to maximally 1/3 of the renal parenchyma. The field included the abdominal aorta and the apex of the heart.
Chemotherapy: Up to 1985 PVB (cisplatin, vinblastin, bleomycin) was the standard chemotherapy for each cycle. Thereafter BEP (bleomycin, etoposide, cisplatin) chemotherapy was introduced as the standard chemotherapy. In different trials other combinations and various numbers of cycles were explored, using vincristine, carboplatin ifosfamide, in addition to cisplatin. Relapse treatment (after previous chemotherapy) could include limited doses of adriamycin or metotrexate along with ifosfamide.

1.2 Hodgkin's lymphoma

1.2.1 Epidemiology

Hodgkin's lymphoma (HL) accounts for only 0.4% of incident cancer in Norway in 2007, even though HL comprises about 15% of all lymphomas in western countries.[1, 33] The age-adjusted incidence in 2003 – 2007 among men was 2.8 per 100 000 while it was 2.0 per 100 000 in women which is in line with other European countries and the US. The incidence varies considerably between different age groups, demonstrating a bimodal shape (Figure 1). In 2007 114 men and women were diagnosed with HL.[1] Most patients with HL are in their late teens or young adulthood. Thus, it accounts for about 15% of all cancer among young adults (15 – 24 years).[33] The incidence of HL during the past decades has been remarkably stable, in contrast to the increased incidence of Non-Hodgkin's lymphoma.

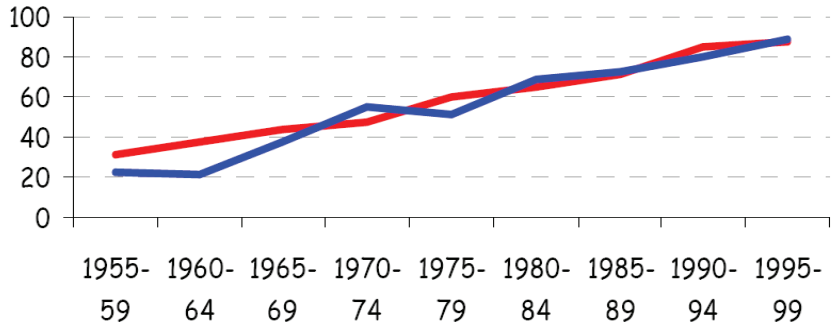
Figure 1 HL incidence according to age registered in 1997 - 2001. The Cancer Registry of Norway (blue line: males; red line: females)

Due to modern radio- and chemotherapy there has been a marked increase in the survival rates over the last 50 years (Figure 2). Overall, the 5 year relative survival rate is approximately 90% according to the Cancer Registry of Norway.[33]

The most frequently identified risk factors of HL are Epstein-Barr virus (EBV), human immunodeficiency virus (HIV), autoimmune disorders, allogeneic bone marrow

transplantation and multiple genetic factors.[33] Interestingly, elevated IL-6 levels linked to genetic polymorphism of the IL-6 gene has been associated with increased risk of HL.[34]

Figure 2 Five year relative survival rate. The Cancer Registry of Norway (blue line: males, red line: females).



1.2.2 Histopathology and staging

HL is divided in to major types called nodular lymphocyte predominant HL (NLPHL) and classical HL (CHL). Among the four subtypes of CHL, nodular sclerosis CHL (NSCHL) constitutes about 70% and affects primarily young adults from developed countries and involves mediastinum in 80% of cases.[35] The origin of HL is B – lymphocytes which in most cases are not able to produce immunoglobulin, at least for CHL.[36] Importantly, most lymph nodes or extra-nodal organs involved by HL contain relatively few neoplastic cells, but are dominated by reactive inflammatory cells.[35, 37]

Basic evaluations performed in patients with HL include a detailed medical history with focus on pre-existing medical conditions, the presence of night sweats, fever, weight loss (B-symptoms) and a medical examination. In many cases the patient presents with a localized painless and firm mass lesion in the neck or supraclavicular fossa.[38] Routine blood tests have included sedimentation rate, full blood counts, liver- and renal function tests including LDH and serological tests for relevant viral infections as they have become available over the last decades (Hepatitis B and C, HIV, EBV).[39]

Since the 1980ies, radiological evaluations have relied mostly on CT scans of the neck, thorax, abdomen and pelvis, supplemented with ultrasonography of abdomen. A bone marrow biopsy, performed uni- or bilaterally, has been mandatory except in some cases of stage IA disease on the neck. Lymphangiography has been part of the staging until the 1990ies, but with the advent of better imaging modalites, it has later been omitted. Routine staging laparotomy was abandoned in Norway in 1980 and since that time it has only been used in isolated special cases. Positron emission totmography (PET) alone or combined with CT (PET-CT) has not been used before year 2000..

Staging is performed to decide which treatment to apply to HL patients and to predict prognosis. Over the last decades the Ann Arbor staging system with its different revisions and modifications has been used at the NRH (Table 3).[40] Early stage disease has typically been defined as stage I and II and advanced stages as stages III and IV. In addition to the Ann Arbor staging system, other clinical, laboratory or radiological findings have been used to identify patients within the early stages at higher risk of treatment failure (such as presence of B-symptoms and/or bulky tumor, 4 or more involved regions, infra-diaphragmatic disease, lymphocyte depleted subtype). Bulky disease is a large lymph node mass beyond 6-10 cm, depending on the definition of various investigators, or a mediastinal tumor greater than or equal to one third of the internal transverse diameter measured at level of the T5/6 intervertebral disc on chest radiography. Thus, since 1980, stage I and II disease has been subcategorized into a favorable group without risk factors and an intermediate group with risk factors.

Table 3 Cotswold Modification of the Ann Arbor Staging Classification[40]

Stage I Stage IE	Involvement of a single lymph node region OR Involvement of a single extralymphatic site
Stage II Stage IIE	Involvement of two or more lymph node regions on the same side of the diaphragm OR Localized contiguous involvement of only one extranodal organ or site and its regional lymph nodes with or without other lymph node regions on the same side of the diaphragm
Stage III Stage IIIS Stage IIIE Stage IIISE	Involvement of lymph node regions on both sides of the diaphragm POSSIBLY ACCOMPAINED BY Involvement of the spleen OR Localized contiguous involvement of only one extranodal organ site OR Both of the above
Stage IV	Disseminated (multifocal) involvement of one or more extranodal organs or tissues, with or without associated lymph node involvement or isolated extralymphatic organ involvement with distant (non – regional) nodal involvement
Designations applicable to any stage	
A	No symptoms
B	Fever (> 38 ^{o}C), night sweats, unexplained loss of > 10% body weight in previous 6 months
X	Bulky disease
E	Involvement of a single extranodal site that is contiguous or proximal to the known nodal site

1.2.3 Treatment of HL

The treatment strategies for HL at the NRH have followed national and international guidelines, and are summarized in Table 4 for the period 1980-1988.[41-44] Based on the staging the patients are divided into 3 groups; patients with stage I or II without or with risk factors and patients with stage III or IV disease.[45] Disease and treatment characteristics for each patient were obtained from medical records, including type of radiotherapy (mantle field vs. mediastinal field only) and details regarding the use of additional chemotherapy.

Table 4 Treatment of HL at NRH 1980-1988.[46]

	Chemotherapy	**Radiotherapy**	**Dose and fractions of radiotherapy**
Stage I & II			
1980-88	With risk factors*: 4 ABOD, ChlVPP or ABOD/ChlVPP (alternating) before radiotherapy	Extended field radiotherapy (Mantle field /Inverted Y)	2 Gy x 20, from 1982 1,8 Gy x 23
Stage III & IV			
1980-1985	8 MVPP/ChlVPP	To sites of initial bulky tumor or residual mass	2 Gy x 20
1985-1991	8 ChlVPP or 8 ABOD/ChlVPP (alternating)	To sites of initial bulky tumor or residual mass	2 Gy x 20 or 1,8 Gy x 23

* Risk factors are presence of B-symptoms and/or bulky tumor, 4 or more involved regions, infra-diaphragmatic disease, lymphocyte depleted subtype

Table 5 Summary treatment of TCSs and HLSs

Treatment	TCSs	HLSs
Radiotherapy		
- **Dose (Gy)**	30 (30 – 45)	40 (27 – 44)
- **Localization**	Infra-diaphragmatic	Mantle or mediastinal field
Chemotherapy	Cisplatin	Doxorubicin, epirubicin
	Bleomycin, Vinblastin	Bleomycin, Vinblastin
	Etoposid	chlorambucil, procarbazine, prednisone

1.3 Risk factors of cardiovascular disease (CVD) and second cancer in TCSs and HLSs

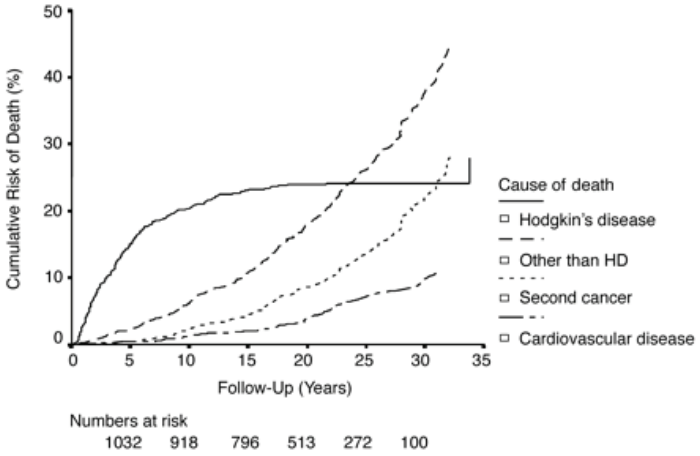
1.3.1 CVD and second cancer in TCSs and HLSs

TCSs are at an increased risk of CVD.[47-51] There has been observed nearly a 2 fold increased risk of CVD median 10 years after treatment in patients treated with either radiotherapy alone, chemotherapy alone or both as compared to only surveillance or the general population.[48, 52] Furthermore, even with the introduction of cisplatin and the reduction in the use of radiotherapy, the risk of non-germ cell cancer in TCSs remains high.[53, 54] The cumulative risk of a second cancer in patients diagnosed with seminoma or nonseminoma 40 years after treatment was 36% and 31% respectively compared with 23% in age matched controls.[54] Cancers located below the diaphragm like colon cancer, bladder cancer, pancreatic cancer and stomach cancer are frequently observed in TCSs, but there is also observed a higher risk of lung cancer and leukaemia compared to the general population.

The mortality due to HL reaches a plateau 10 years after the treatment.[55] Unfortunately, mortality due to especially CVD and a second cancer continues to rise with increasing follow-up time (Figure 3). Several reports indicate that CVD appears about 10 years after treatment, including coronary artery disease (CAD), congestive heart failure due to

cardiomyopathy, valvular disease, and constrictive pericarditis.[56-63] In 1080 HLSs treated in 1969 – 1997 the relative risk of cardiac death was 2.8 15 – 20 years after treatment, and this risk increased to 4.5 for a group observed more than 20 years after treatment.[64] There is also increased risk of Non-Hodgkin's lymphoma but the majority of malignancies are solid tumors, especially breast, lung and gastrointestinal cancer.[65-68] Among 32591 HLSs the observed to expected ratio for a second malignancy was 2.3 (95% CI 2.2 – 2.4), and almost 1/3 had been followed for more than 25 years.[65]

Figure 3 The actuarial risk for major disease categories in HL.[55]



1.3.2 Radiotherapy and CVD

Mechanisms

The acute effects of radiotherapy are supposed to cause small vessel thrombosis due to endothelial cell swelling and endothelial inflammation .[69] The irradiated endothelium is characterized by activation of NF-αB, alterations in the expression of adhesion molecules, chemotactic cytokine production and leukocyte infiltration into tissues.[70] This response is sustained for several weeks. Radiotherapy is also believed to cause fibrosis and endothelial damage through generation of reactive oxygen species.[71] A study was performed on cervical arteries after neck dissection in 17 patients previously irradiated for malignant neck tumor.[72] The tissue was harvested 4-6 weeks after radiotherapy. The irradiated cervical arteries as compared to contra-lateral non-irradiated vessels did not

express eNOS and had impaired vasodilatation upon acetylcholine stimulation. Another study in patients treated with mediastinal radiotherapy for breast cancer demonstrated decreased endothelium-dependent vasodilatation with the use of acetylcholine in the irradiated axillary arteries compared to the non-irradiated arteries in the same patients.[73] An in vitro study has shown that the endothelial marker vWF is released from isolated vessels early after irradiation. Radiotherapy may thus lead to endothelial dysfunction as demonstrated by reduced flow-mediated dilatation and increased intima-media thickness.[74, 75] These adverse effects of radiotherapy may initiate or accelerate atherosclerosis directly or through interference by established cardiovascular risk factors like hypercholesterolemia, hypertension and the metabolic syndrome which are frequently observed among TCSs.[76, 77] A self-perpetuating process of endothelial inflammation may be the results of these stimuli. Eventually, these processes are also believed to be responsible for the damage of coronary arterioles and capillaries, and may lead to myocarditis, pericarditis and coronary ischemia.[78] In summary, the observed increase in CVD among TCSs and HLSs may be due to a direct and localized effect of radiotherapy and/or an indirect effect mediated by the activation of the immune system associated with the increase in the inflammatory markers. The more frequent occurrence of CVD in HLSs compared to TCSs most likely represent both a dose-effect relationship and a direct toxic effect on the heart caused by mediastinal radiotherapy. TC patients received about 30 Gy of infra-diaphragmatic radiotherapy while HL patients received about 40 Gy of mediastinal radiotherapy.

CVD associated with radiotherapy

An increased cardiovascular mortality in TCSs has been reported by Zagars who studied 453 patients with seminoma stage I or II treated with infra-diaphragmatic radiotherapy after orchiectomy.[51] Seventy-one (16%) patients had received additional mediastinal radiotherapy. In those with an observation time exceeding 15 years the cardiac standardized mortality ratio (SMR) was higher among those who had received infra-diaphragmatic radiotherapy (SMR = 1.75) and those who received additional mediastinal radiotherapy (SMR = 2.33) compared to the general population (US males). In contrast, van den Belt-Dusebout et al did not observe any increased risk of cardiovascular events

in TCSs treated with infra-diaphragmatic radiotherapy alone while mediastinal radiotherapy was followed by a nearly four fold risk of a composite end point of myocardial infarction, angina pectoris and heart failure..[50] Fosså et al showed that TCSs younger than 35 years and treated with surgery combined with radiotherapy had a standardized mortality ratio of 1.70 for all CVD compared to the general population.[52]

The main cause of cardiovascular disease in HLSs is related to the treatment with mediastinal radiotherapy. Cardiac mortality has been shown to be significantly increased at doses beyond 30 Gy.[79] Aleman et al observed also a 3.6-fold increased risk of myocardial infarction and a 4.9-fold increased risk of cardiac heart failure in 1474 HLSs with a follow-up of 18.7 years as compared to the general population.[57] Mediastinal radiotherapy increased the risks of MI, angina pectoris, CHF, and valvular disorders by 2- to 7-fold. The risk for MI became significant at 10 years after treatment and remained elevated up to 25 years after treatment. There has been observed a relative risk of death from myocardial infarction at 2.5 in 7033 HLSs with a median follow-up of 9.9 years compared to the general population.[63] The risk of death and myocardial infarction was elevated from the first year after treatment and remained elevated up to 25 years after treatment. Mantle field radiotherapy without chemotherapy was associated with increased risk of death due to myocardial infarction (relative risk 3.2 (2.3 – 4.3)). CAD is frequently observed even in asymptomatic HLSs.[59] Stress echocardiography and radionuclide perfusion imaging were performed in 294 patients treated with mediastinal radiotherapy who had no symptoms of heart disease. Based on the results from the imaging 40 patients underwent angiography and 22 patients had coronary stenosis of ≥ 50%. Furthermore, radiotherapy is known as a cause of restrictive cardiomyopathy.[56]

The cardiac valves are included in the mediastinal radiotherapy field, and it has been shown that the incidence of valvular dysfunction increased during the second decade after treatment with mediastinal radiotherapy for HL.[56, 58, 61] After 20 years, 6 – 15% of HLS have moderate or severe valvular regurgitation in the aortic or mitral valve. The actual risk of valvular dysfunction in patients treated for HL is under debate. Aleman et al. observed a hazard ratio of 7.0 and a cumulative incidence of approximately 10% for

having clinically diagnosed valvular disorder after a median observation time of 13 years in HLSs treated with mediastinal radiotherapy.[57] Hull et al. reported that 6% of 415 HLSs had clinically important valvular disorder 20 years after radiotherapy.[61] Heidenreich performed a cross-sectional follow-up investigation in 73 patients 20 years after radiotherapy. Twelve (16%) had aortic stenosis, 11 (15%) had moderate or severe aortic regurgitation, and 3 (4 %) had mitral regurgitation.[58] However, the cause of the enhanced susceptibility of valvular dysfunction in some of the HLSs is unknown.

In patients treated with mantle field radiotherapy the pre-cranial arteries are exposed to irradiation. Accordingly, an increased risk of carotid artery stenoses and stroke have been reported in HLSs.[61, 80-82] Radiotherapy was associated with a subsequent stroke in 24 of 1926 HLSs compared to 9 of 3846 healthy siblings after a follow-up of 17.5 years (RR = 5.6). A Dutch group reported a relative risk of stroke and transient ischemic attack at 2.2 (95% CI 1.7 – 2.8) and 3.1 (95% CI 2.2 – 4.2) respectively in 2201 HLSs after a median follow-up of 17.5 years.[82] The cumulative incidence of stroke or transitory ischemic attack (TIA) 30 years after the treatment was 7%. Cardio-embolism and large-artery atherosclerosis were the sources of an event in 24% and 36% of the patients respectively. Hull et al observed an actuarial incidence of stroke, TIA, carotid artery stenosis and subclavian stenosis at 3 % at 10 years and 7 % at 20 years.[61] However, it was suggested from that study that patients who developed TIA or stroke did so because radiotherapy accelerated the atherosclerotic process in these patients who were already 51 years old at the time of radiotherapy and who experienced an event within 6 years after treatment. The same study showed that 30 of 404 (7.4 %) had carotid or subclavian disease after a median of 11.2 years after radiotherapy.[61] Younger patients who were on average 20 years when treated with radiotherapy demonstrated isolated carotid or subclavian stenosis after a median follow-up of 21 years.

