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# ACCELERATED AGING AFTER HEMATOPOIETIC STEM CELL TRANSPLANTATION IN CHILDHOOD

*Early Menopause, Premature Cardiovascular Aging and Frailty*

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**ACADEMIC DISSERTATION**

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*To my father*

# Table of Contents

# List of Original Publications

This thesis is based on the following publications:

I Vatanen A, Wilhelmsson M, Borgström B, Gustafsson B, Taskinen M, Saarinen-Pihkala UM, Winiarski J, Jahnukainen K. Ovarian function after allogeneic hematopoietic stem cell transplantation in childhood and adolescence. *Eur J Endocrinol* 2013 Dec 27;170(2):211-8. doi:10.1530/EJE-13-0694.

II Vatanen A, Sarkola T, Ojala TH, Turanlahti M, Jahnukainen T, Saarinen-Pihkala UM, Jahnukainen K. Radiotherapy-related arterial intima thickening and plaque formation in childhood cancer survivors detected with very-high resolution ultrasound during young adulthood. *Pediatr Blood Cancer* 2015 Jun 5;62:2000-2006. doi:10.1002/pbc.25616.

III Vatanen A, Ojala TH, Sarkola T, Turanlahti M, Jahnukainen T, Saarinen-Pihkala UM, Jahnukainen K. Left ventricular mass and ambulatory blood pressure are important follow-up markers in childhood cancer survivors. *BMT* 2016 Feb 1;00:1–3. doi:10.1038/bmt.2015.355.

IV Vatanen A, Hou M, Huang T, Söder O, Jahnukainen T, Kurimo M, Ojala TH, Sarkola T, Turanlahti M, Saarinen-Pihkala UM, Jahnukainen K. Accelerated aging and shortened telomeres among long-term survivors of childhood high-risk neuroblastoma. Submitted.

The publications are referred to in the text by the above Roman numerals. These articles were reprinted with the permission of their copyright holders. In addition, some previously unpublished data are also presented.

# Abstract

Hematopoietic stem cell transplantation (HSCT) is used to treat many non-malignant and malignant pediatric diseases. A growing number of survivors of pediatric HSCT enter adulthood and middle age. There is increasing evidence that exposure to irradiation and DNA damaging agents in childhood accelerates aging. Increased risk of premature ovarian failure, early atherosclerosis and frailty has been reported among adult survivors of childhood cancer. Less is known about the effects of pediatric HSCT on long-term organ functions and aging.

The first part of this thesis was designed to retrospectively evaluate the effects of allogeneic HSCT in childhood and adolescence on long-term ovarian function. The second part was designed to investigate cardiovascular risk factors, endothelial function, arterial morphology and stiffness, left ventricular (LV) mass and function, and physical fitness and frailty in adult and adolescent survivors of high-risk neuroblastoma (HR NBL) after autologous HSCT.

The objective of this thesis was to study whether long-term survivors of pediatric HSCT show signs of premature vascular, reproductive and physical aging and whether these signs of aging associate with HSCT therapy, cardiovascular health, markers of inflammation or telomere length.

The first study population included a cohort of 92 female long-term survivors who were less than 20 years of age when receiving allogeneic HSCT at the Children's Hospital, Helsinki University Hospital, Helsinki, Finland, or Karolinska University Hospital Huddinge, Stockholm, Sweden, between 1978 and 2000. The follow-up data were collected from medical records and included signs of spontaneous puberty, age at menarche, the use of hormone replacement therapy, pregnancies, and information about pubertal or postpubertal serum FSH levels.

The second study population included the Finnish national cohort of 19 (79%) very long-term HR NBL survivors after autologous HSCT, born in 1996 or earlier and treated at any of the five University Hospitals in Finland between 1980 and 2000. One high-risk retinoblastoma survivor treated similarly was included as well. Twenty healthy individually age- and sex-matched controls were recruited as controls. Clinical examinations included anthropometrics, blood samples, 24h ambulatory blood pressure (BP), very-high-resolution vascular ultrasound, 3D echocardiography and Tissue Doppler Imaging cardiac ultrasounds, flow-mediated-dilatation test, body composition measured with dual-energy X-ray absorptiometry, physical performance tests, questionnaire and interview. Medical history was collected from medical records.