1.3.3 Chemotherapy and CVD

Mechanisms

Chemotherapy with the use of either cisplatin or anthracyclines has become the corner stone in the treatment of TC and HL respectively. These agents mediate their anti-cancer effects by different mechanisms and have different long-term adverse effects.

Cisplatin-based chemotherapy used in the treatment of TC induces apoptosis through its cross-linking action on DNA.[83] There is evidence of cisplatin in plasma even 20 years after treatment which may partially explain the long-term toxic effects of cisplatin.[84] In vitro studies of the endothelial function after administration of cisplatin and bleomycin have clearly demonstrated endothelial dysfunction, inflammatory activation by increased levels of IL-1, IL-6, TGF-beta and inhibited proliferation of endothelial cells.[85, 86] Furthermore, TC patients treated with chemotherapy have reduced flow-mediated dilatation and increased intima-media thickness.[87, 88] Thus, chemotherapy may cause CVD through endothelial dysfunction and inflammation.

Anthracyclines applied to HL patients, may induce cardiomyopathy and aggravate cardiac disorders by various mechanisms.[89-92] The most important mechanism is considered to be the transfer of single electrons from the electron transport chain in mitochondria to doxorubicin giving rise to oxygen radicals.[93] But also the release of cytochrome c from the mitochondria and the subsequent apoptosis and transcriptional changes in intracellular ATP production in cardiac myocytes may play an important role.[92] Left ventricular shortening fraction (LV-FS) related to impaired contractility and reduction in ventricular mass and wall thickness has been observed 12 years after treatment with anthracyclines for childhood acute lymphoblastic leukemia.[94]

Cardiovascular disease associated with chemotherapy

Meinardi demonstrated an observed-to-expected ratio of 7.1 (95% CI 1.9 – 18.9) for CVD in 87 TCSs treated with cisplatin-based chemotherapy 10 years after the treatment as compared to the general population.[49] Even though this was the first study to compare CVD events in TCSs to the general population, the study is of limited value. The sample

size was small with only 5 events in the 87 patients. van den Belt-Dusebout demonstrated a 2 fold increased risk after treatment with chemotherapy (BVP-regimen) compared to patients treated with only surgery.[50] Fosså et al showed that men treated with chemotherapy had higher mortality from CVD median 10 years after treatment with a standardized mortality ratio of 1.58 compared to the general population.[52]

Chemotherapy may also directly influence cardiovascular disease in HLSs.[63, 95] The risk of anthracycline induced heart failure was estimated to 4-5% 15 – 20 years after treatment.[96, 97] In HLSs the risk of heart failure is relatively low if the cumulative dose of doxorubicin is less than 300 mg/m^2.[89, 95] Aleman reported that the addition of anthracyclines to mediastinal radiotherapy gave a 3-fold increase in the risk of cardiac heart failure and a 2-fold increase in the risk of valvular disorders compared to mediastinal radiotherapy alone.[57]

1.3.4 Treatment-related second cancer

In TCSs the risk is of having a non-germ cell cancer is elevated in patients treated with radiotherapy alone or chemotherapy alone.[98] Etoposide is a risk factor of acute myelogenic leukaemia.[99] HLSs have an elevated risk of developing second malignancies.[55, 60, 65, 100] In the first decade after treatment the appearance of leukemia probably due to alkylating chemotherapy is a feared malignancy.[64] Recently it was shown that HLSs had approximately 30-fold increased risk of malignant mesothelioma due to radiotherapy.[101] The risk of second malignancy is probably due to both radiotherapy and chemotherapy, although there is evidence that while the combined treatment with radiotherapy and chemotherapy gives an additive increase in the risk of lung cancer, the risk of post-HL breast cancer is reduced following chemotherapy.[66-68] The explanation for the latter is probably that the decreased ovarian function caused by alkylating agents abort the hormonal stimulation that may be of importance in mediating radiation induced breast cancer.

1.3.5 Traditional risk factors

Atherosclerosis is a multi-factorial disease involving oxidized LDL, HDL, inflammation, endothelial dysfunction and platelet activation and aggravated by traditional risk factors like hypertension, diabetes, hyperlipidemia and smoking.[102] Hypertension, hypercholesterolemia and the metabolic syndrome are frequently observed among TCSs treated with cisplatin which may contribute to the increased risk of CVD.[76, 77] A study by Sagstuen et al revealed that TCSs treated with cisplatin-based chemotherapy had higher levels of blood pressure, a higher prevalence of hypertension and a excessive weight gain compared with controls.[76] Hypercholesterolemia (79%) and hypertension.(39%) were observed among 62 patients treated with chemotherapy for metastatic TC, [49] There is evidence that not only TC treated with chemotherapy but also patients treated with orchiectomy only are at increased risk of the metabolic syndrome.[77]

Traditional risk factors are also of importance in HLSs. Hypercholesterolemia, hypertension, diabetes mellitus and smoking have been identified as risk factors of coronary and non-coronary disease in HLSs.[57, 61, 81] Potentially, a high cholesterol may aggravate the adverse atherosclerotic processes associated with radiotherapy or chemotherapy in both TC and HL patients.

1.3.6 Hypogonadism

Impaired spermatogenesis is associated with TC. The treatment with bleomycin, cisplatin and radiotherapy is associated with hypogonadism.[103-106] Hypogonadism is observed among TCSs and has been demonstrated in every fifth patient with TC before the start of treatment.[107, 108] Likewise, it has been suggested that post-treatment gonadal dysfunction may be associated with pre-treatment reduced fertility in HLSs.[109, 110] Alkylating agents and high-dose chemotherapy in male HLSs have been associated with hypogonadism.[111] Hypogonadism and low testosterone are associated with endothelial dysfunction, the metabolic syndrome, inflammation and CVD.[112, 113]

1.3.7 Inflammatory markers

Indices of inflammation (e.g., high-sensitivity-C-reactive protein [hsCRP]), platelet-mediated inflammation (e.g., soluble CD40 ligand [sCD40L]), and of endothelial cell activation (e.g., von Willebrand factor [vWF]) have all been shown to predict cardiovascular events.[114-116] Chronic subclinical inflammation may be associated with cancer as a consequence of invasive tumor growth and secondary activation of the immune system. Furthermore, inflammatory markers like CRP, vWF and sCD40L may be associated with future cancer and a pre-cancerous condition.[117-119]

C-reactive protein (CRP) is an acute phase protein produced by the liver in response to inflammatory stimuli.[120] Infections, surgery and advanced cancer cause marked elevations of CRP. Chronic minor elevation in CRP identifies subclinical inflammation which has been suggested to be instrumental in driving atherosclerosis, but this hypothesis remains undefined.[120] CRP is established as a predictor of cardiovascular events in patients with known CVD as well as in asymptomatic individuals with risk factors for CVD.[121, 122] It has been shown that CRP is associated with advanced HL, the presence of B symptoms and subsequent relapse of the disease.[123] However, there is evidence suggesting that CRP is not merely a marker of a manifest cancer but is also able to predict first-onset cancer. [124-127] Recently, CRP levels above 3 mg/l were identified as a marker of incident lung cancer in the general population in comparison to individuals with CRP < 1 mg/L.[124] Although elevated levels of CRP have been associated with future colorectal cancer, Rifai et al were not able to demonstrate that increasing quartiles of CRP were associated with future cancer among more than 28000 women older than 45 years, however the observation time was relatively short (58 months).[128]

CD40 ligand is expressed on T-lymphocytes, monocytes and activated platelets while CD40 is expressed on the endothelium.[115, 129, 130] It amplifies the endothelial cell response to inflammation partly by upregulating various CAMs (cellular adhesion molecules) and contribute to the regulation of hemostasis. This will lead to an increased transfer of LDL through the endothelium. An increased expression of CD40/CD40L has been seen in

patients with hypercholesterolemia.[130] Elevated serum concentrations of CD40L in apparently healthy women predicted cardiovascular events.[115]

Platelets are attached to the vessel wall through the binding of von Willebrand factor (vWF), which is localized beneath the endothelium and exposed in the case of endothelial injury.[118] The Monica study revealed that vWf measured on populations in different European countries was significantly associated with number of cardiovascular events.[131]

An association between sCD40L or vWF and cancer has also been suggested.[132, 133] There is a lack of studies on the use of these markers as prognostic indicators.

The evidence regarding inflammatory markers are mainly related to TCSs. Inflammatory markers and endothelial function were measured before and within 10 weeks after cisplatin-based chemotherapy in 65 TC patients with stage I to IV disease.[87] There was an increase in the levels of vWF and carotid intima-media thickness compared to pre-treatment values. Another study revealed increased levels of CRP, vWF, fibrinogen, plasminogen activator inhibitor (PAI-I) and tissue-type plasminogen activator (tPA) in 90 patients treated with chemotherapy 7 years earlier compared to 47 healthy men.[134] A third study by Vaughn et al compared 24 patients who had received cisplatin with 15 chemotherapy naive patients.[88] Chemotherapy decreased flow-mediated dilatation in the brachial artery and increased the level of soluble intercellular adhesion molecules in patients treated more than 2 years ago.

1.4 Preliminary conclusion and aims

Radiotherapy and chemotherapy may cause CVD and a second cancer through various mechanisms. The adverse effect of radiotherapy is probably dependent on both the localization and size of the radiation field, the total dose applied and indirect effects caused by a general inflammatory activity. The adverse effects of chemotherapy may be related to the mode of action of the used chemotherapeutic agent and their metabolism. Furthermore, the treatment with radio- and chemotherapy may have additive effects on the incidence of CVD and second cancer.

In order to reveal some insight in these mechanisms we investigated the presence of inflammation, lipid-related markers, blood pressure and hypogonadism in TCSs and if some of these markers (and especially CRP) may serve as predictors of future CVD and second cancer. In HL we focused on the development of valvular and myocardial dysfunction, the presence of coronary artery disease and atherosclerosis and focused on whether there were important predictors of cardiovascular adverse effects of mediastinal radiotherapy. We were able to perform studies on TCS and HL with an observation time exceeding 20 years for most of the participants.

The aim of each separate paper:

- Assess treatment-related differences in cardiovascular risk factors and inflammatory markers in testicular cancer survivors 11 years after treatment (Paper I).
- Explore if high sensitivity (hs) CRP predict subsequent cardiovascular events or non-germ cell cancer in testicular cancer survivors (Paper II).
- Evaluate the evolution and development of valvular and myocardial dysfunction in Hodgkin's lymphoma survivors treated with mediastinal dysfunction (Paper III).
- Assess the relation between premature coronary artery disease and coronary artery calcium score in Hodgkin's lymphoma survivors (Paper IV).
- Examine the interaction between radiotherapy and traditional risk factors on premature atherosclerosis in Hodgkin's lymphoma survivors (Paper V).

2 SUBJECTS AND METHODS

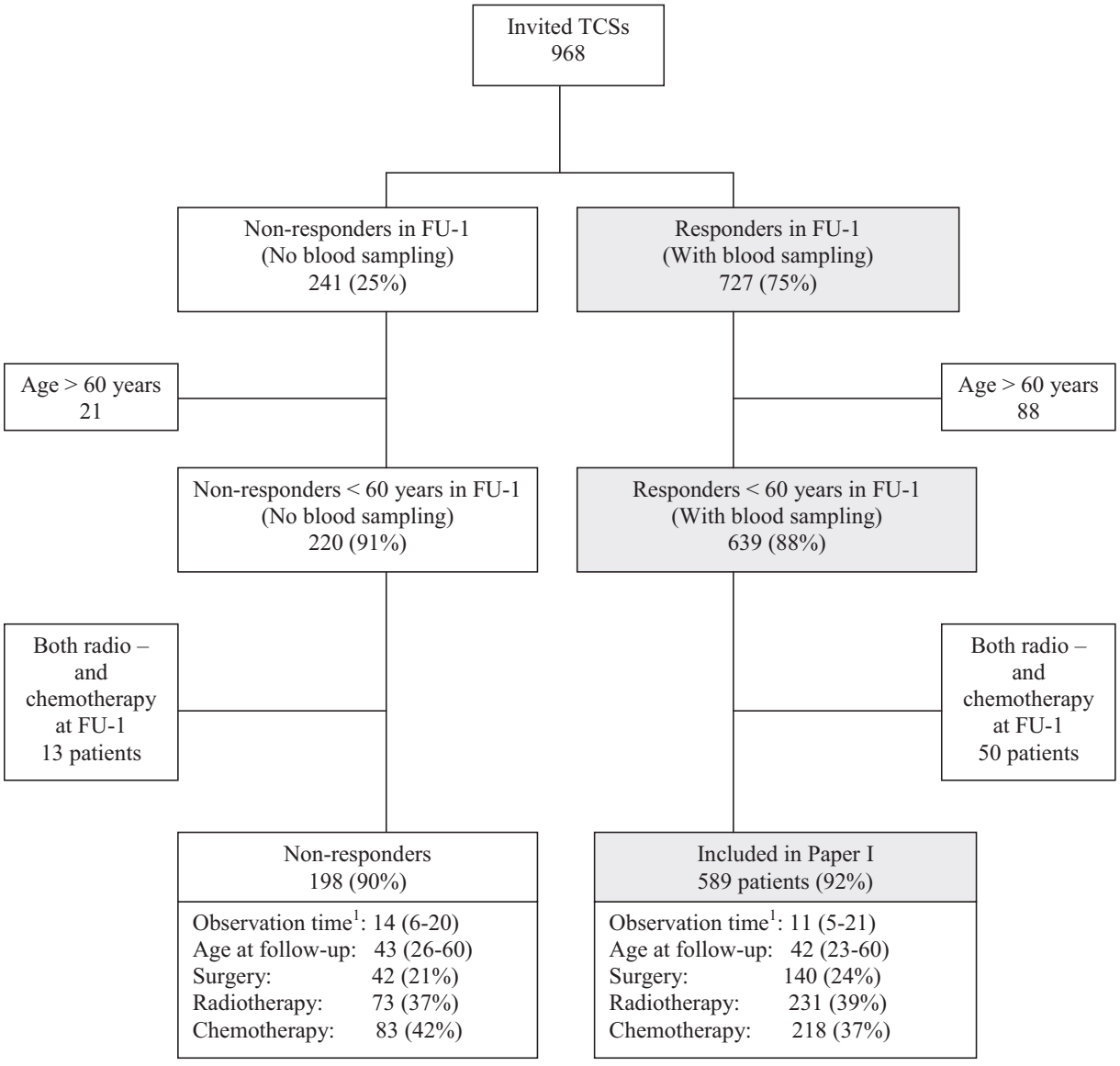
2.1 Study populations and design

TCSs (Paper I and II)

All surviving and disease-free men treated for unilateral TC in Norway during the period 1980-1994 were invited to participate in a national survey conducted from 1998 to 2001 (n = 1814). Patients with extragonadal germ cell tumors, bilateral TC, secondary malignancy except skin tumors and mental retardation were excluded. Patients treated for unilateral TC received a 219-item questionnaire and attended the outpatient department of the regional oncological unit for a clinical examination and blood testing.[104] At the time of assessment; all men were free of any apparent infections or any other incidental disease. Furthermore, in some patients audiometry, spirometry and semen analysis were assessed. Institutional and regional ethical committees approved of this study, and all participants gave their written consent.

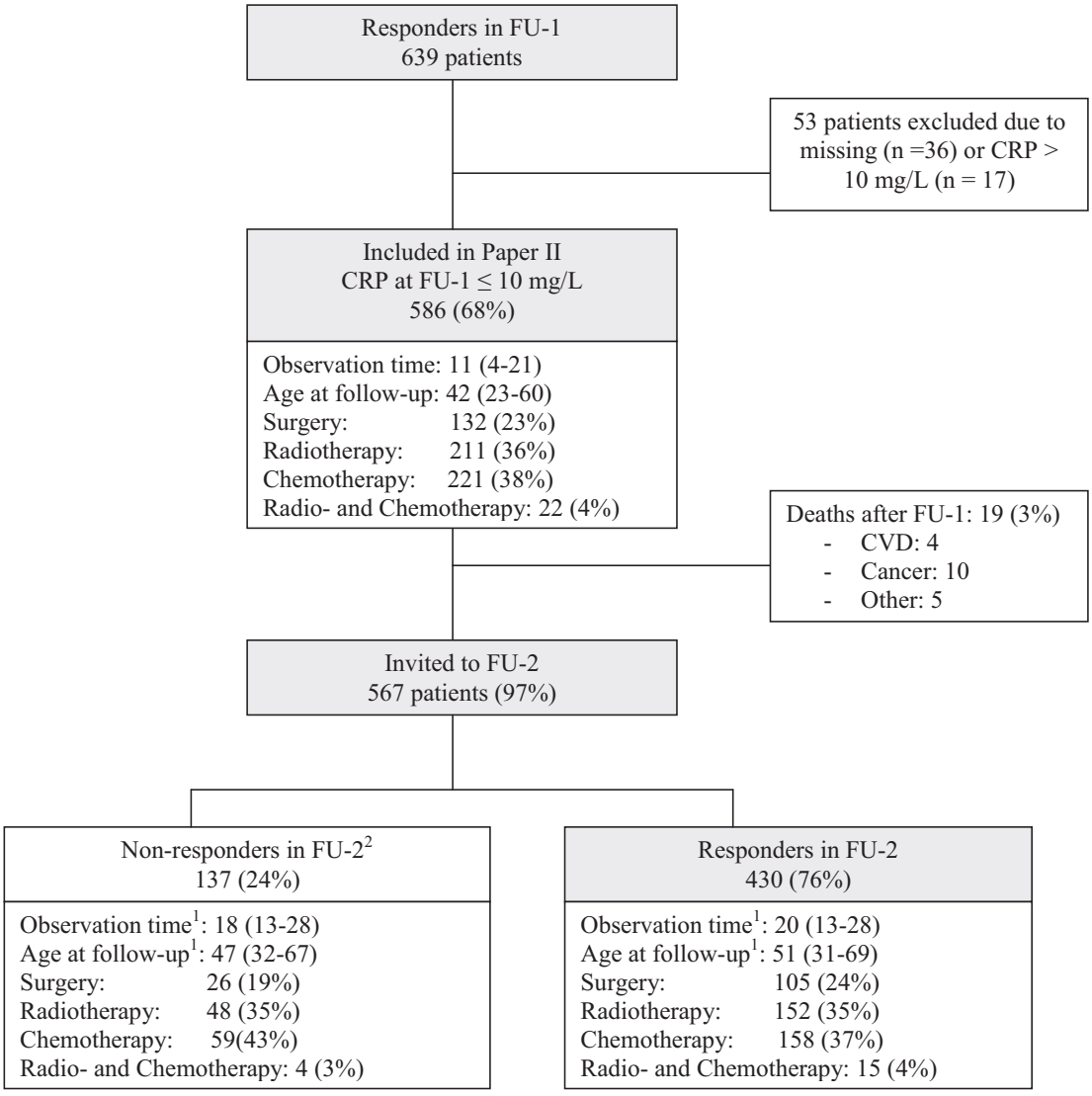
Paper I and II are restricted to patients seen at the Norwegian Radium Hospital (NRH; 968 of 1814 patients). Of these 968 patients, 727 (75%) men participated while 241 men declined or failed to show up for their scheduled outpatient visits (Figure 4). Eighty-eight men were excluded from our analyses because they were over 60 years of age at the time of the first follow-up and 50 were excluded because they were treated with both radio- and chemotherapy. Thus, a total of 589 men participated in the first follow-up (FU-1) presented in Paper I. The responders and the non-responders who were potentially eligible for the study were comparable in age at diagnosis and FU-1 and also with respect to the distribution of the histological diagnoses. The non-responders had a longer observation time (median 3 years older) than the responders. A later quality check revealed that 22 of the 50 patients believed to have received both radio- and chemotherapy actually had been treated with only chemotherapy.