The first study showed that older age at HSCT and total body irradiation and busulfan-based conditionings were risk factors for early ovarian aging. Leukemia survivors with previous cranial radiotherapy or transplanted after disease relapse were at high risk of premature ovarian failure. The second study showed a higher frequency of carotid and femoral arterial plaques, thickened intima layer in radial and femoral arteries, increased carotid intima-media thickness and stiffness, decreased carotid compliance and arterial lumen diameters in brachial and femoral arteries and increased cardiovascular risk profile in the HR NBL survivors when compared to the controls. The third study showed that the HR NBL survivors had increased LV mass and decreased systolic and diastolic LV function when compared to the controls. Poor LV function associated with cardiac biomarkers, poor physical performance and increased BP. The fourth study showed shorter telomere length and increased frequency of frailty phenotype including decreased lean mass, slowness, weakness and low physical activity among the HR NBL survivors when compared to the age-matched controls. The frailty phenotype associated with cardiovascular health and chronic inflammation.

In conclusion, our study shows that adult and adolescent survivors after HSCT at a young age are at risk of early reproductive and vascular aging and frailty. The survivors of pediatric HSCT require regular follow-up in adulthood and interventions for declining ovarian function, cardiovascular risk factors, high BP, subclinical signs of atherosclerosis and decreased cardiac function. Since lifestyle choices can influence cardiovascular health and frailty status, a healthy lifestyle, non-smoking and physical activity should be advocated among all survivors who have received HSCT in childhood.

# Tiivistelmä

Hematopoieettista kantasolusiirtoa käytetään hoitona useissa hyvän- ja pahanlaatuisissa taudeissa. Yhä useampi kantasolusiirtohoidetuista potilaista paranee ja saavuttaa aikuisiän. Ensimmäiset kantasolusiirtohoidetut potilaat ovat pian keski-ikäisiä. Altistuminen lapsena DNA:ta vaurioittavalle säde- tai solunsalpaajahoidolle altistaa ennenaikaiselle vanhenemiselle. Aikuistuneilla lapsisyöpäpotilailla on todettu verrokkeja useammin ennenaikaisia vaihdevuosia, verisuonien vanhenemista ja raihnaisuutta. Tietoa hematopoieettisen kantasolusiirtohoidon pitkäaikaisista vaikutuksista elinten toimintaan ja vanhenemiseen on toistaiseksi vähän.

Tutkimuksen ensimmäisen osan tavoitteena oli selvittää pitkäaikaista munasarjatoimintaa lapsuus- ja nuoruusiässä saadun allogeenisen kantasolusiirron jälkeen. Tutkimuksen toisen osan tavoitteena oli selvittää sydän- ja verisuoniriskitekijöiden esiintymistä, valtimoiden rakennetta ja jäykkyyttä, endoteelitoimintaa, vasemman kammion massaa ja toimintaa sekä fyysistä kuntoa ja raihnaisuutta aikuisiässä korkean riskin neuroblastoomasta selvinneillä. Näillä tutkimuksilla pyrittiin selvittämään, todetaanko lapsena kantasolusiirtohoidon saaneilla ikäverrokkeja useammin raihnaisuutta tai suonten tai sukurauhasten vanhenemista. Pyrimme lisäksi arvioimaan raihnaisuuden ja kardiovaskulaarisen vanhenemisen yhteyttä sydän- ja verisuoniterveyteen, tulehdukseen ja telomeerin pituuteen.

Ensimmäinen tutkimuskohortti käsitti 92 parantunutta naispotilasta, jotka olivat alle 20-vuotiaita saadessaan allogeenisen kantasolusiirron Lastenklinikalla Helsingin yliopistollisessa sairaalassa Helsingissä tai Karoliinisessa yliopistosairaalassa Tukholmassa vuosina 1978-2000. Tiedot puberteetista, kuukautisten alkamisiästä, hormonikorvaushoidon tarpeesta, raskauksista ja seerumin FSH-tasoista kerättiin sairaskertomuksista.