Figure 4 Study population Paper I



Observation time and age at follow-up are presented as median (range). Other values are number (%).
[1] p:<0.01, all other comparisons p:>0.05.[2] No returned questionnaire

Figure 5 Study population Paper II



Observation time and age at follow-up are presented as median (range). Other values are number (%).
[1] p:<0.01, all other comparisons p:>0.05.[2] No returned questionnaire

A second follow-up questionnaire-based survey (FU-2) was performed in 2007-2008 median 8 (range 6 – 9) years after FU-1. Surviving TCSs were asked to have a clinical examination and blood sampling at their family doctor's office. The blood samples were immediately mailed to the NRH where all biochemical analyses were performed. Of the 567 remaining patients who were invited to FU-2, 430 (76%) returned the questionnaire (Figure 5). At the time of FU-2, 19 men had died; 4 had died from CVD and 10 from cancer.

In Paper II all patients who participated in FU-1 were considered for inclusion as they were aged ≤ 60 years at FU-1, had no known second cancer and no apparent acute or chronic infections at blood sampling (FU-1). A criterion for inclusion was a valid CRP at FU-1. Thus, 36 patients in whom CRP was missing due to logistic reasons were excluded. In order to be certain of excluding all patients who may have an underlying infection, men with CRP > 10 mg/L (n = 17) were excluded. After the exclusion of 53 men, 586 patients were included in the study and were eligible for analyses of a second cancer or a cardiovascular event (myocardial infarction, stroke, revascularization, and hospitalization for heart failure) after FU-1. A total of 15 patients who in the questionnaire reported a cardiovascular event prior to FU-1 were excluded. Thus, 571 patients were eligible for the analysis of post-FU-1 cardiovascular events.

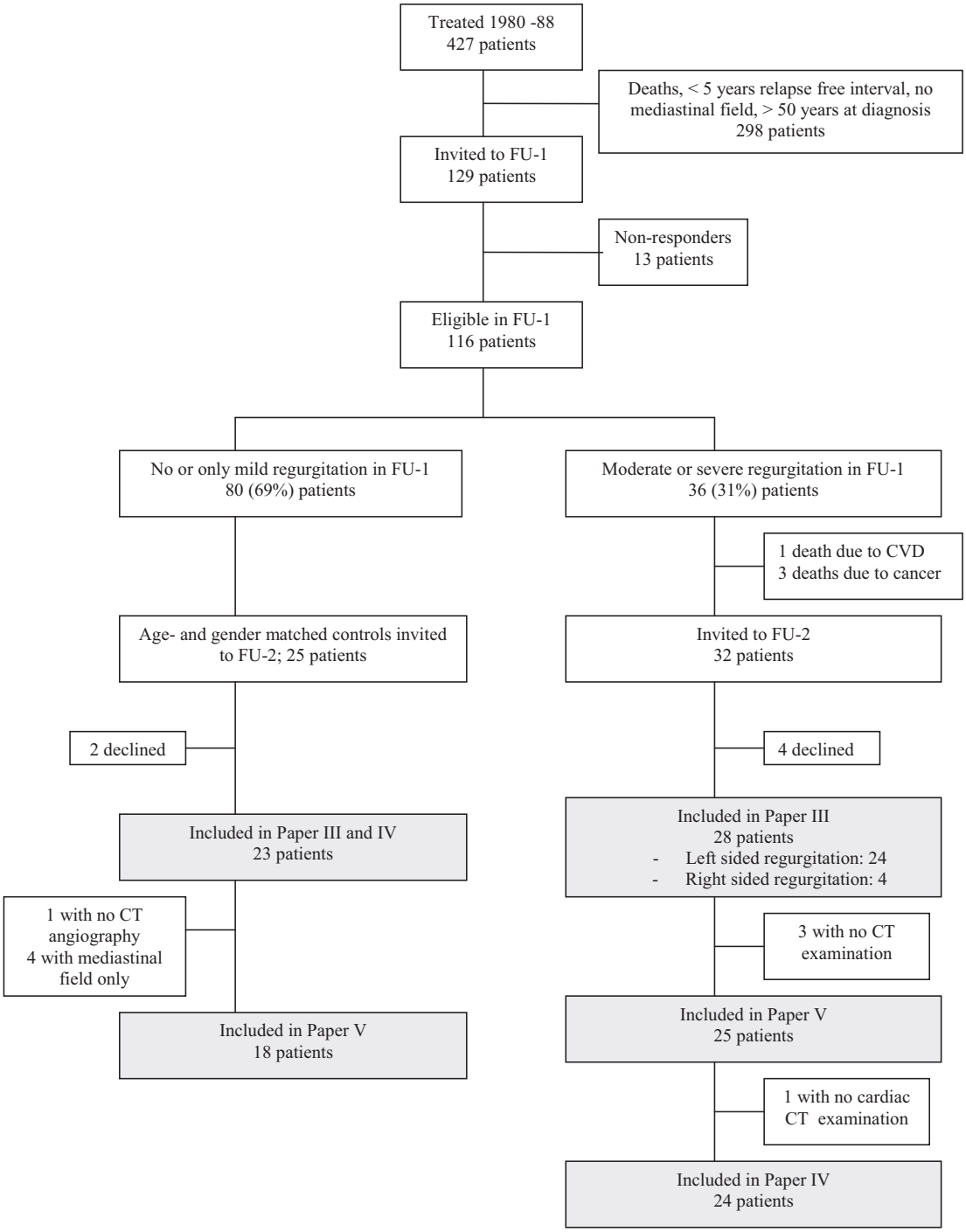
HLSs (Paper III – V)

Paper III is a longitudinal study assessing the development and evolution of valvular and myocardial dysfunction related to treatment with mediastinal radiotherapy and anthracyclines in HLSs. Between 1980 and 1988 427 patients were treated for HL at the NRH. In 1993 our group invited HLSs to perform echocardiography (FU-1) if they fulfilled the following criteria; mediastinal radiotherapy (with or without chemotherapy), age < 50 years at diagnosis and relapse-free survival for > 5 years (Figure 6).[62] The median observation time at FU-1 was 10 years. All of the 32 surviving patients with moderate and severe regurgitations at FU-1 were in 2005 invited to participate in a second echocardiographic examination (FU-2). From the group of 80 patients without or with only mild valvular regurgitation we invited 25 age- and gender-matched survivors

for comparison. Six patients declined. Of the 51 remaining participants who were included in Paper III, 28 had at least one valve with moderate or severe regurgitation at FU-1. Four patients had either moderate pulmonal (n = 2) or tricuspid (n = 2) regurgitation but no or only mild aortic or mitral regurgitation. Thus, 24 patients had moderate mitral and/or aortic regurgitation. The remaining 27 patients demonstrated no (n =14) aortic or mitral regurgitation or only mild (n =13) aortic and/or mitral regurgitation. Prior to the re-examination at FU-2 three patients had received mechanical valve replacement (for aortic regurgitation, aortic stenosis and mitral regurgitation respectively), and for these patients the last preoperative echocardiography was included in FU-2 as the second follow-up examination. A cardiovascular event was in Paper III-V defined as myocardial infarction, percutaneous coronary intervention or coronary artery bypass graft surgery, valvular surgery, stroke or TIA.

In Paper IV the cross sectional study aimed to quantify coronary artery calcium in HLSs and relate it to the presence and absence of CAD. All patients who were included in paper III were eligible for the study of coronary calcification. In order to be included for the analysis of coronary calcifications the participants needed a valid CT examination of the heart. Four patients were excluded; one had undergone a CT angiography of the neck vessels, but unfortunately no CT examination of the heart had been performed while three patients did not attend the CT examination. Two of the three patients who did not attend the CT examination had already received a mechanical valve and one was living abroad. In total, 47 patients with a valid CT examination of the heart with the estimation of coronary artery calcium score (CACS) were included.

Figure 6 Study populations in Paper III, IV and V



Paper V is based on longitudinal assessments. The aim was to examine whether traditional risk factors and inflammatory markers could predict atherosclerosis and the presence of peripheral endothelial dysfunction in HLSs. Furthermore, we examined if atherosclerosis in the irradiated arteries was associated with peripheral endothelial dysfunction and markers of inflammation. A criterion for inclusion was that all patients had received radiotherapy against the neck vessels. Thus, 43 of the 51 patients included in Paper III and who had been treated with mantle field radiotherapy were eligible for the study of extra-coronary atherosclerosis as assessed by the CT angiography of the mediastinum and neck vessels.

The Health Study of Nord-Trøndelag

For comparison of lipid-related markers and the frequency of the metabolic syndrome, 15 individuals from the second Health Study of Nord-Trøndelag County, Norway (HUNT-2) were randomly selected for each patient included in this study, matched for age and gender.[135] The HUNT study consists of 3 surveys and is a longitudinal population-based epidemiological study in Nord-Trøndelag for the assessment and development of somatic and psychological issues. The compliance rate in HUNT 2 was 71% and contained about 65 000 participants aged 20 years and older. The control group was considered to be representative for age-matched Norwegian males.[135] We retrieved data on systolic and diastolic blood pressure, the use of antihypertensive medication, body mass index, HDL-cholesterol and LDL-cholesterol. Blood samples were not available for the control subjects for evaluation of serum indices for inflammation and endothelial dysfunction.

2.2 Treatment of TC and HL

Principles for treatment of TC during 1980 – 1994 and HL during 1980 – 1988 in Norway are described in chapter 1.

According to the treatment policies TC patients were divided into four groups:

1) Surgery only.

2) Infradiaphragmatic therapy with the use of the dog-leg technique.

3) Cisplatin-based therapy only

4) Cisplatin-based chemotherapy and infra-diaphragmatic radiotherapy. Only one patient had received additional mediastinal radiotherapy.

Our HLSs had all received mantle field or mediastinal field only at a dose of median 40.0 (range 27 – 44) Gy. No subcarinal blocks or cardiac shields were used (Figure 5). In Paper III, IV and V 40, 36 and 36 patients respectively were diagnosed with stage I or II HL. Fourteen patients were treated with only radiotherapy while the remaining patients with risk factors had also received concurrent chemotherapy (ChlVPP/ABOD or ChlVPP or ABOD alone). In Paper III, IV and V only 11, 11 and 8 patients respectively had been diagnosed with stage III and IV HL. They had been treated with ChlVPP and ABOD or ChlVPP only. Table 5 summarizes the different treatment principles in TCSs and HLSs.

2.3 Methods

Questionnaire

The questionnaire mailed to TCSs and HLSs consisted of validated instruments and sample items, all selected to allow comparison with the general population in Norway. Information was obtained on smoking habits, physical activity, educational level, family status, medication and the occurrence of diabetes mellitus type I and II. Patients were categorized as current smokers or persons who had quit or never smoked, and as physically active or inactive. Two questions were used to assess low physical activity (for example walking) and high physical activity associated with breathlessness and sweating. Physical inactivity was defined as the absence of high physical activity and less than 1 hour of low physical activity during one week. Patients were categorized to the presence or absence of education at university level and into whether they lived alone or were married/cohabitant. The patient was categorized to have diabetes according to the questionnaire.

In Paper II we extracted information from the questionnaire answered at FU-2 about first-time post-FU-1 cardiovascular events (myocardial infarction, stroke, revascularization, and hospitalization for heart failure). All self-reported cardiovascular events were validated by the patient's hospital medical record.

The Cancer Registry of Norway

With permission from the Regional Ethical Committee, the Norwegian Directory of Health and the Data Inspectorate we received data (type of cancer, date of diagnosis) about non-germ cell cancer diagnosed after FU-1 among our eligible TCSs as registered by December 2008. Furthermore, there were also data on all deaths as well as the cause of death registered after FU-1. Thus, we were able to identify those who died from cardiovascular causes. The diagnosis of localized prostate cancer (n = 5) was not considered to be a valid event as this malignancy is increasingly detected by PSA screening and many of these patients are not at risk of developing invasive cancer and die from other causes.

Clinical examinations

Blood pressure was measured manually or with automatically devices. Weight with only light clothing and without shoes and height were registered. Body mass index was calculated as weight in kilograms divided by the square of height in meters.

Biochemical analyses

Non-fasting blood was sampled between 9 am and noon into pyrogen-free, pre-cooled vials without additives (serum) or with EDTA as anticoagulant (plasma). The tubes were centrifuged at 1,000g for 10 minutes within 30 minutes (plasma) or allowed to clot before centrifugation (serum). Because of in vitro production of pro-inflammatory biomarkers the application of EDTA was added to all samples in order to impair ex vivo production of inflammatory markers. Serum levels of sCD40L were determined by enzyme immunoassay (EIA, R&D Systems). Plasma levels of vWF were determined by EIA as reported.[136] High sensitivity CRP in plasma was determined by a high-sensitive particle-enhanced immunoturbidimetric assay (Roche Diagnostica) and was analyzed both in FU-1 and FU-2. Plasma levels of leptin were analyzed by radio immunoassay (Linco Research, Inc.). Plasma levels of total cholesterol, low-density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol and triglycerides were measured enzymatically on a Roche/Hitachi 917 analyzer (Roche Diagnostics). Serum levels of apolipoprotein A-1 (apo A-1) and apolipoprotein (apo B) were analyzed on a Behring

Nephelometer Analyzer II (Behringwerke). Plasma homocysteine concentration was determined by HPLC.[137] Lp(a) in plasma was determined by an immunoturbidimetric assay on a Modular platform (Roche Diagnostica). Serum levels of luteinizing hormone (LH), testosterone and sex hormone binding globulin (SHBG) were analyzed by immunoassays.[104] Serum levels of free testosterone were calculated as reported elsewhere.[138]

Echocardiography

Echocardiography was used to determine valvular dysfunction and cardiac function including ventricular dimensions in both systole and diastole. We performed 2-dimensional (2D) transthoracic echocardiography with a Vivid 7 scanner (GE Vingmed Ultrasound). Three consecutive heart cycles from the parasternal, long axis, short-axis, apical four chamber, and subcostal views were obtained. The digital loops were stored and analyzed by EchoPac software (GE Vingmed Ultrasound). In M-mode we recorded measurements of left atrial diameter (LA), left ventricular end-diastolic and end-systolic diameters (LVEDD and LVESD, respectively), left ventricular posterior wall (LVPW), interventricular septum thickness (IVSd), and LV-SF. Cardiac index (CI) was calculated from cardiac stroke volume, heart rate, body height, and body weight. Left ventricular ejection fraction (LVEF) was assessed by the modified Simpson's rule. LVEF and left ventricular end diastolic volume were measured only in ECHO 2005. 2D and pulsed Doppler echocardiography were used to estimate valvular regurgitation and stenosis (mild, moderate, or severe) according to published recommendations.[139, 140] Left ventricular remodeling was defined as a thinning of ventricular walls and dilatation of the ventricle between FU-1 and FU-2. All examinations were carried out according to a standardized protocol by physicians and sonographers at the Department of Cardiology, and all the data were interpreted by a single experienced cardiologist (Thor Edvardsen), blinded for patient history (including the results from FU-1) and treatment.

The degree of valvular dysfunction was classified according to recommendations from the American Heart Association and the European Society of Cardiology.[139, 140] Aortic stenosis is also graded as mild, moderate or severe based on the valve area, mean pressure gradient in systole and the jet velocity during systole.[139]

To analyze changes in the myocardial function we calculated the percent change in LVEDD, LVESD, IVSd, LVPW, LV-SF and CI from the first to the second follow-up. LV-SF is defined as the percent change in the internal diameter from diastole to systole. CI is defined as stroke volume multiplied with the heart rate divided by body surface area.