Toinen potilasryhmä oli vuosina 1980-2000 Suomessa hoidettu kansallinen kohortti, joka koostui 19:stä (79 % koko kohortista) autologisella kantasolusiirrolla korkean riskin neuroblastoomasta parantuneesta potilaasta. Lisäksi tutkittiin yksi samaa hoitoa saanut korkean riskin retinoblastoomasta parantunut potilas sekä 20 tervettä ikä- ja sukupuolivakioitua verrokkia. Kliininen tutkimus sisälsi pituuden ja painon mittaukset, verikokeet, 24 tunnin verenpainemittauksen, valtimoiden korkeataajuisen ultraäänitutkimuksen, sydämen 3D- ja kudosdopplerultraäänen, endoteelifunktion tutkimuksen, kehon koostumuksen mittauksen DXA-laitteella, kuntotestit sekä kyselykaavakkeen ja haastattelun. Sairaushistoria kerättiin sairaskertomuksista.

Ensimmäisessä tutkimuksessa todettiin, että korkeampi ikä kantasolusiirron hetkellä ja koko kehon sädetys tai busulfan-pohjainen esihoito ennustivat munasarjatoiminnan ennenaikaista loppumista ja munasarjojen vanhenemista. Leukemiasta parantuneilla pään sädehoito ennen kantasolusiirtoa tai kantasolusiirto taudin uusimisen jälkeen lisäsivät riskiä munasarjojen toiminnan hiipumiseen. Toisessa tutkimuksessa todettiin, että korkean riskin neuroblastoomapotilaiden valtimoissa todetaan useammin plakkeja, paksuuntunut intima, alentunut valtimoiden joustavuus, pienentynyt läpimitta sekä enemmän sydän- ja verisuoniriskitekijöitä kuin ikävakioiduilla verrokeilla. Kolmannessa tutkimuksessa todettiin, että korkean riskin neuroblastoomapotilailla vasemman kammion massa on lisääntynyt, systolinen ja diastolinen toiminta on huonontunut, sydämen merkkiaineet veressä ovat koholla ja fyysinen suorituskyky on alentunut verrattuna ikävakioituihin verrokkeihin. Lisäksi todettiin yhteys kohonneen verenpaineen ja lisääntyneen vasemman kammion massan välillä. Neljännessä tutkimuksessa todettiin, että korkean riskin neuroblastoomapotilailla on lyhyemmät telomeerit kuin ikävakioiduilla verrokeilla, minkä lisäksi heillä todetaan useammin raihnaisuuteen liittyviä löydöksiä, kuten vähentynyttä lihasmassaa, hitautta, heikkoutta ja vähäistä fyysistä aktiivisuutta. Raihnaisuus liittyi huonoon sydän- ja verisuoniterveyteen sekä krooniseen tulehdukseen.

Johtopäätöksenä todetaan, että lapsuuden kantasolusiirtohoitoon liittyy riski verisuonten ja sukurauhasten ennenaikaiseen vanhenemiseen ja raihnaisuuteen. Lapsena kantasolusiirtohoidon saaneita tulee seurata säännöllisesti aikuisiässä mahdollisesti heikkenevän sydän- ja munasarjatoiminnan, kohonneen verenpaineen ja kehittyvän ateroskleroosin vuoksi. Koska elämäntapavalinnoilla voidaan vaikuttaa sydän- ja verisuoniterveyteen ja raihnaisuuteen, tulee kaikkia lapsuudessa kantasolusiirron saaneita aktiivisesti ohjata terveellisiin elämäntapoihin, tupakoinnin välttämiseen ja kuntoiluun.