Multidetector computed tomographic scan (MDCT) of the heart

Coronary artery calcium correlates closely with overall burden of atherosclerotic plaques. Quantification of coronary artery calcium has been suggested as a surrogate endpoint of coronary artery disease.[141-144] Their importance as a risk factor of coronary artery disease in in HLSs has not been properly defined. Therefore, all HLSs were examined in a 64-detector computed tomography unit (GE LightSpeed 64 VCT, GE Healthcare, Milwaukee, WI) including a dedicated work-station (Advantage Window 4.3.3). The scan was performed with ultra-fast acquisition time and is electrocardiographic triggered near the atrial systole in order to achieve a scan when the heart is moving minimally. The acquisition time is only 100 ms producing 3 mm non-overlapping slices. The quantification of coronary artery calcium is done by a computerized program (SmartScore 3.5, GE Medical Systems, Milwaukee, WI) giving the CACS as both Agatston score and volume score. In the 3 mm thick non-overlapping slices a hyperattenuated lesion above a threshold of 130 Hounsfield Units indicates the presence of coronary artery calcium. Additionally this lesion has to cover an area of at least 1 mm^2.[145] The Agatston score was calculated by multiplying the lesion area in every 3 mm slice by a density factor derived from the maximal Hounsfield unit following manner: 1 for lesions whose maximum density were 130 to 199, 2 for lesions 200 to 299, 3 for lesions 300 to 399, and 4 for lesions >400.[146] With the volume score the input data is sampled at several intermediate cross sections between the original 3 mm slices in order to reconstruct the precise volume of the atherosclerotic plaques. Total volume or Agatston score were calculated as the sum of scores in the left main artery (LMA), left anterior descending artery (LAD), circumflex artery (CX) and right coronary artery (RCA). The patients were categorized according to the following ranges for volume

score; 0, 1-199, 200-999 and ≥ 1000. Even if the methods are slightly different, the correlation between the two methods is excellent.[147]

The assignment of segments to coronary arterial territories was done in accordance with clinical recommendations.[148] Three patients underwent percutaneous coronary intervention with implantation of a stainless steel stent in 1 coronary artery. Arterial segments with stent were excluded from CACS calculation because of the stent artefact. The scoring was carried out independently by two experienced radiologists (Rune Andersen and Anne Günther). The interobserver variability was 4.7%.

CT angiography of pre-cranial arteries

Estimation of carotid stenoses by CT angiography has shown a strong correlation with the gold-standard conventional catheter angiography.[149-151] Furthermore, CT angiography displays all extra-coronary arteries included in the radiation field and gives a detailed picture of the atherosclerotic burden in HLSs. All patients were examined in a 64-detector computed tomography scanner (GE LightSpeed 64 VCT, GE Healthcare, Milwaukee, WI). During an i.v. bolus injection of contrast medium (Visipaque 320mg/ml, 60 ml), a CT volume scan covering the anatomy from the aortic arch to the skull base was performed. The native slice thickness of 0.625 mm was reformatted to 2.5 mm axial, 1 mm coronal and sagittal maximal intensity projection (MIP) slices. The degree and extent of atherosclerosis was assessed by an experienced neuroradiologist (B.N). For each individual patient, the sites of detectable atherosclerotic abnormalities were compared to the radiation field margins by Bård Nedregaard, Alexander Fosså and Torgeir Wethal and scored as within field (>1 cm from field boundary) or outside field. Vessel lumen diameter measurements were performed in all participants. The minimal luminal diameter at the site of an atherosclerotic lesion was measured together with the normal luminal diameter of the vessel at the closest adjacent segment to the atherosclerotic lesion, from which the percent of luminal narrowing was calculated. Atherosclerotic plaques with or without calcifications were specifically registered without regard to stenoses.

In an effort to provide an estimate of the amount of atherosclerosis in the irradiated pre-cranial arteries we developed a pre-cranial atherosclerosis score (PAS) representing a

semi-quantification of pre-cranial artery atherosclerosis. The following pre-cranial arteries were scored; the brachiocephalic trunk, the subclavian arteries, the common carotid arteries, the carotid bifurcations, the internal carotid arteries, and the vertebral arteries. In each of these 11 arteries we calculated if the atherosclerotic lesion had less than 5% luminal narrowing (score 1), with 5-30% narrowing (score 2), with 30-50% narrowing (score 3) and the presence of >50 % lumen reduction (score 4). A total score was then calculated for each patient, ranging between a score of 0 to 44.All arteries were equally weighted without respect to differences in the length and normal luminal diameter of the vessels included. The subclavian arteries were scored before the departure of the vertebral artery. As an alternative measure for pre-cranial artery atherosclerosis we dichotomized the patients according to the presence or absence of any degree of atherosclerosis in both the internal carotid artery including the carotid bifurcation and at the origin of the great arteries from the aorta.

Strain-gauge plethysmography

Peripheral endothelial function was assessed with a mercury-in-silastic strain gauge plethysmography (Hokanson EC6, Bellevue, Washington). This is a non-invasive method and several studies have demonstrated an impairment of the endothelial function in patients with cardiovascular risk factors.[152-154] The endothelium of the arterial vasculature is a highly specialized and active tissue which modulates the vascular tone, and release signal substances activated during inflammation.[155] Endothelial dysfunction precedes the development of atherosclerosis and serves as an early marker of CVD.[156, 157] Strain-gauge plethysmography measured forearm blood flow (ml/100 ml forearm/min). In the supine patient the left arm was elevated about 5 centimeters above the heart level. A gauge was placed around the widest and most muscular part of the forearm and a cuff was placed proximal for the gauge. A venous occlusion pressure of 50 mm Hg was used to measure the resting blood flow which was taken as the average of 5 consecutive measurements. The cuff was inflated to suprasystolic pressures in exactly 5 minutes to induce ischemia. Peak reactive hyperemia was measured immediately (within 4 seconds) after cuff deflation using venous occlusive stasis. Peak reactive hyperemia was caused by instant relaxation of peripheral resistance arteries mediated by local vasoactive ischemic

metabolites like adenosine where contributing factors are NO, prostaglandins and activation of ATP sensitive K+ channels [158, 159]. The available amount of NO is dependent on asymmetric dimethylarginine (ADMA), an inhibitor of endogenous nitric oxide synthase. The decreased peripheral resistance leads to an increased shear stress caused by the blood flow leading to a secondary release and production of NO. The early peak reactive hyperemic flow was taken as a measure of endothelial function.

Composite measures

We examined other possible risk factors like the metabolic syndrome and hypogonadism. The metabolic syndrome has been associated with CVD. [160, 161] The metabolic syndrome was defined according to the presence of 3 or more of the following 4 criteria**:** (1*)* systolic blood pressure ≥130 mm Hg, diastolic pressure ≥85 mm Hg, or use of antihypertensive medication, (2) serum triglycerides ≥1.70 mmol/L, (3) HDL cholesterol <1.04 mmol/L, and (4) body mass index ≥30 kg/m^2 (a modified definition based on the Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III), 2001).[162] The measurement of glucose was not included in our modified definition of the metabolic syndrome because of non-fasting values. Additionally, TCSs were classified as having hypogonadism if any of the following 3 conditions were met: serum testosterone <8 nmol/L, serum LH >12 U/L, or regular use of exogenous testosterone.[104] Testosterone/LH ratios were calculated for use as an additional indicator of gonadal function.

2.4 Statistical analyses

Data were analyzed using SPSS 12.0 - 14.0 (SPSS Inc) and R 2.1.1 for PC. Mann-Whitney and Kruskal-Wallis tests were used to compare skewed data, and Chi-square or Fisher's exact tests were used to compare categorical data. The Student's t test was used to compare normally distributed continuous data. The Mann-Whitney test was used for highly skewed continuous data. Pearson or Spearman correlation analysis was used to investigate the relationships between continuous data (i.e. age-related variables,

echocardiographic parameters, lipid-related markers, inflammatory markers, peripheral endothelial dysfunction, CACS and PAS).

In Paper I the inter-group comparisons of the inflammation indices hsCRP, vWF and sCD40L were adjusted for age, gonadal hormones, lipid-related indices, body mass index and systolic and diastolic blood pressure by application of a general linear model that used Gamma distributed residuals and a logarithmic link-function.[163] This test can be described as an expanded ANOVA which takes into consideration possible confounders.

Binary logistic regression was performed to reveal the association between the metabolic syndrome (Paper I), aortic stenosis (Paper III), new onset valvular regurgitation (CAD (Paper IV), the presence of extra-coronary atherosclerosis (Paper V) and the various treatment-related, clinical-related or biochemical indices. Linear regression analyses were performed with the percent changes in each of the 6 echocardiographic variables (Paper III) and PAS and CACS (Paper V) as dependent variables. Parameters with a p-value <0.10 in univariate analyses were included in a final multiple logistic or linear regression analysis. A p-value <0.05 was considered statistically significant. Results were given as β or odds ratios (ORs) with 95 % confidence intervals.

ROC analyses were used in Paper II to determinate the optimally discriminating CRP value for the composite end point of CV event and a second cancer. The identified cut-off level was used to construct groups of patients with CRP < 1.5 mg/L and ≥ 1.5 mg/L. The cumulative incidence of CVD and a second cancer as separate end-points were then computed for these two groups using Kaplan-Meier plots. Additional Kaplan-Meier plots were drawn to illustrate the impact of different treatment modalities, separating irradiated patients (+/- chemotherapy) from those having surgery and/or chemotherapy. Differences in time to event between CRP groups or treatment groups were assessed using log-rank tests. Uni- and multiple Cox-regression models were fitted to explore the relation between clinically important predictors of a second cancer and cardiovascular events. The limited number of each of these end-points allowed only three variables to be included in the multiple Cox-regression analyses, all meeting the proportional Hazard assumption.

Results of the Cox-regression analyses were presented as hazard ratios (HRs) with 95 % confidence intervals (CI). A two-sided p-value <0.05 was considered statistically significant.

3 RESULTS

Paper I

In TCSs we examined the implication of the treatment on the inflammatory markers CRP, vWF and sCD40L and lipid-related markers (total cholesterol, LDL – cholesterol, HDL – cholesterol and triglycerides), which are associated with increased cardiovascular risk

All patients treated with radiotherapy and with a diagnosis of seminoma, had higher levels of CRP and soluble CD40 ligand compared to the SURG group. After adjustment for cardiovascular risk factors (age, blood pressure, lipid-related markers, gonadal function) CRP and sCD40L was respectively, 0.35 (± 0.13) mg/L and 0.27 (± 0.08) pg/mL ($p < 0.01$) higher in the radiotherapy group as compared to patients treated with surgery only.

Patients who had received chemotherapy had lower levels of high density lipoprotein cholesterol and an increased apolipoprotein B/apolipoprotein A-1 ratio than those treated with surgery only ($p < 0.001$ and $p = 0.01$ respectively). In the chemotherapy group the number of men with HDL cholesterol levels less than 1.04 mmol/L exceeded that observed in the age-matched reference control group (41.4 vs. 29.6 %, $p<0.001$. The prevalence of the metabolic syndrome was only 6.3% in patients treated with surgery. In contrast; this prevalence was 15.2% in the radiotherapy group and 17.6% in patients treated with chemotherapy. Using the matched control groups as a reference, we observed an elevated prevalence of the metabolic syndrome among chemotherapy-treated patients ($p = 0.07$) and a low prevalence among those who were treated with surgery only ($p <0.01$). Additionally, hypogonadism was significantly more prevalent in the chemotherapy group (13%) compared to the surgery group (5%, $p = 0.01$).

Paper II

The aim was to examine if inflammatory markers, lipid-related markers or traditional clinical risk markers measured during the follow-up of TCSs were able to predict cardiovascular events and second cancer. The post-FU-1 development of a non-germ cell

cancer or a first-time cardiovascular event represented the end-point. After FU-1 31 (5.3%) of 586 patients developed non-germ cell cancer (excluding localized prostate cancer) while 28 (4.9%) developed CVD. Cox regression analyses showed that patients with CRP ≥ 1.5 mg/L had 2.21 (95% CI 1.04 – 4.70) times higher risk of developing non germ cell cancer and 2.79 (95% CI 1.22 – 6.34) times higher risk for CVD compared to patients with a CRP < 1.5 mg/L at FU-1. Four patients developed non-germ cell cancer within 2 years after the measurement of CRP. The estimated risk of a second cancer was 2.16 (95% CI 0.97 – 4.82) after excluding these patients. There was a strong relationship between CRP-levels at FU-1 and FU-2 (Spearman $r = 0.59$, $p < 0.001$).

Radiotherapy was associated with 2.56 (95% CI 1.19 – 5.51) times higher risk for developing non-germ cell cancer in comparison to patients treated with surgery with or without chemotherapy. Additionally, after the exclusion of the 22 patients who were registered to be treated with both chemotherapy and radiotherapy, treatment with infra-diaphragmatic radiotherapy only was associated with increased risk of a second cancer (HR 2.29 (95% CI 1.03 – 5.07)). Radiotherapy was not associated with CVD (HR 1.40 (95% CI 0.67 – 2.95).

Paper III

This paper tested if the long-term development and evolution of valvular dysfunction in irradiated patients were independent of a previous diagnosis of valvular regurgitation observed during follow-up of HLSs. Furthermore, we tested if the use of anthracyclines was associated with the development of myocardial dysfunction even between FU-1 (median 10 (5 – 13) years after treatment) and FU-2 (median 22 (11 – 27) years after treatment). Median age at diagnosis was 26 years (range 14 – 42 years), and 67% of the participants were women. Thirty-six patients received additional chemotherapy. Antracyclines were given to 28 (55%) of the patients

The second echocardiographic study at FU-2 demonstrated that 10 out of 27 (37%) patients with only mild or no aortic or mitral regurgitation at FU-1 had developed moderate regurgitation in either or both the aortic or mitral valve. Eight of 24 (33%)

patients with moderate (n=23) or severe (n=1) regurgitation in the aortic or mitral valve at FU-1 had progressed to severe regurgitation, developed moderate regurgitation in a previously normal or mild regurgitant valve or had received valvular replacement. A total of 20 of all patients (39%) patients had developed mild to severe aortic stenosis and three patients had received valvular replacement. Of those who had aortic stenosis 13 were mild, 3 moderate and 4 severe. The estimated aortic valve area among the 20 patients was median 1.6 (range 0.6 – 3.2) cm^2, and the median valvular gradient was 11 (range 5 - 45) mm Hg.

The appearance of both the aortic and mitral dysfunctional valve was dominated by different degrees of degeneration including leaflet thickening, reduced leaflet movements and calcification. Calcification was observed among those with moderate/severe stenosis or regurgitation. Regurgitation was observed in the first decade after treatment, while aortic stenosis first became apparent during the second decade after treatment. New-onset valvular regurgitation did not demonstrate any relation to clinical or treatment related parameters. Binary logistic regression with the presence of aortic stenosis as the dependent variable failed to show any relationship with chemotherapy, the presence or absence of valvular regurgitation at FU-1 or any of the registered clinical parameters.
In a multiple linear regression the use of anthracyclines predicted left ventricular remodeling between FU-1 and 2 compared to those who did not receive anthracyclines. Left ventricular end systolic diameter increased (ß 0.09 (95% CI 0.01 – 0.17), p = 0.04) while the thickness of the left ventricular posterior wall (ß - 0.18 (95% CI -0.33 – -0.03), p = 0.02) and interventricular septum (ß – 0.16 (95% CI - 0.30 – -0.03), p = 0.02) decreased.

Paper IV

This study examined the relationship between CAD and CACS in forty-seven HLSs treated with mediastinal radiotherapy 22 ± 3 years earlier. Total volume score was higher in 7 patients (15%) with verified CAD (median 439 (range 8 – 2057) compared to those without (median 68 (0 – 767), p = 0.022). LMA, LAD and CX had a higher CACS in patients with verified CAD compared to patients without, whereas CACS for RCA was

comparable in the 2 groups. Ten patients had volume score above 200; five had undergone revascularization of the coronary arteries.

Multiple logistic regression analysis adjusting for the presence of aortic stenosis, pro-BNP, gender and age at diagnosis of HL revealed that CACS was the only parameter associated with verified CAD with an odds ratio of 2.1 (95% confidence interval 0.98 – 4.94, $p = 0.057$) for every 200-U increase in CACS. Use of an Agatston score yielded similar results, providing an excellent correlation with volume score ($r = 0.996$, $p < 0.001$).

Paper V

The objective was to examine the association between the extent of atherosclerosis within the radiation field including the coronary arteries, peripheral endothelial dysfunction, circulating markers of inflammation and traditional risk factors measured at FU-1. A total of 141 atherosclerotic lesions were found within the radiotherapy field in the 43 HLSs and 120 (85%) of the lesions were calcified.

Multiple linear regression analyses showed that only cholesterol measured at FU-1 was a predictor of both PAS (β 3.29 (95% CI 1.90 – 4.69), $p < 0.001$), CACS (β 308 (95%CI 213 – 403), $p < 0.001$) and a decrease in peripheral endothelial function (β 2.74 (95%CI 0.47 - 5.01, $p = 0.02$). Multiple logistic regression analyses showed that cholesterol measured at FU-1 was the only predictor of pre-cranial atherosclerosis (OR 3.86 (95% CI 1.24 – 12.01), $p = 0.02$).

Multiple linear regression analyses showed that for every increase in CACS by 100 units there was an increase in PAS by 0.72 (95%CI 0.43 – 1.01), $p < 0.001$). Furthermore, every increase in peripheral endothelial function by 1 ml/100 ml forearm/min was also associated with a decrease in PAS by 0.21 (95%CI 0.03 – 0.38), $p = 0.02$). At FU-2 an increase in CACS by 100 units (OR 2.64 (95% CI 1.33 – 5.26, $p = 0.01$)) was associated with the presence of pre-cranial atherosclerosis.

4 DISCUSSION

4.1 Methodological considerations

4.1.1 Epidemiological studies

Epidemiology deals with the variations of incidence and prevalence of diseases in different populations and aims to identify etiological factors which determine this variation.[164] Epidemiologic studies such as cross-sectional studies, longitudinal studies and case-control studies are used to measure the effect of individual and population based interventions. Cross sectional and longitudinal studies are observational, i.e. we do not control for any other risk factors when the patients are included except for the exposure that we are interested in. A cross sectional design is useful to determine the prevalence of a relatively frequent disease or define risk factors for a disease across groups. Any causal relationship can not been defined. Nevertheless, cross sectional studies are useful to identify possible risk factors and generate hypotheses about causes of a disease. Risk factors may be difficult to identify because of recall bias. Longitudinal studies are suitable to estimate the incidence of a disease and establish a causal relationship between exposure and disease.

4.1.2 Systematic errors (Bias)

Our results may be hampered by bias which is divided into selection bias, information bias and confounders. Together they constitute the internal validity of the studies. Internal validity describes whether the results can be representative for the study population.

4.1.2.1 Selection bias

In Paper I we included all surviving and disease free men who had been treated for unilateral TC at the NRH and had not developed another malignancy at the time of FU-1. TCSs above the age of 60 years and those treated with both radiotherapy and chemotherapy were excluded from Paper I. Of those eligible for the study 75% participated. Non-responders and responders displayed similar baseline characteristics (age, treatment, histology indicating that the participants were representative for the cohort of TCSs treated at the NRH. However, non-responders may differ from responders

on characteristics not measured at baseline or follow-up. Non-responders may express more morbidity with impaired quality of life and depression than responders and are less likely to appear at a follow-up study. This phenomenon is well known as up to 10% of the non-responders in the HUNT survey did not attend due to immobilization.[135]
In Paper II we included patients treated with chemotherapy (n=221), radiotherapy (n=211) and the combination (n=22). The latter group was difficult to analyze separately due to low sample size and the possible interaction between chemotherapy and radiotherapy. Since the goal in Paper II was to examine the predictive value of hsCRP independent of the treatment group we included this small group as well. Patients without a CRP value did not differ from those with a CRP with respect to age at FU-1, type of cancer treatment, systolic and diastolic blood pressure and current smoking. We therefore believe that the results reported by this study may be representative for Norwegian TCSs. The participants in Paper I and II represented over 50% of the Norwegian TCSs included in a nation wide study and are comparable to the whole cohort of Norwegian TCS.[76]

In Paper III we included HLSs who had no, mild or moderate valvular regurgitation at FU-1. This may have caused a selection of those who also otherwise are vulnerable to radiotherapy and likely to develop atherosclerosis, cardiovascular risk factors and cardiovascular disease itself. However, the progression and development of valvular regurgitation and stenosis were independent of their valvular status at FU-1. Furthermore, the prevalence of CVD in our 51 HLSs were comparable to those presented by other studies.[57, 59, 63]

The external validity of the results is probably high, both for TCS and HLSs. The age at which the cancer was diagnosed and the symptoms and the histology of the cancer showed typical characteristics, and the treatment they have received were according to the guidelines at that time as presented in the Method section. Nevertheless, the patients in the presented Papers consist of a homogenous ethnic group (Caucasians). Furthermore, also socioeconomic factors are important and interact with the incidence and prevalence of TC and HL, the histopathology, the response to treatment and may eventually have an impact on the long-term consequences of the treatment. The HLSs in the present studies

are mainly women making it difficult to generalize the results to men and the small sample size indicate that we have to be careful to make conclusions for HLSs in general. In paper I we used controls from the Nord–Trøndelag County. Even though it has been reported that the controls are fairly representative of the Norwegian population, they did differ from our population of TCSs who more often had higher education, were non-smokers and were less likely to be living alone.[135] This may have caused an overestimation of the presence of risk factors in controls compared to our TCSs. The controls were characterized by low HDL cholesterol and an increased prevalence of the metabolic syndrome compared to patients treated with only surgery.
In Paper III, IV and V there was no control group of healthy individuals. In the Framingham population between 40 and 60 years old the prevalence of moderate or severe mitral regurgitation in both men and women was 1%, and the prevalence of moderate or severe aortic regurgitation in men and women was only 0.4 and 0.1%, respectively.[165] In Lund et al's study reporting on the results from FU-1 31% HLSs had developed a moderate regurgitation while none of the 40 age- and gender matched controls displayed moderate valvular regurgitation.[62]

4.1.2.2 Information bias

A systematical misclassification of measurements is regarded to be of minor importance with variables measured at the out-patient clinic or at hospital. These variables included weight, height (for the calculation of body mass index) and biomarkers (CRP, vWF, sCD40L, cholesterol, gonadal hormones etc) where the issue of reliability probably is more important. All non-fasting samples were stored at -70°C and thawed less than three times. This procedure decreased the influence which may be caused by temperature, storage time and freeze and thaw cycles as biomarkers are relatively unaffected by up to three freeze and thaw cycles. Enzyme linked immunosorbent assays (ELISAs) were measured with both inactive and active forms of sCD40L and vWF and the measured levels can not be compared with the levels measured in other studies as the unique recognition profile of the antibodies will differ between kits from different manufactures. The intra- and inter-assay coefficients of variation obtained in our laboratory were less than 10% for all biochemical assays.

In TCSs the blood pressure was measured only once while the blood pressure measured in the controls from HUNT2 was given as the mean of the 2nd and 3rd measured systolic and diastolic blood pressure. This may cause an overestimation of the blood pressure among TCSs when compared to the controls. An additional problem with TCSs compared to controls is that there are preliminary results indicating that TCSs more frequently visit the general practitioners compared to the general population with the consequence that the use of hypertensive medication (and possibly cholesterol medication) is more frequent among TCS than the controls (Dahl, in preparation).

Information on education, smoking, physical activity and diabetes was assessed only by the questionnaire for TCSs and by a personal systematic interview of HLSs. Generally people often describe themselves as healthier than they actually are and the expressions of risk factors in the individuals may change over time; some smokers quit, other becomes more or less physical active. Additionally, the problem of misclassification may be due to poorly defined questions in the questionnaire. For example, the questions regarding physical activity could be more specified.

During the work with the results from FU-1 in the TCSs we decided to analyze whether there were any treatment related differences in the clustering of cardiovascular risk factors. Therefore, we decided to use a modified definition of the metabolic syndrome. Since the blood samples were not collected in the fasting state we did not use elevation of serum levels of fasting glucose as a criterion. The use of other criteria for this syndrome had perhaps yielded other estimates of the prevalence of this syndrome and the use of non-fasting triglyceride values may have caused an overestimation of the metabolic syndrome incidence. However, this would be expected to introduce similar amounts of error into the analyses of both our cancer survivors and the HUNT control group since only non-fasting triglyceride values was available for the controls.

In Paper II we used are under the curve analyses to find the optimal cut off for the level of hsCRP which was best suited to identify cardiovascular disease and second cancer. A pitfall with this assumption is that the cut off is “designed” for this study population and

may not be applicable to other groups of TCSs. However, previous studies and guidelines have demonstrated increased risk of CVD or cancer with the use of a cut-off ranging from 1 - 3 mg/L which make our cut-off at 1.5 mg/L reasonable.[121, 122, 166] In Paper II the use of the alternative cut offs 1 and 3 mg/L yielded similar results for the prediction of cardiovascular events, but there was only the cut off at 1.5 mg/L which was able to identify TCSs with a higher risk a second cancer.

In Paper III the classification of valvular regurgitation was performed according to recommended guidelines which have mainly been unchanged since 1993.

In Paper V it is important to emphasize that the presented score only gives a semi-quantitative estimate of the pre-cranial atherosclerotic burden. PAS does not take into account that the volume of the arterial segments included in the score is very different. For example, a defined plaque lesion may be given a low score if present in the volumetric large common carotid artery but a high score in the volumetric small vertebral artery. PAS can not be regarded as a parameter to define those who are at an elevated risk of cerebrovascular disease, since all arteries are equally weighted and a relatively small soft non-calcified plaque in the vertebral artery may be more clinically important than a calcified lesion causing stenosis in the common carotid artery. However, we consider PAS to give an approximate estimate of the atherosclerotic burden in the pre-cranial arteries, which is of importance in the risk estimation of CVD. Importantly, although not validated, the use of either a score or a dichotomization of atherosclerotic lesions to describe the extent of extra-coronary atherosclerosis yielded similar results.

Peripheral endothelial function was assessed by strain-gauge plethysmography. The method is often combined with the use of intra-arterial administration of endothelial dependent and independent vasodilators. Nevertheless, our use of an ischemic stimuli and the subsequent measurement of peak reactive hyperemia without intra-arterial infusion of vasoactive substances was simple and reproducible. The measurement of flow-mediated vasodilatation in the brachial artery by ultrasound could have increased the diagnostic

sensitivity of endothelial function and is today regarded as the most sensitive method to define endothelial dysfunction in conduit vessels.[167]

4.1.2.3 Confounders

Before attributing any difference in outcome between the exposure groups to the exposure itself it is important to examine whether the exposure-outcome association has been affected by other factors that differs between the exposure groups and which may affect the outcome.[168] Such factors are said to confound the association of interest. To solve the problem with confounding multiple regression analyses or stratification are used.

Hypogonadism and low testosterone are associated with endothelial dysfunction, the metabolic syndrome, inflammation and cardiovascular disease.[112, 113] Low testosterone correlated with the metabolic syndrome and a reduced HDL cholesterol. Because it has been demonstrated that reduced fertility and hypogonadism may be evident in TCSs even before the diagnosis and treatment of TC, we can not rule out that atherogenic lipid changes and inflammation observed in TCSs during follow-up may be due to the phenotype and not to the treatment. Several possible confounders that were not adjusted for (education, smoking, physical activity, civil status) may have affected the differences between HUNT controls and TCSs in the prevalence of the metabolic syndrome and its components.

The fact that HLSs were informed about their valvular regurgitation in FU-1 may theoretically influence the patient's behavior and caused life-style changes thus reducing their risk of further progress of valvular dysfunction. We have no information about eventual post-FU-1 life style adjustments of life style or medical treatment in our patients. No known life-style changes will change the natural course of valvular disease. Treatment of hypertension has been suggested to slow the progression of aortic stenosis. Conversely, patients without or with only mild regurgitation in 1993 may have paid less attention to lifestyle and cardiac symptoms, provoking an acceleration of the disease course. This suggestion is not supported by our data: Patients without or mild compared

to moderate regurgitation showed similar characteristics regarding comorbidites, cardiac risk factors and intervention for hypertension and hypercholesterolemia. We therefore believe that our longitudinal findings to a large extent mirror the "natural course" of valvular function after mediastinal radiotherapy for HL. Additionally, the small sample size of HLSs gave some restrictions on the ability to adjust for confounding variables.

4.1.3 Reliability

The precision or the possibility of random error is small with a large sample size. In Paper I 589 TCS were analyzed for inflammatory and lipid-related markers. The intra- and inter-essay coefficients of variations for the measured biomarkers were less than 10% for all assays. The use of MDCT has demonstrated a mean inter-examination variability of 4 ± 2%, and our interobserver variability was only 4.7%.[169] As previously stated CT angiography has demonstrated a strong correlation with conventional cathether angiography in the estimation of carotid lesions.[170] With respect to strain-gauge plethysmography the intraobserver variability for the resting values was 14%. Taken together, the reliability of the applied methods was considered as good.

4.2 Appraisal of the main findings

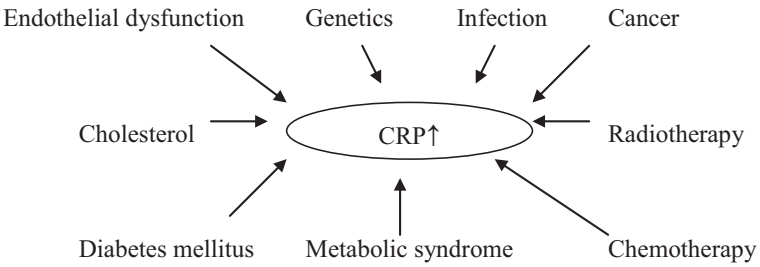
4.2.1 Cancer treatment and atherosclerosis

Radiotherapy

In Paper I the treatment of TC can be considered to be related to long-term biochemical cardiovascular risk factors by different pathways: Radiation treatment was followed by elevated serum markers of chronic inflammation and endothelial dysfunction, whereas chemotherapy was followed by the development of atherogenic lipid changes and of the metabolic syndrome. In Paper V HLSs treated with radiotherapy 20 years earlier were characterized by extensive and typically calcified atherosclerotic lesions affecting all arteries within the radiation field accompanied by peripheral endothelial dysfunction. These findings were presaged by elevated cholesterol measured 12 – 15 years earlier at the first follow-up.

Several studies have demonstrated long-term regional effects of radiotherapy on the vascular system, including accelerated development of atherosclerosis and fibrosis in the irradiated arterial segments and also activated endothelium and decreased endothelium-dependent vasodilatation.[69, 71, 171] We demonstrate that inflammatory indices in our study were elevated up to 20 years after large-field radiotherapy possibly triggered by the radiation causing a self-perpetuating process of inflammation in the endothelium. CRP, a marker of general inflammation, was elevated above 2 mg/L in 24 (51%) of our HLSs. Furthermore, CRP was higher in TCSs treated with radiotherapy as compared to those treated with chemotherapy or only surgery. The level of CRP was not associated with in-field atherosclerosis and peripheral endothelial function in HLSs. A possible relationship can not be excluded because of the small sample size. The augmentation of CRP could be enhanced by several other risk factors as outlined in Figure 7.

Figure 7 Conditions associated with increased CRP



vWF is regarded as a marker of endothelial damage. An in vitro study has shown that vWF is released from isolated vessels early after irradiation but there is conflicting evidence whether vWF is a marker or a cause of CVD.[116, 172-174] We observed an association between vWF and peripheral endothelial dysfunction as well as pre-cranial atherosclerosis. In the TCSs vWF was elevated in patients treated with radiotherapy 11 years after the treatment compared to patients treated with chemotherapy or only surgery. However, this association was not significant after adjusting for other risk factors.

An indirect toxic effect of radiotherapy is suggested by the long-term increase in CRP, vWF and sCD40L. Elevated levels of sCD40L found in TCSs indicate that this process may also include platelet activation and thus not only limited to irradiated sites. vWF may be an important link to explain the association between in-field atherosclerosis and peripheral endothelial dysfunction outside the radiation field. In-field endothelial inflammation with the induction of various cytokines and vWF may cause long-term endothelial dysfunction outside the radiotherapy field. Previous studies have shown impairment of endothelial function in irradiated arteries a few weeks after irradiation for head and neck cancer as well as several years after irradiation for breast cancer, but the impairment was only found within the radiation fields.[72, 73] We demonstrate an association between atherosclerosis within the radiotherapy field and peripheral out-of-field endothelial dysfunction more than 20 years after mantle-field radiotherapy. Although the clinical significance of the peripheral endothelial dysfunction measured in our cohort of HLSs is difficult to assess, it may shed light on mechanisms involved in radiation induced atherosclerosis. It is tempting to speculate that local inflammatory and endothelial processes within the radiotherapy field cause systemic effects mediated by elevated circulating molecules or that it is associated with the finding of previously elevated cholesterol levels.

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Traditional risk factors and especially cholesterol may be of importance to explain why some HLSs seem to be at an increased risk of developing atherosclerosis. The finding that elevated cholesterol levels a few years after radiotherapy predicted later atherosclerosis in HLSs, affecting both the coronary and pre-cranial arteries, is of particular importance. Aleman et al found a significant association between hypercholesterolemia and the risk of CAD in HLSs.[57] Furthermore, it has been demonstrated that statins may reduce the inflammatory markers and improve the endothelial function in patients treated with radiotherapy.[175, 176] This may imply that HL patients should be screened for hypercholesterolemia and receive statins early prior to radiotherapy. Furthermore, there was a close association between cholesterol at FU-1 and endothelial dysfunction at FU-2. This may suggest that the combination of high cholesterol and endothelial dysfunction in irradiated patients reflects a phenotype with an

increased risk of atherosclerosis. Radiotherapy may further aggravate this disposition as demonstrated in this study.

There is an ongoing debate whether measurements of individual CRP values will give additional information beyond what is provided by the traditional risk scores. Recently, there has been demonstrated that biomarkers like CRP and N-BNP were not better than traditional risk factors.[177] In contrast, our study suggests that CRP obtained 6-9 years earlier may assist in identifying TCSs with increased risk of developing CVD. A CRP level ≥ 1.5 mg/L was associated with almost three times higher risk of CVD compared to survivors without elevated CRP levels during a follow-up of 10 years. CRP was superior to traditional risk factors in predicting cardiovascular events. Although, the positive predictive value of CVD was only 10%, the high negative predictive value of 97% may indicate the usefulness of CRP as a first screening test to identify those with an elevated risk of CVD. Used together with other risk markers of CVD it may be possible to improve the test battery to identify individuals at risk of premature CVD who might benefit from primary prophylaxis with statins. Oncologists have increasingly discussed the need of life-long monitoring of TCSs, at least of high-risk TCSs. However, no biomarker has been identified in TCSs at risk of a second solid cancer or a CVD event. Paper II suggests that CRP may be such a predictive factor.

Radiotherapy contributes to CVD also when given only infra-diaphragmatically and has been shown to be the most important etiological factor for a second malignancy.[48, 52] Although we demonstrated increased levels of inflammatory activation in radiotherapy-treated TCSs, we were unable to show that radiotherapy was associated with the incidence of CVD. This may be explained by the fact that the radiotherapy field only covers the apex of the heart. Thus, the heart receives limited radiation and the doses are lower as compared to HL patients who had been treated with mediastinal radiotherapy at a dose of approximately 40 Gy. Furthermore, the limited number of cardiovascular events within each treatment group and the short follow-up since CRP measurement perhaps reduced the possibility to reveal an association between radiotherapy and CVD.