# Abbreviations

| | |
|---|---|
| 24DIA% | proportion of 24-hour diastolic blood pressure exceeding limits |
| 16SDI | 16-segment dyssynchrony index |
| 24SYS | mean 24-hour systolic blood pressure |
| 24SYS% | proportion of 24-hour systolic blood pressure exceeding limits |
| 3DE | three-dimensional echocardiography |
| ABPM | ambulatory blood pressure measurement |
| ALL | acute lymphoblastic leukemia |
| AML | acute myeloid leukemia |
| BMI | body mass index |
| BP | blood pressure |
| BSA | body surface area |
| CNS | central nervous system |
| CRT | cranial radiotherapy |
| ECG | electrocardiogram |
| EDV | end-diastolic volume |
| EF | ejection fraction |
| ESV | end-systolic volume |
| FMD | flow-mediated dilatation |
| FSH | follicle stimulating hormone |
| GHbA1c | glycosylated hemoglobin A1c |
| GVHD | graft-versus-host disease |
| fTBI | fractioned total body irradiation |
| HDTx | high-dose therapy |
| HR NBL | high-risk neuroblastoma |
| hsCRP | high sensitivity C-reactive protein |
| HSCT | hematopoietic stem cell transplantation |
| IGF-1 | insulin-like growth factor 1 |
| IMT | intima-media thickness |
| LD | lumen diameter |
| LV | left ventricle |
| LVM | left ventricular mass |
| LVMI | left ventricular mass index |
| NBL | neuroblastoma |
| proPNB | N-terminal pro-B-type Natriuretic Peptide |
| PW | Pulse Wave Doppler |
| PW E | early mitral peak flow velocity |
| RT | radiotherapy |
| SAA | severe aplastic anemia |
| SD | standard deviation |
| SDS | standard deviation score |
| sTBI | single fraction total body irradiation |
| TBI | total body irradiation |
| TDI | Tissue Doppler Imaging |
| TDI E’ | early diastolic myocardial relaxation velocity |
| TDI S’ | systolic myocardial velocity |
| TNF | tumor necrosis factor |
| TNI | total nodal irradiation |
| VHRU | very-high-resolution ultrasound |

# 1 Introduction

Hematopoietic stem cell transplantation (HSCT) is used in many high-risk malignant and non-malignant diseases affecting hematopoietic or immune systems. Patients with metastatic, refractory, or recurrent solid tumors are treated with intensive high-dose therapies (HDTx) with autologous HSCT as a stem cell support. In Finland, approximately 40 children receive HSCT every year.

HSCT was first investigated as a protective treatment for those exposed to accidental high-dose irradiation, such as atomic bomb exposure. In the 1950s total body irradiation (TBI) was shown to eradicate leukemic hematopoietic cells and HSCT was introduced as a treatment in hematologic malignancies. First HSCTs were performed between identical twins (Thomas et al. 1959). TBI by itself was not proven to be good in long-term leukemia control, and cyclophosphamide was introduced in combination with TBI in the late 1970s.

Dose intensification of conditioning regimens and exploring new drugs to reduce the risk of relapse or rejection were under research in the 1980s. HDTx with autologous HSCT support was introduced to increase dose intensity and response in chemosensitive tumors. In Finland, single dose TBI (sTBI) was replaced with fractionated TBI (fTBI) in 1984 to reduce toxicity.

In the 1990s, standard, intensified and reduced conditioning regimens were introduced. Patients with low-risk disease but at high risk for treatment-related toxicity were given different combinations of drugs and radiotherapy (RT) than patients with high-risk disease and low risk for toxicity. Due to the increasing evidence of TBI-related long-term toxicity and adverse effects on growth, endocrine and other organ systems, TBI was no longer used to treat small children.

During the 2000s effective primary therapies, with conventional methods, have significantly reduced the use of HDTx in high-risk solid tumors and the use of allogeneic HSCT in the first leukemia remission. Indications of allogeneic HSCT have become wider and it is increasingly used to treat immunodeficiencies, metabolic diseases and hemoglobinopathies. The future development of HSCT therapy aims at the further individualization of conditioning therapies, novel graft product engineering, and improved graft-versus-host disease (GVHD) prophylaxis.

Less treatment-related morbidity and improved quality of life after HSCT are other significant future aims. Nearly all children treated with HSCT will experience at least one late effect (Wilhelmsson et al. 2015). Information from long-term studies is emerging, suggesting a significant rise in organ dysfunctions with increasing age. Any acute major life event, such as severe illness, is known to accelerate human aging. Suboptimal lifestyle choices may further speed up this process. Young adult

childhood cancer survivors have been shown to be at an increased risk of frailty (Ness et al. 2015) but the frequency of frailty after pediatric HSCT is not known.

This study was initiated to investigate the frequency and etiology of ovarian failure, vascular aging and frailty among adult survivors of HSCT in childhood.