Prior radiotherapy was a risk factor of a second cancer development in TCSs. TCSs with a CRP level ≥ 1.5 mg/L at FU-1 had more than twice as large risk for developing non-germ cell cancer compared to those with CRP < 1.5 mg/L. The mechanism for a second cancer is not clearly defined but may be due to radiation-induced genetic mutations and long-term subclinical inflammation induced by infra-diaphragmatic radiotherapy in TCSs .[178] Our results may be confounded by patients who already had asymptomatic cancer at the time of CRP measurement or incidental elevation of CRP due to environmental infections. Allin et al demonstrated that the association between CRP and cancer was attenuated after excluding patients diagnosed within 2 years after CRP measurement.[124] In our study only 4 patients were diagnosed with non-germ cell cancer within 2 years after CRP measurement. After the exclusion of these patients CRP ≥ 1.5 mg/L was still associated with future cancer, even if this association was attenuated.

Chemotherapy - cisplatin

Cisplatin-based chemotherapy is today considered to be a possible factor in the development of premature atherosclerosis and CVD.[48, 52, 77] Our data on the lipid-related markers and the metabolic syndrome is in accordance with other studies demonstrating that hypertension, hypercholesterolemia and the metabolic syndrome are frequently observed among TCSs treated with cisplatin and may contribute to the increased risk of CVD.[76, 77] Criticism has recently been raised against the concept of the metabolic syndrome, because there are no clear additive effects between the different descriptors of the syndrome beyond the contribution of diabetes.[179, 180] In the present study the metabolic syndromes was primarily characterized by reduced HDL cholesterol levels. Low testosterone was found to correlate significantly with the metabolic syndrome. In the study group as a whole we found a significant correlation between serum levels of testosterone and HDL cholesterol, suggesting that low testosterone levels in TCSs at least partly can contribute to the metabolic disturbances in these individuals.

The role of inflammatory markers with regard to atherosclerosis after treatment with chemotherapy is still undefined. In contrast to our study, previous studies have observed that chemotherapy and not radiotherapy is associated with inflammatory markers in

TCSs.[87, 88] An increase in vWF and intima-media thickness has been observed within 10 weeks after the completion of chemotherapy.[87] Furthermore, another study showed that after a median follow-up of 7 years CRP and vWf levels tended to be higher after chemotherapy combined with surgery than after surgery alone.[134] However, these studies are hampered by small sample size and/or short observation time. TCSs treated with radiotherapy were not included in these studies. We can not rule out that chemotherapy may induce an inflammatory process in TCSs as suggested by other studies.[88, 134] TCSs treated with chemotherapy was in our study characterized by low HDL cholesterol. HDL has anti-inflammatory and endothelial protective properties. Low HDL may reflect a dyslipidemic genotype or be secondary to chemotherapy and subsequently aggravate endothelial dysfunction.[85, 181] Thus, it may be hypothesized that persistent subclinical endothelial inflammation after radiotherapy and/or chemotherapy, with on-going activation of cytokines, may represent a common link between local or systemic cytotoxic treatment and the development of cardiovascular events also in TCSs.

The role of cisplatin-based chemotherapy in the development of second solid cancer is suggested in a previous study but is less obvious compared to the increased risk of second cancer observed after radiotherapy; probably due to short observation times.[54] Cisplatin can be demonstrated in serum or urine of TCSs more than 10 years after their chemotherapy.[84, 182] We were not able to demonstrate that the TCSs treated with chemotherapy were at risk of developing a second cancer compared to those treated with only surgery.

4.2.2 Cancer treatment and cardiovascular disease

Both inflammatory markers and traditional risk factors may be of importance when considering the increased risk of CVD in TCSs as well as HLSs..[47-51].[56-63] Even though, several of these reports indicate that CVD more often affects HLSs who have an increased risk of CAD, congestive heart failure, valvular disease and constrictive pericarditis. HLSs remain at increased risk of CVD more than 20 years after the treatment and our prevalence of CAD (15%) is comparable with that observed in other studies. [183-186] Radiotherapy may exert a direct toxic effect on the heart and the great arteries which

is aggravated by the concurrent use of anthracyclines. The infra-diaphragmatic radiotherapy used to treat TC includes only the apex of the heart and the patients receive lower doses of irradiation. However, the inclusion of the apex in the infra-diaphragmatic field may to some extent explain the increased risk of cardiovascular disease after infradiaphragmatic radiotherapy for TC.[47, 48, 51]

Coronary artery disease

There is evidence that CACS is an independent and reliable predictor of CAD in asymptomatic individuals although absence of calcification does not exclude coronary events.[187, 188] Paper IV demonstrates a strong relationship between the severity of coronary artery calcification and CAD requiring treatment in HLSs. A volume score > 200 was associated with verified CAD in 5 of 10 individuals. Coronary angiography was not performed as a routine in asymptomatic patients. Therefore our number of patients with verified CAD may underestimate the true proportion of HLSs with CAD. Asymptomatic patients with a score above 200 had calcium deposits mainly in RCA and CX, vessels which are less likely to cause symptoms. Coronary angiography in these patients may have diagnosed more patients with angiographically significant coronary artery stenoses. Coronary artery calcification after radiation therapy for HL has previously been quantified in only 9 HLSs with no history of CAD. Volume score was above 200 in 5 of these patients and angiography confirmed > 50% stenosis in 4 of these.[189].Two patients in our study with verified CAD and a CACS at 8 and 46 respectively could not be properly scored because of a stent implantation which makes the culprit vessel unsuited for calcium quantification. Therefore the most pathologic segment of the coronary tree of these patients was excluded from CACS. Inclusion of these patients before stenting of the stenosed artery would have strengthened the correlation between CACS and CAD. Only 17% of our patients (8 of 47 HLSs) presented with a coronary calcium score of zero. In a slightly older population referred to MDCT angiography, 46 % had zero coronary calcium [190]. Our patients without coronary artery calcium did not report symptoms of CAD. This may suggest a favourable prognosis but a low CACS does not exclude CAD as has been shown in non-irradiated patients [191].[192]

However, this may be different in irradiated patients where coronary calcification seems to be a more prominent feature.

Stroke

Seven significant stenoses (≥ 50% of luminal narrowing) were revealed in 6 of 43 (14%) HLSs studied in the present study. Although only 3 patients reported a previous history of cerebral ischemia or stroke, a long-term increase in the incidence of stroke can not be excluded. A recent study by de Bruin et al demonstrated an incidence ratio of stroke at 2.2 17 years after treatment in HLSs compared to the general population.[82] Our findings are in line with studies demonstrating increased intima-media thickness and more subclavian and carotid stenosis in HLSs.[193, 194] Also for head and neck cancer patients treated with radiotherapy, an increased incidence of stroke has been reported, but these patients are generally older and have received higher doses of irradiation than HL patients.[195] It is important to emphasize that a total of 85% of the atherosclerotic lesions in the pre-cranial arteries were calcified and that the coronary arteries in HLSs contained highly calcificated plaques. The association between radiotherapy and the presence of calcified atherosclerotic lesions may suggest a more clinically stable stenosis with less risk of a rupture, which may explain the low number of strokes among our HLSs.

Congestive heart failure

Our results consistently demonstrated that treatment with low to intermediate doses of anthracyclines were associated with thinning of the ventricular walls and ventricular dilatation compared to radiotherapy alone or radiotherapy combined with chemotherapy without anthracyclines. This is of relevance for the treatment principles of stage I and II HL today, as short term anthracycline-based chemotherapy followed by involved field radiotherapy (frequently encompassing parts of the mediastinum) is considered standard treatment for early stage disease in most Western countries.[196] To our knowledge this is one of the first studies to document a progressive unfavorable effect of anthracyclines on cardiac function two decades after the treatment of adult onset cancer. Studies are underway which will evaluate the time-dependent changes in cardiac function and

examine the effect of an ACE-inhibitor on myocardial performance, mainly in children with different types of leukemia and lymphomas.[197, 198]

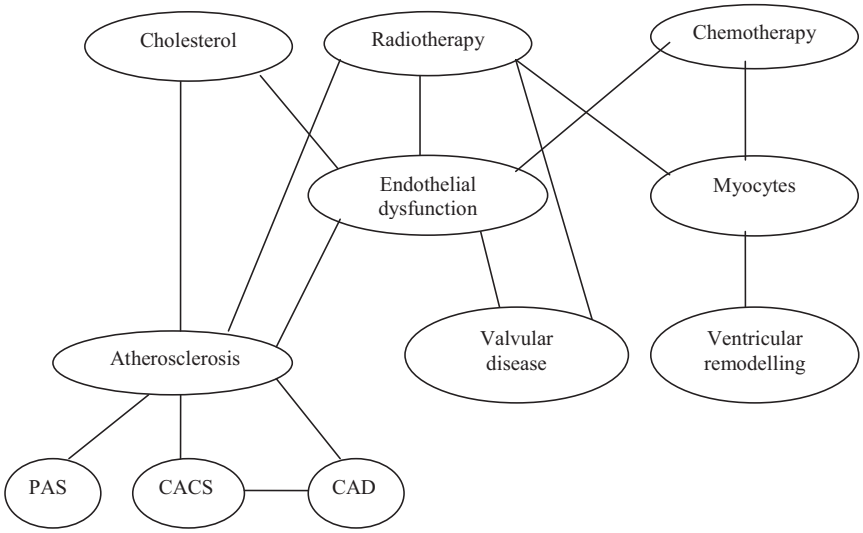
Valvular dysfunction

In Paper III we demonstrate a progressive valvular deterioration after mediastinal radiotherapy. Aortic and mitral regurgitation was a dominant finding in the first decade after treatment while aortic stenosis seems to develop in the second decade after treatment for HL. The rates are higher compared to other studies and may be due to differences in the treatment, classification of valvular dysfunction and the length of the follow-up.[56, 58, 61] Nevertheless, our data suggest that deterioration of valvular regurgitation and the development of aortic stenosis are slow dynamic processes that may appear more than 10 years after the diagnosis of HL. Importantly we could show that aortic stenosis developed during the second and third post-treatment decade. No clear associations were observed between the development of valvular dysfunction and the cholesterol level measured at FU-1 and the cholesterol level and inflammatory markers measured at FU-2. Previous studies have documented an association between cholesterol and aortic stenosis but the SEAS study did not observe any beneficial effect of statin treatment on the progression of aortic stenosis.[199] However, the occurrence of CVD was decreased. It is therefore likely that radiotherapy was the dominant cause of valvular dysfunction in the present study and that cholesterol is a more important contributor of atherosclerosis in the irradiated coronary and pre-cranial arteries.

Radiotherapy may directly damage the valves and cause fibrotic thickening, retraction, and calcifications.[69] More recently decreased levels of endothelial progenitor cells have been suggested to reduce endothelial regenerative capacity and to contribute to the progression of degenerative aortic stenosis.[200] It is unknown whether radiotherapy may reduce endothelial regenerative capacity in HLSs. Though valves are normally avascular, cellular injury may cause fibrosis and stimulate secondary angiogenesis and calcification. Our results suggest that valve retraction is the predominant early change that causes valvular regurgitation, and it takes as long as 20 years to develop thickened, calcified valves that finally may result in stenosis. Higher pressure on the left side of the heart

most likely explains the observation that the aortic and mitral valves were more often affected than the tricuspid or pulmonary valves, and that stenosis is primarily observed in the aortic valve.[56, 58, 61] Previous reports have demonstrated that valvular calcification is a dominant finding after mediastinal radiotherapy for HL.[80, 201] The present study suggests that this occurs late in the development of valvular pathology. Leaflet thickening and reduced movements without calcification were seen among those with no or only mild valvular stenosis/regurgitation. Valvular calcification was seen among those with moderate/severe valvular regurgitation/stenosis. CRP was elevated in our group of HLSs reflecting that a lasting inflammatory condition was initiated by radiotherapy. This was independent on whether the patients developed valvular dysfunction or not. The present study demonstrated no additional effect of anthracyclines on the development of valvular dysfunction while this has been reported by another study.[57] Neither were we able to document an association between aortic stenosis and cholesterol.

Figure 8 Possible connections between radiotherapy, cholesterol, atherosclerosis within the radiation field and peripheral endothelial dysfunction



5 CONCLUSIONS

Paper I

Infra-diaphragmatic radiotherapy was associated with a low grade increase in markers of inflammation and endothelial dysfunction. Chemotherapy was associated with atherogenic lipid changes.

Paper II

TCSs with a CRP level ≥ 1.5 mg/L median 11 years after treatment had a more than twice as large risk for developing non-germ cell cancer and almost three times as high risk of CVD compared to survivors without elevated CRP levels during the eight consecutive years. Prior radiotherapy represented an added risk factor for cancer development.

Paper III

Hodgkin lymphoma survivors who were treated with mediastinal radiotherapy had a high risk of developing progressive valvular dysfunction and subsequent aortic stenosis during the second or third decade after treatment. Additional treatment with anthracyclines was associated with left ventricular remodeling.

Paper IV

There is a strong relationship between elevated coronary artery calcium score and clinical significant coronary artery disease in Hodgkin lymphoma survivors treated with mediastinal radiotherapy > 15 years earlier.

Paper V

In-field atherosclerotic lesions are frequent in Hodgkin lymphoma survivors treated with mediastinal radiotherapy. The atherosclerotic lesions in the coronary and pre-cranial arteries were predicted by elevated cholesterol and characterized by impaired peripheral endothelial function suggesting a target population that may benefit from statin treatment.

6 IMPLICATIONS FOR FOLLOW-UP AND FUTURE RESEARCH

There are several similarities between TC and HL. The patients with TC and HL are typically young adults, the prognosis is excellent due to modern treatment with radiotherapy and multi-agent chemotherapeutic regimens and radiotherapy, and they are at risk of long-term adverse effects of the treatment. A diagnosis of cancer is always dramatic and has long-term psychological effects. Therefore it is important that the treatment is effective and has minimal acute and long-term side effects. The development and knowledge about anti-cancer drug treatment and their long-term adverse effects will allow us to develop strategies to minimize these effects and reduce the uncertainty among young cancer survivors. A thorough examination of TCSs and HLSs will allow us to reveal the presence of long-term adverse effects of the treatment where the most important concerns are the development of a second cancer and cardiovascular disease. The studies presented in Paper I – V demonstrate the presence and development of cardiovascular risk factors and disease in long-term cancer survivors. Based on the results from these studies we have some suggestions which are of relevance for health care and research.

1) Primary prevention of CVD in TCSs and HLSs.

It seems reasonable to recommend a regular follow-up of all TCSs and HLSs every 5 years with the measurement of lipid-related markers and CRP (in TCSs) as well as assessment of other traditional risk factors like hypertension, smoking, waist circumference, physical activity and hypogonadism. Regarding HLSs treated with radiotherapy to the mediastinum or neck, the Norwegian Lymphoma group recommends regular examinations by the general practitioner from 10 years after treatment with the aim to prevent and early diagnose treatment-related cardiovascular adverse effects.

- Further, studies should be undertaken to confirm if CRP is able to predict CVD and a second cancer in TCSs and examine if CRP may be of importance in larger cohorts of HLSs. Furthermore, there is a need to identify other predictive markers of a second cancer and CVD which can be of benefit in TCSs and HLSs.

- In HLSs especially the identification and treatment of high cholesterol may be of importance to prevent atherosclerosis and subsequent CVD. Though demanding from a practial point of view, our results warrant prospective studies to examine if treatment with statins before radiotherapy may prevent late adverse effects of radiation

2) Reveal and treat premature CVD and a second cancer in TCSs and HLSs.

- It is mandatory that these patients are referred to further examinations when they experience symptoms of CVD or a second cancer as these conditions may occur at a younger age than the general population.
- The presence of a systolic or diastolic murmur in HLSs should call for an echocardiographic examination.
- The clinical role of coronary calcification needs to be defined in a larger prospective study. This may be more important in this group than in the general population as arterial calcifications are prominent after mediastinal radiotherapy. The use of multi-detector CT may be useful as a screening procedure in the follow-up of HLSs in detecting premature subclinical cardiac disease. However; determination of CACS can not represent a "stand alone screening procedure" and the role of CACS in HLSs follow-up remains to be solved.

3) Information to the patients, their family doctors and specialists in oncology and cardiovascular medicine.

Knowledge about the increased incidence of CVD and second cancer in cancer survivors are necessary to elevate the awareness of these conditions. The Norwegian Health Directorate informed all Norwegian family doctors on the increased risk of CVD in irradiated HLSs. These initiatives were motivated partly by the findings in Paper III. However, also the patients have to be informed about symptoms of the adverse treatment related effects and their family doctors should have a low threshold for referring these patients to further examinations. Oncologists and cardiologists have to work in close cooperation to take care of these patients who often face multiple health problems like CVD, second cancer, infertility, hypothyreodism, muscular weakness etc. We have

recently constructed information leaflets for patients treated for lymphoma with mediastinal and or/neck radiotherapy to inform about their increased risk.

Finally, it is important to keep in mind that even though these studies are performed in patients partly receiving “old-fashioned” treatment these patients will still be a part of the population for several decades. Furthermore, even modalities like involved-field radiotherapy to the mediastinum and/or the neck in HL patients may in many cases include the pre-cranial great arteries and the coronary arteries and these patients may also in the future be at increased risk. Thus, it is likely that today’s patient population with HL and TC cannot entirely avoid the risk of long-term treatment-related adverse effects.

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I

II

C- reactive protein; a potential marker of second cancer and cardiovascular disease in testicular cancer survivors?

Running title: Prediction of cardiovascular disease and second cancer

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ABSTRACT

Purpose: C-reactive protein (CRP) is a marker of cardiovascular risk both in patients with cardiovascular disease (CVD) and in presumably healthy patients with normal LDL cholesterol while there is a conflicting evidence regarding CRP as a marker of future cancer. The aim was to assess whether CRP predicts CVD and consecutive cancer in testicular cancer survivors (TCSs).

Methods: During 1998 – 2001, 586 TCSs with a high sensitivity CRP ≤ 10 mg/L were identified median 11 (4 – 21) years after treatment (FU-1). A second follow-up survey (FU-2) was conducted median 8 (6 – 9) years after FU-1. At FU-2 we obtained information about post-FU-1 CVD (cardiovascular death, nonfatal myocardial infarction, stroke, revascularization or heart failure). Information about post-FU-1 non-germ cell cancer and cardiovascular death in all 622 patients were retrieved from the Cancer Registry of Norway.

Results: After FU-1 31 (5.3%) of 586 patients developed non-germ cell cancer (excluding localized prostate cancer) while 28 (4.9%) developed CVD. Cox regression analyses showed that patients with CRP ≥ 1.5 mg/L had 2.21 (95% CI 1.04 – 4.70) times higher risk of developing non germ cell cancer and 2.79 (95% CI 1.22 – 6.34) times higher risk for CVD compared to patients with a CRP < 1.5 mg/L at FU-1. Radiotherapy was associated with 2.56 (95% CI 1.19 – 5.51) times higher risk for developing non-germ cell cancer in comparison to patients treated with surgery with or without chemotherapy.

Conclusion: In long-term TCSs CRP may serve as a potential marker of cardiovascular events and a second cancer.

Introduction

C-reactive protein (CRP) is established as a predictor of cardiovascular events in patients with known cardiovascular disease (CVD) as well as in asymptomatic individuals with risk factors for cardiovascular disease.[1,2] CRP is an acute phase protein produced by the liver in response to inflammatory stimuli.[3] Atherogenesis involves the tight interaction between lipids and inflammation and the ability of CRP to predict forthcoming cardiovascular events relies on its function as a stable and reliable marker of low-grade upstream inflammation. Chronic subclinical inflammation may also be associated with cancer for example as a pathogenic mediator in colon cancer, cervical cancer or gastric carcinoma.[4] This hypothesis is supported by the observation of elevated CRP in patients with prevalent cancer as well as in patients who develop cancer several years after the registration of an elevated CRP.[4-7] Recently, a CRP level ≥ 3 mg/l was identified as a marker of incident lung cancer in the general population in comparison to individuals with CRP < 1 mg/L.[5] However, the association between CRP and cancer is still controversial. [7-9]

Long-term testicular cancer survivors (TCSs) treated with radiotherapy and/or chemotherapy have an increased risk of second malignancies and cardiovascular morbidity.[10-13] Elevated levels of CRP and other markers of inflammation have been observed in TCSs several years after treatment with radiotherapy and chemotherapy.[14,15] Thus, we have previously shown that TCSs treated with radiotherapy had elevated levels of CRP median 11 years after treatment compared with TCSs treated only with surgery or chemotherapy.[15]

However, it is still unknown whether elevated CRP levels seen in TCSs identify individuals at risk for developing a second cancer or CVD. The aim of this study was primarily to explore the hypothesis that elevated high sensitivity (hs) CRP levels determined at one occasion during long-term follow-up of TCSs may predict subsequent major cardiovascular events (i.e. cardiovascular death, nonfatal myocardial infarction, stroke, revascularization or heart failure) or non-germ cell cancer in TCSs. Secondly, we wanted to study if treatment with radiotherapy shown to be associated with raised CRP levels was associated with an increased risk of CVD or a consecutive cancer.

Methods

Patients

Surviving TCSs treated for unilateral testicular cancer at the Norwegian Radium Hospital from1980 to1994 participated in a questionnaire-based survey conducted during 1998-2001 (FU-1).[16] They also underwent an out-patient clinical examination at the hospital with determination of several biomarkers, among them CRP. Post-orchiectomy treatment consisted of retroperitoneal lymph node dissection alone or surveillance (n = 140), cisplatin-based chemotherapy with or without additional surgery (n = 240) or abdominal radiotherapy alone (n = 231, median dose 35 Gy). Only one patient received mediastinal radiotherapy. Twenty-eight patients received combined chemotherapy and radiotherapy. The chemotherapeutic agents used were cisplatin in the combination with bleomycin and vinblastin (before 1985) or etoposid (after 1985). Detailed treatment strategies of the surveyed men have been reported previously.[15,16] At FU-1 patients were categorized as smokers versus never or previous smokers and as physically active or inactive.

Eligibility criteria for the present study were aged ≤ 60 years at FU-1, no known second cancer and no apparent acute or chronic infections at blood sampling thus excluding men with CRP > 10 mg/L and those in whom CRP was missing due to logistics reasons (Figure 1).

A second questionnaire-based survey (FU-2) was conducted in 2007-2008 median 8 (range 6 – 9) years after FU-1. Surviving TCSs were asked to have a clinical examination and blood sampling performed at their family doctor's office.

The blood samples were immediately mailed to the NRH where all biochemical analyses were performed. At the time of FU-2, 19 men died; 4 died from CVD and 10 from cancer. The post-FU-1 development of a non-germ cell cancer or a first-time cardiovascular event represented the end-point of our analyses.

Post-FU-1 non-germ cell cancer and CVD

From the Norwegian Cancer Registry we received data (type of cancer, date of diagnosis) about non-germ cell cancer diagnosed after FU-1 among our eligible TCSs as registered by December 2008. The diagnosis of localized prostate cancer (n = 5) was not considered to be a valid event as this malignancy is increasingly detected by PSA screening. From the questionnaire at FU-2 we extracted information about first-time post-FU-1 cardiovascular events (myocardial infarction, stroke, revascularization, and hospitalization for heart failure) after exclusion of 15 men who had reported such an event prior to FU-1. All self-reported CV events were validated by the patient's hospital medical record. The study was approved by Institutional and Regional Ethical Committees and funded by the University of Oslo.

Biochemical analyses of serum and plasma

At FU-1 non-fasting blood was sampled between 9 am and noon into pyrogen-free, pre-cooled vials without additives (serum) or with EDTA as anticoagulant (plasma). The tubes were centrifuged at 1,000g for 10 minutes within 30 minutes (plasma) or allowed to clot before centrifugation (serum). All samples were stored

at -70°C and thawed < three times. High sensitivity CRP in plasma was determined by a high-sensitive particle-enhanced immunoturbidimetric assay (Roche Diagnostics, Basel Switzerland). Plasma levels of total cholesterol, low- and high density lipoprotein (LDL and HDL) cholesterol and triglycerides were measured enzymatically with a Roche/Hitachi 917 analyzer (Roche Diagnostics). Serum levels of luteinizing hormone (LH) and testosterone were analyzed by immunoassays.[16] Patients were classified as having hypogonadism when any of the following 3 conditions were met: serum testosterone <8 nmol/L, serum LH >12 U/L, or regular use of exogenous testosterone.[16] The intra- and inter-assay coefficients of variation were less than 10% for all assays.

Statistical analyses

Continuous variables were described with median (range or interquartile range; IQR) and categorical with proportions (percentages). Spearman's correlation coefficient was computed to assess the correlation between hsCRP at FU-1 and -2. Mann-Whitney Wilcoxon and Kruskal-Wallis tests were used to compare continuous data with skewed distributions. Chi-square test or Fisher's exact test were applied to compare categorical data. To determine the optimally discriminating CRP, we depicted the area under the curve (AUC) for the composite end- point of CV event and a second cancer and calculated that a cut point of CRP level at 1.5 mg/L reached sensitivity of 65% and specificity of 58% (Online Figure 1). This cut-off level was used to construct groups of patients with CRP < 1.5 mg/L and ≥ 1.5 mg/L. The cumulative incidences of CVD and a

second cancer as separate end-points were computed for those two groups using Kaplan-Meier plots. The time to event was calculated from FU-1 to the date of the end-point, the date of FU-2 (for CVD events) or December 31, 2008 (for second cancer), whatever occurred first. Differences in time to event between groups were assessed using the log-rank test. Additional Kaplan-Meier plots were drawn to illustrate the impact of treatment modalities for which the log-rank test could not be performed. Therefore, to fulfil the proportional hazard assumption patients treated with only surgery and patients treated with chemotherapy were considered as one group in the Cox-regression models. Univariate and multiple Cox-regression models were fitted to explore the relation between clinically important predictors of second cancer or cardiovascular event. The limited number of events for each end-point allowed only three variables to be included in the multiple Cox regression model. Based on the univariate analyses and the main purpose of the study CRP, treatment (surgery ± chemotherapy versus radiotherapy ± chemotherapy) and age were entered into the multiple model. The categorical variables current smoking, physical active, education, hypogonadism and diabetes mellitus were not included in the analyses partly because there was no association between these variables and the end- points and additionally because they did not fulfil the proportional hazards assumption. There were significant correlations between systolic and diastolic blood pressure as well as between the total-, LDL- and HDL-cholesterol. To avoid multi colinearity, only systolic blood pressure and LDL-cholesterol were selected for the multiple Cox regression. A two-sided p-value <0.05 was

considered statistically significant. Data were analyzed using SPSS 14.0 (SPSS Inc).

Results

Overall, 586 patients were included in FU-1 at a median of 11 (range 4 – 21) years after treatment. All were eligible for analyses of a second cancer while 571 patients were eligible for analyses of CVD between FU-1 and FU-2. Patients who had been treated with radiotherapy (alone or with additional chemotherapy) were older both at diagnosis and at FU-1 and had a higher level of CRP at FU-1 compared to the other two treatment groups (Table 1). There was a strong relationship between CRP-levels at FU-1 and FU-2 (Spearman $r = 0.59$, $p < 0.001$).

Non-germ cell cancer

In total, 31 (5.3 %) of 586 patients developed non-germ cell cancer after FU-1, excluding 5 patients who were diagnosed with localized prostate cancer (Table 2). Gastrointestinal cancer, kidney cancer, bladder cancer and skin cancer were the most frequent consecutive cancer types seen in TCSs. The cumulative incidence of non-germ cell cancer was significantly increased among TCSs with a CRP ≥ 1.5 mg/L as compared to those with CRP < 1.5 mg/L (Figure 2a; $p = 0.01$). The multiple Cox regression analyses revealed that patients with a CRP level ≥ 1.5 mg/L at FU-1 had a 2.21 (95% CI 1.04 – 4.70) higher risk of developing non-germ cell cancer after adjustment for age and treatment (Table 3). Systolic blood pressure was not associated with non-germ cell cancer after adjustment for age. Four patients developed non-germ cell cancer within 2 years after the measurement of CRP. The estimated risk of a second cancer was 2.16

(95% CI 0.97 – 4.82) after excluding these patients. Non-germ cell cancer and in particular gastrointestinal cancer were more prevalent and with a higher cumulative incidence in patients treated with radiotherapy as compared to patients treated with surgery only or chemotherapy (Figure 2c). Cox regression analyses demonstrated that patients treated with infra-diaphragmatic radiotherapy alone or in combination with chemotherapy had 2.56 (95% CI 1.19 – 5.51) higher risk of developing a second cancer as compared to patients treated with surgery with or without chemotherapy (Table 3). Additionally, after the exclusion of the 22 patients who were registered to be treated with both radiotherapy and chemotherapy, treatment with only infra-diaphragmatic radiotherapy was associated with increased risk of a second cancer (HR 2.29 (95% CI 1.03 – 5.07)).

CVD

Twenty-eight (4.9 %) TCSs were diagnosed with post-FU-1 cardiovascular event (Table 2). The most frequent CV events were myocardial infarction and revascularization. At FU-1 CRP was generally higher among patients who eventually developed CVD compared to those without events (median 1.9 (IQR 1.2 – 3.6) mg/L vs median 1.2 (IQR 0.7 – 2.1) mg/L, $p = 0.005$). The positive predictive value of CRP ≥ 1.5 mg/L at FU-1 was only 10% while the corresponding negative predictive value was 97%. The cumulative incidence of CV events was increased among TCSs with a CRP ≥ 1.5 mg/L as compared to those with CRP < 1.5 mg/L (Figure 2b). Cox regression analyses demonstrated

that patients with a CRP ≥ 1.5 mg/L at FU-1 had 2.79 (95% CI 1.22 – 6.34) times higher risk of developing CV events after adjustment for age (Table 3). Systolic blood pressure and LDL-cholesterol were not associated with non-germ cell cancer after adjustment for age. The cumulative incidence of CVD was no different in the 3 treatment groups (Figure 2d). Cox-regression analyses did not reveal any influence of treatment modality on cardiovascular outcome.

Discussion

TCSs included in our study with a CRP level ≥ 1.5 mg/L had more than twice as large risk for developing non-germ cell cancer and almost three times as high risk of CVD compared to survivors without elevated CRP levels. Prior radiotherapy represented an additional risk factor for cancer development. On a group level the hsCRP obtained median 11 years after the testicular cancer diagnosis remained relatively stable for the consecutive 8 years, suggesting that this may represent an inflammatory phenotype of these patients.

The increased risk of non-germ cell cancer and CVD in TCSs is documented with several studies.[11-13,17,18] Radiotherapy has been shown to be the most important etiological factor for a second malignancy but it also contributes to CVD even if only administered infra-diaphragmatically.[11,12] Second cancer is only partly explained by radiation-induced genetic mutations which lead to cancer by supplementary environmental influence. Long-term subclinical inflammation caused by infra-diaphragmatic radiotherapy in TCSs with release of cytokines may be of importance in mediating second cancer.[15] The role of cisplatin-based chemotherapy in second solid cancer development is suggested but is less obvious; probably due to short observation times.[18] On the other hand, cisplatin, which may be an etiological factor for both second cancer and CVD, can be demonstrated in serum or urine of TCSs more than 10 years after their chemotherapy.[19,20] Oncologists have increasingly discussed the need of life-long

monitoring of TCSs, at least of high-risk TCSs. However, no biomarker is accepted for identification of TCSs at particular high risk of a second solid cancer or a CVD event. With this background we suggest that hsCRP might represent such a predictive factor.

In patients with manifest cancer elevated CRP indicates a poor prognosis.[21-23] However, hsCRP may also predict a malignancy as cancer may etiologically be related to chronic infections by viral or bacterial agents (cervical cancer: human papilloma virus; gastric carcinoma: Helicobacter pylori).[4-7]. The majority of evidence is related to patients with colorectal cancer and lung cancer as demonstrated by recent studies and meta-analyses, although not all studies have supported this view.[5-8] The results from the abovementioned studies may be confounded by patients who already had occult cancer at the time of CRP measurement or incidental elevation of CRP. Allin et al demonstrated that the association between CRP and cancer was attenuated after excluding patients diagnosed within 2 years after CRP measurement. In our study only 4 patients were diagnosed with non-germ cell cancer within 2 years from CRP measurement. After the exclusion of these patients, hsCRP ≥ 1.5 mg/L was still associated with future cancer, even though this association was attenuated, possibly due to the limited number of events among our TCSs.

Cisplatin-based chemotherapy is today considered to be a possible factor in the development of premature atherosclerosis and CVD.[11,12, 24] Hypertension, hypercholesterolemia and the metabolic syndrome are frequently observed

among TCSs and may contribute to the increased risk of CVD.[24,25] Cisplatin-based chemotherapy may lead to an increase in inflammatory markers and endothelial dysfunction as demonstrated by reduced flow-mediated dilatation and increased intima-media thickness.[14,26] Large- field radiotherapy has similar effects predominantly on the vessels within the target field. [27] Previously we demonstrated elevated levels of hsCRP and von Willebrand factor in irradiated TCSs, most of them included in the present study.[15] Other groups have observed similar alterations of inflammatory serum markers after chemotherapy.[14,26] It is tempting to hypothesize that persistent subclinical endothelial inflammation after radiotherapy and/or chemotherapy, with on-going production of cytokines, may represent a common link between local or systemic cytotoxic treatment and the development of cardiovascular events in TCSs. Large studies with long follow-up have to confirm this hypothesis.

After the demonstration that a hsCRP level above 2 mg/L identified individuals with increased risk of coronary heart disease, hsCRP has gained much interest as a potentially important tool in primary prevention of CVD.[1,28] This is reflected in the recent JUPITER trial where cardiovascular morbidity and mortality were reduced by 44% in presumably healthy individuals with normal LDL-cholesterol but a CRP-level above 2 mg/L.[2] There is an ongoing debate whether measurements of individual hsCRP values will give additional information beyond what is provided by the traditional risk scores. In the present study, a CRP level ≥ 1.5 mg/L was better in predicting CVD than traditional risk factors. Recently, it has been demonstrated that biomarkers like CRP and N-BNP were not better

than traditional risk factors.[29] In contrast; our study suggests that hsCRP may assist in identifying TCSs at increased risk of developing CVD. However, the positive predictive value was only 10% but the high negative predictive value of 97% may indicate the usefulness as a first screening test to identify those with an elevated risk of CVD. If used together with other markers of future CVD it would be possible to compose test battery to identify individuals at risk of premature CVD who might benefit from primary prophylaxis with statins.

The strength of our study is the prospective design, with the use of high sensitivity analyses of CRP applied for the first time in follow-up of TCSs. Furthermore, only 23 % were lost during follow-up, making selection bias unlikely. Previous studies and guidelines have demonstrated increased risk of CVD or cancer with the use of a cut-off at 1 mg/L, 2 mg/L or 3 mg/L which makes our cut-off at 1.5 mg/L reasonable.[1, 2, 28] Nevertheless, some limitations have to be pointed out: The total number of our patients under observation and the number of TCSs who experienced an end-point was limited which gave some restrictions to our Cox regression analyses. The cardiovascular events in those alive are self-reported suggesting that the incidence of cardiovascular events might be underestimated, but all events reported by the patients were validated by medical records. We did not have an age-matched male control group available to investigate whether determination of elevated CRP is of greater value in TCSs than in the general population. We conclude that hsCRP might be a useful marker of cardiovascular events and non germ cell cancer among TCS irrespective of cancer treatment modality. Future large-scaled studies should

evaluate if CRP can be a useful marker in the screening of TCSs for the prevention of a second cancer and CVD.