"A Man is as Old as His Arteries"

Thomas Sydenham, M.D. (1624-1689)

# 2 Review of the Literature

## 2.1 Hematopoietic Stem Cell Transplantation in Childhood

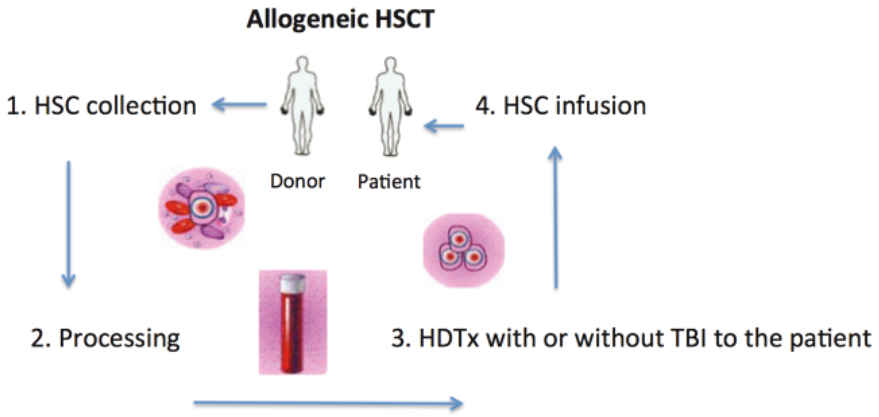


**Figure 1.** Patient receiving hematopoietic stem cells (HSC) from a healthy donor in allogeneic hematopoietic stem cell transplantation (HSCT) after high-dose therapy (HDTx) with or without total body irradiation (TBI).

### 2.1.1 Allogeneic HSCT

In severe hematological diseases, the purpose of allogeneic HSCT is to replace the defective bone marrow with a healthy one, and in malignant hematological diseases, to harness alloimmunity for the prevention of disease recurrence. In allogeneic HSCT, patients are treated with chemo- or radiotherapy in order to cause bone marrow failure and to destroy unwanted cancer cells. Stem cells from a closely matched donor are infused and bone marrow is replaced (Figure 1). The donor can be a sibling, other relative or an unrelated donor from registries. The stem cell source can be bone marrow, peripheral blood or cord blood. In general, criteria that have to be taken into account in donor selection are human leukocyte antigen type, cytomegalovirus status, gender, health status, and donor age. Indications for allogeneic HSCT include hematological malignancies (Acute Lymphoblastic Leukemia (ALL), Acute Myeloid Leukemia (AML), Chronic Myeloid Leukemia, Myelodysplasia), malignant solid tumors (Non-Hodgkin Lymphoma), non-malignant hematological diseases (Severe Aplastic Anemia (SAA), Osteopetrosis, Diamond-

Blackfan Anemia, Fanconi Anemia, Sickle Cell Anemia, Thalassemia Major, Chronic Granulomatous Disease), immune deficiencies (Severe Combined Immunodeficiencies, Wiskott-Aldrich syndrome, X-Linked Lymphoproliferative Disease) and inborn errors of metabolism (Lysosomal Storage Diseases).

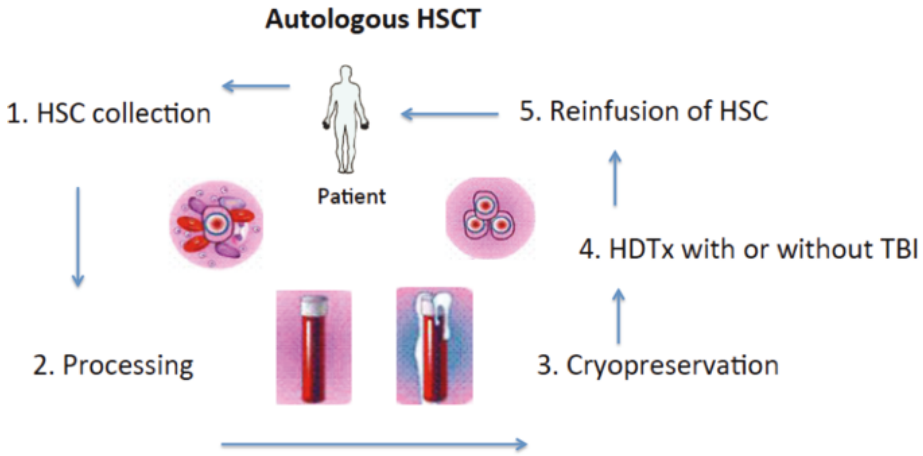


**Figure 2.** Patient receiving his or her own hematopoietic stem cells (HSC) collected before high-dose therapy (HDTx) with or without total body irradiation (TBI) in autologous hematopoietic stem cell transplantation (HSCT).

### 2.1.2 Autologous HSCT

In autologous HSCT, the patient receives his or her own cryopreserved bone marrow stem cells or CD34+ stem cells collected from peripheral blood before intensive therapy (Figure 2). Stem cells are infused to support hematological recovery after HDTx. HDTx is used to increase dose intensity and response in chemosensitive tumors. In previous decades, tandem or even triple HDTx have been used. Current indications for using autologous HSCT are high-risk neuroblastoma (HR NBL), Hodgkin's disease, germ cell tumors and Ewing sarcoma. HDTx is also used to avoid irradiation in infants and small children and in bilateral Wilms' tumor.

### 2.1.3 HSCT in Treatment of High-Risk Neuroblastoma

Neuroblastoma (NBL) is the most common extracranial malignant solid tumor in pediatric patients. NBL is a disease of the sympaticoadrenal lineage of the neural crest. Tumors arise from the adrenal medulla, cervical, thoracic or lumbar areas. Disseminated disease can be found in cortical bone, bone marrow, liver, skin, lymph nodes, lungs and the central nervous system (CNS). Staging of NBL is based on the International Neuroblastoma Staging System (INSS) (Brodeur et al. 1988, Brodeur et al. 1993) and International Neuroblastoma Risk Group Staging System (INRG) (Cohn et al. 2009) (Table 1). The annual incidence of NBL in Finland is approximately nine new cases per year (Madanat-Harjuoja et al. 2014), which corresponds to an incidence of nine per million children. Approximately one-half of the NBL patients present with high-risk features, which are: age over 18 months at diagnosis, stage 4 disease, MYCN amplification and stroma-poor histology in Shimada classification. All stage 4 NBL patients over 18 months at diagnosis, patients with NBL with MYCN amplification (except stage 1 and stage 4S) and those with stage 3 NBL with unfavorable histology are regarded to have HR NBL.

The treatment of HR NBL with intensive induction chemotherapy, surgery, RT, single or multiple HDTx with autologous HSCT and post-transplant therapy with isotretinoin (13-cis retinoic acid) therapy has resulted in event-free survival up of to 40-50%. However, more than half of the patients experience disease recurrence. Myeloablative therapy has shown to improve event-free survival (Saarinen-Pihkala et al. 2012, Yalcin et al. 2015). However, for overall survival, there is currently no evidence of an improving effect when additional follow-up data are included (Yalcin et al. 2015). A possible explanation can be that treatment options for progressive disease or relapse are decreased in patients who have received myeloablative therapy, or there is increased mortality in HSCT-related late effects. In the newest HR NBL treatment protocols novel immunotherapies have been introduced, including anti-GD2 monoclonal antibody, granulocyte-macrophage colony-stimulating factor (GM-CSF) and aldesleukin (interleukin-2).

**Table 1.** Neuroblastoma staging according to INSS and INRG systems.

| | |
|---|---|
| **INSS classification system** | |
| **Stage 1** | Localized tumor with complete gross excision, with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumor microscopically (nodes attached to and removed with the primary tumor may be positive). |
| **Stage 2A** | Localized tumor with incomplete gross excision; representative ipsilateral nonadherent lymph nodes negative for tumor microscopically. |
| **Stage 2B** | Localized tumor with or without complete gross excision, with ipsilateral nonadherent lymph nodes positive for tumor. Enlarged contralateral lymph nodes must be negative microscopically. |
| **Stage 3** | Unresectable unilateral tumor infiltrating across the midline, with or without regional lymph node involvement; or localized unilateral tumor with contralateral regional lymph node involvement; or midline tumor with bilateral extension by infiltration (unresectable) or by lymph node involvement. |
| **Stage 4** | Any primary tumor with dissemination to distant lymph nodes, bone, bone marrow, liver, skin, and/other organs (except as defined for stage 4S). |
| **Stage 4S** | Localized primary tumor (as defined for stage 1, 2A, or 2B), with dissemination limited to skin, liver, and/or bone marrow (limited to infants, 1 year of age). |
| **INRG staging system** | |
| **L1** | Localized tumor not involving vital structures as defined by the list of IDRFs and confined to one body compartment. |
| **L2** | Loco-regional tumor with the presence of one or more IDRFs. |
| **M** | Distant metastatic disease (except stage MS). |
| **MS** | Metastatic disease in children younger than 18 months with metastases confined to skin, liver, and/or bone marrow. |