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Table 1 Demographic and clinical characteristics among the 622 patients at FU-1 (median 11 years after diagnosis)

	Surgery only	Chemotherapy	Radiotherapy ± chemotherapy
Number of patients (n=622)	132	221	233
Age (years)[1]			
at diagnose	29 (24 – 35)	28 (23 – 33)	34 (29 – 38)
at follow up	40 (36 – 47)	40 (34 – 45)	45 (40 – 51)
Current smoker, n (%)	42 (32)	83 (38)	82 (35)
Physical active, n (%)	107 (81)	186 (84)	191 (82)
Education at university level, n (%)	44 (33)	90 (41)	94 (40)
Gonadal function			
Hypogonadism, n (%)	7 (5)	31 (14)	21 (9)
Lipid-related markers			
Total cholesterol (mmol/L)	5.6 (4.8 – 6.4)	5.6 (4.9 – 6.3)	5.6 (5.0 – 6.3)
LDL cholesterol (mmol/L)	3.7 (3.1 – 4.3)	3.6 (3.0 – 4.2)	3.6 (3.1 – 4.2)
HDL cholesterol (mmol/L)	1.2 (1.0 – 1.4)	1.1 (1.0 – 1.3)	1.1 (1.0 – 1.3)
Triglycerides (mmol/L)	1.3 (0.9 – 2.0)	1.6 (1.0 – 2.5)	1.6 (1.0 – 2.4)
Clinical markers			
Body mass index (kg/m^2)	26.3 (24.4 – 28.6)	25.7 (23.7 – 27.9)	26.2 (24.1 – 28.4)
Systolic blood pressure (mm Hg)	120 (115 – 130)	125 (120 – 140)	130 (120 – 140)
Diastolic blood pressure (mm Hg)	80 (70 – 85)	80 (70 – 90)	80 (75 – 85)
Diabetes mellitus I or II, n (%)	2	6	8
Inflammatory marker			
hsCRP (mg/L)	1.2 (0.6 – 2.0)	1.2 (0.7 – 2.4)	1.5 (0.9 – 2.6)

[1]Values are median (interquartile range) unless otherwise specified

Table 2 Clinical end points registered after FU-1 according to treatment

	Surgery	Chemotherapy	Radiotherapy ± chemotherapy	Total	P-value
A: Second cancer					
Eligible patients (FU-1)	132	221	233	586	
Gastrointestinal cancer	2 (1.5)	1 (0.5)	7 (3.0)	10 (1.7)	
Kidney - and bladder cancer	0	4 (1.8)	3 (1.3)	7 (1.2)	
Prostate cancer	1 (0.8)	1 (0.5)	4 (1.7)	6 (1.0)	
Melanoma	1 (0.8)	0	2 (0.9)	3 (0.5)	
Other*	0	2 (0.9)	8 (3.4)	10 (1.7)	
All second cancer	4 (3.0)	8 (3.6)	24 (10.3)	36 (6.1)	0.003
All, except localized prostate cancer	3 (2.3)	7 (3.2)	21 (9.0)	31 (5.3)	0.004
B: Cardiovascular events**					
Eligible patients (FU-2)	130	216	225	571	
Nonfatal myocardial infarction	2 (1.5)	5 (2.3)	6 (2.6)	13 (2.3)	
Nonfatal stroke	2 (1.5)	2 (0.9)	2 (0.9)	6 (1.1)	
Revascularization	3 (2.3)	6 (2.8)	6 (2.6)	15 (2.6)	
Heart failure	0	2 (0.9)	1 (0.4)	3 (0.5)	
Death from cardiovascular disease	0	1 (0.5)	3 (1.3)	4 (0.7)	
Any	5 (3.8)	10 (4.6)	13 (5.8)	28 (4.9)	0.70

* Pulmonary cancer (1), brain cancer (1), peripheral nerve system, pelvis (1), pancreatic cancer (1), laryngeal cancer (2), cancer in the lymphatic system (1), peritoneal cancer (1), squamous cell carcinoma (2)

** Fifteen patients with CVD before FU-1 were excluded. Cardiovascular events registered after FU-1 included death due to CVD, nonfatal myocardial infarction, nonfatal stroke, coronary revascularization and heart failure.

Data are numbers (%)

Table 3 Cox regression with non-germ cell cancer (left panel) and cardiovascular disease (right panel) as the dependent variable

	Univariate analyses			Multiple results*			Univariate analyses			Multiple results**		
	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
Age at FU-1	1.08	1.03 – 1.13	0.001	1.07	1.02 – 1.12	0.01	1.12	1.06 – 1.18	< 0.001	1.11	1.06 – 1.17	< 0.001
Lipid-related markers												
Total cholesterol	1.26	0.94 – 1.68	0.13				1.36	1.00 – 1.84	0.05			
LDL cholesterol	1.08	0.75 – 1.55	0.67				1.52	1.06 – 2.18	0.02			
HDL cholesterol	1.00	0.28 – 3.59	0.99				0.51	0.12 – 2.08	0.35			
Clinical markers												
Body mass index	1.01	0.92 – 1.11	0.85				1.04	0.95 – 1.15	0.42			
Systolic blood pressure	1.02	1.00 – 1.04	0.02				1.03	1.01 – 1.04	0.004			
Diastolic blood pressure	1.03	1.01 – 1.06	0.04				1.03	1.00 – 1.06	0.10			
Inflammatory markers												
hsCRP	1.08	0.91 – 1.29	0.39				1.17	0.99 – 1.38	0.07			
CRP ≥ 1.5 mg/L	2.60	1.22 – 5.51	0.01	2.21	1.04 – 4.70	0.04	3.27	1.44 – 7.42	0.005	2.79	1.22 – 6.34	0.02
Treatment												
Surgery ± chemotherapy	1.00	Reference		1.00	Reference		1.00	Reference				

Radiotherapy ± chemotherapy	3.46	1.62 – 7.35	< 0.001	2.56	1.19 – 5.51	0.02	1.40	0.67 – 2.95	0.37

Hazard ratios (HR) and 95% confidence interval (CI) for different predictors of non germ cell cancer and cardiovascular disease

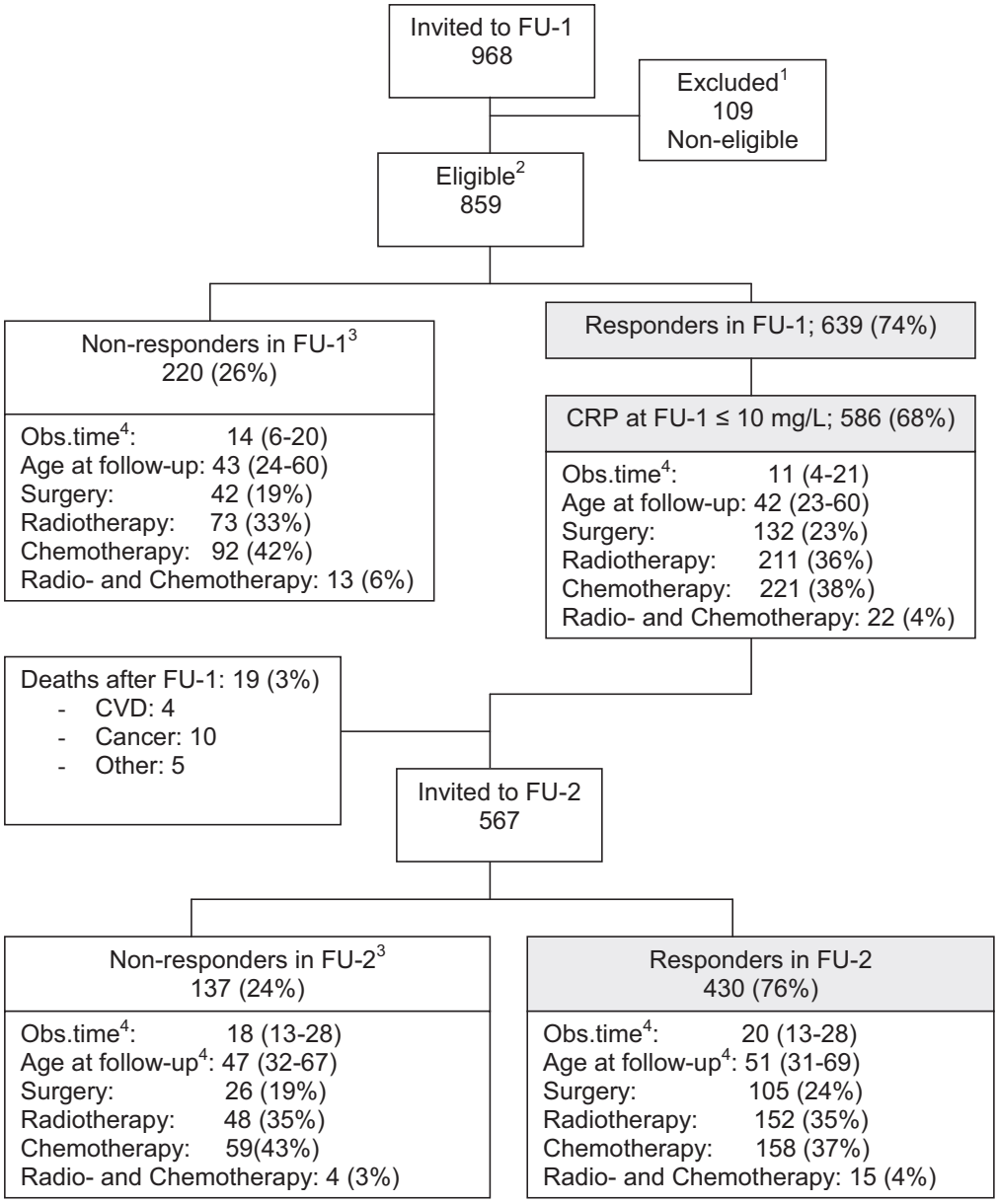
HR = 1 (Reference) for the following categorical variables; a CRP level below 1.5 mg/L and treatment with surgery

* For the analysis of second cancer age at FU-1, CRP ≥ 1.5 mg/L and treatment were included.

** For the analysis of CV events age at FU-1 and CRP ≥ 1.5 mg/L were included.

Due to multi co-linearity we could possibly choose systolic or diastolic blood pressure and one of the lipid-related markers for the multiple Cox-regressions. For the analysis of second cancer systolic blood pressure was regarded as possible confounder. For the analysis of CV events LDL-cholesterol and systolic blood pressure were possible confounders. After adjustment for age no significant associations between these markers and a second cancer or CVD were revealed.

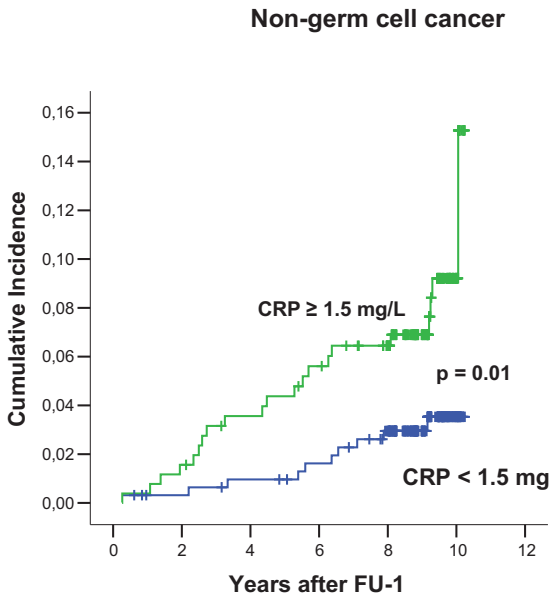
Figure 1 Flow-diagram of the recruited patients



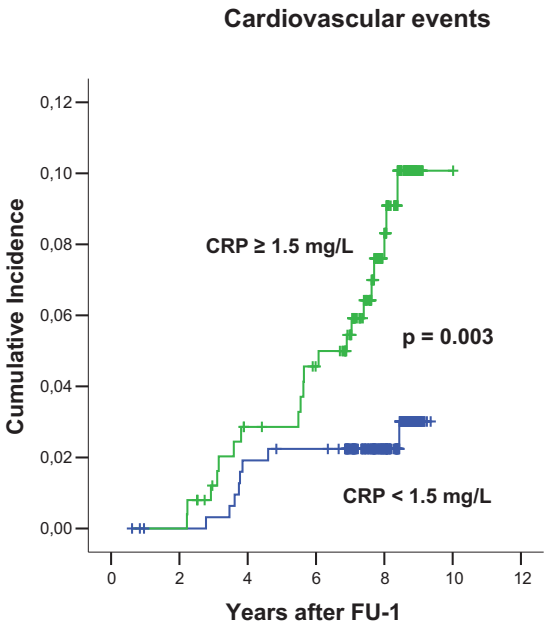
Abbreviations: FU-1: first follow-up survey; FU-2: second follow-up survey; CVD: cardiovascular disease
[1]Age at survey >60 years; [2]Invited patients eligible for FU-1; [3]Without out-patient visit, questionnaire or lack of blood sampling; [4] p:<0.01, all other comparisons p:>0.05; Observation time and age at follow-up are presented as median and range. Other values are number (%).

Figure 2 Cumulative Incidence of non-germ cell cancer (Panel A) and cardiovascular disease (Panel B) according to CRP-level ≥ or below 1.5 mg/L. Cumulative Incidence of non germ cell cancer (Panel C) and CVD (Panel D) according to treatment group

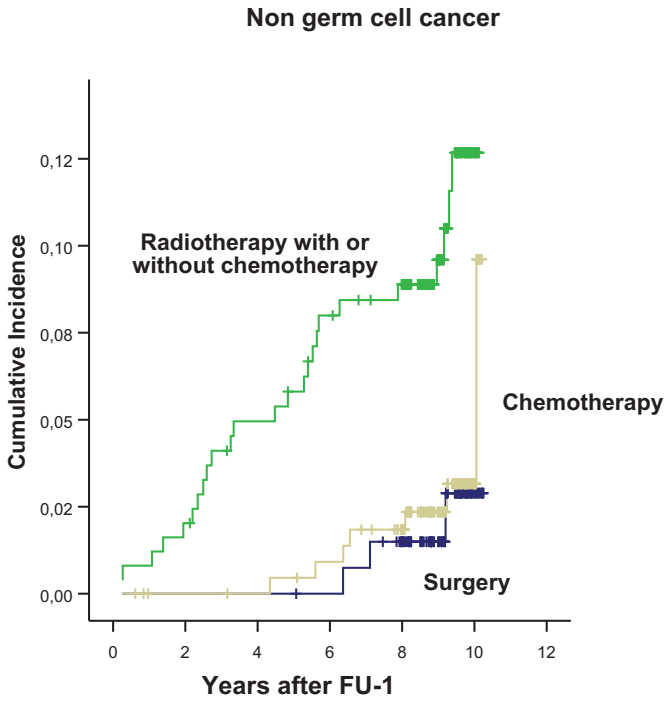
Panel A



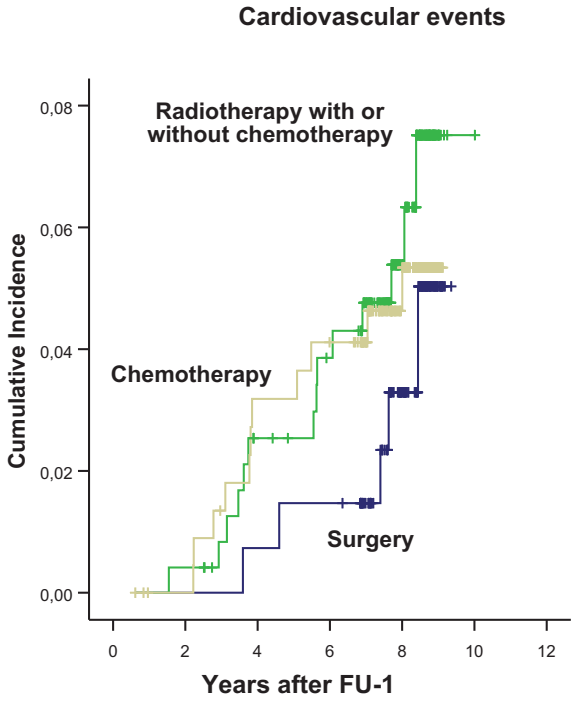
Panel B


Cardiovascular events
Cumulative Incidence
0,12
0,10
0,08
0,06
0,04
0,02
0,00
CRP ≥ 1.5 mg/L
p = 0.003
CRP < 1.5 mg/L
0
2
4
6
8
10
12
Years after FU-1

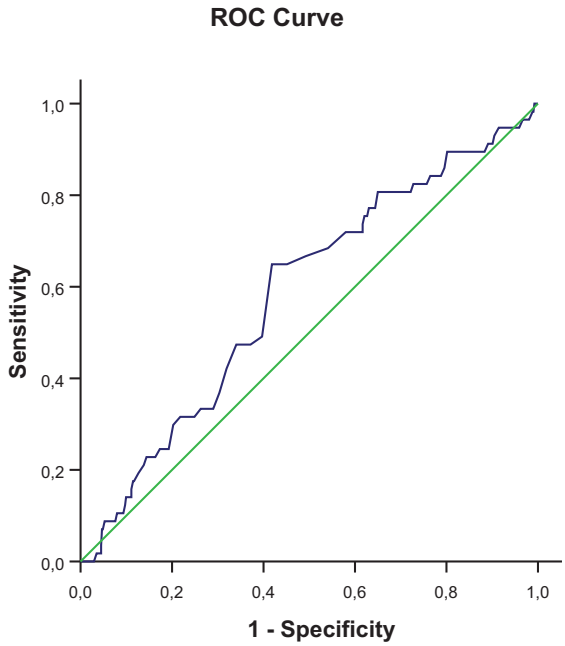
Panel C


Non germ cell cancer
Cumulative Incidence
0,12
0,10
0,08
0,05
0,02
0,00
Radiotherapy with or without chemotherapy
Chemotherapy
Surgery
0
2
4
6
8
10
12
Years after FU-1

Panel D


Cardiovascular events
Radiotherapy with or without chemotherapy
Chemotherapy
Surgery
Cumulative Incidence
0,08
0,06
0,04
0,02
0,00
0
2
4
6
8
10
12
Years after FU-1

Online Figure 1 Area under the curve for CRP and second cancer or cardiovascular disease. CRP ≥ 1.5 mg/L had a sensitivity of 65% and specificity at 58% to predict cardiovascular disease or second cancer. Area under the curve: 0.58 (95% CI 0.51 – 0.66), p = 0.04).



III

IV