IDFR, Image Defined Risk Factors; INRG, International Neuroblastoma Risk Group Staging System; INSS, International Neuroblastoma Staging System

Modified from detailed criteria of staging (Brodeur et al. 1988, Brodeur et al. 1993, Cohn et al. 2009).

## 2.2 Late Effects after HSCT in Childhood

### 2.2.1 Acute Complications after HSCT

Acute complications after HSCT include conditioning regimen-related toxicity, infections and GVHD. Acute toxicity includes sinusoidal obstruction syndrome, veno-occlusive disease in the liver, hemorrhagic cystitis and transplant-associated microangiopathy. After HSCT, the recipient's immune system is reconstituted. Patients are at risk for bacterial infections, viral infections, infections with respiratory viruses, fungal infections and reactivation of herpes viruses, such as cytomegalovirus. Acute and chronic GVHD results from alloreactivity between donor and recipient. Acute GVHD develops after a "cytokine storm", usually one to 14 weeks after allogeneic HSCT. Chronic GVHD is usually defined when GVHD occurs after day 100 post-transplant. The classic target organs are the skin, liver, and gastrointestinal tract (Ferrara et al. 2009).

### 2.2.2 Late Complications of HSCT

#### *2.2.2.1 Chronic GVHD, Late Infections, and Immune Deficiency*

Chronic GVHD with immune deficiency is the primary cause of transplant-related mortality late after HSCT and contributes directly or indirectly to most non-malignant complications. The incidence of chronic GVHD after sibling-matched related and unrelated transplantation lies between 27% to 50% and 42% to 72% (Lee et al. 2002). Higher donor and recipient age, prior acute GVHD, use of alloimmune female donors, type of GVHD prophylaxis, and history of recipient herpes virus infection are factors which further increase the incidence of chronic GVHD (Socie et al. 2003, Lee et al. 2002). Skin changes, extensive skin involvement, elevated bilirubin, progressive onset, thrombocytopenia, and prior steroid refractory/dependent acute GVHD are known to predict poor survival of patients suffering from chronic GVHD (Lee et al. 2002).

#### *2.2.2.2 Late Ocular Effects*

Ocular complications include keratoconjunctivitis sicca, conjuctival GVHD, cataracts, corneal infections, microvascular retinopathy, optic disk edema, hemorrhagic complications and infectious retinitis (Coskuncan et al. 1994). The incidence of cataract may reach up to 80% in long-term follow-up (Socie et al. 2003). Posterior segment complications have been seen in 13% of the patients (Coskuncan et al. 1994).

#### *2.2.2.3 Pulmonary Late Effects*

Sensitivity to cytotoxic agents and irradiation, infections, and immune-mediated lung injury associated with GVHD are the factors contributing to late pulmonary complications. These complications affect 15% to 40% of transplanted patients and include abnormal pulmonary function tests with loss of lung volume and diffusing capacity and chronic obstructive pulmonary disease (Cerveri et al. 2001, Soubani et al. 1996).

#### *2.2.2.4 Late Liver Complications*

Late liver complications include chronic hepatitis and iron overload. Liver biopsies show hemosiderosis in most patients (90%) when performed early after HSCT (Iqbal et al. 1996). A correlation exists between iron accumulation and persistent hepatic dysfunction, presumably as a consequence of intracellular iron and the toxic effect of free radicals.

#### *2.2.2.5 Late Complications of Bones and Joints*

TBI, previous chemotherapy and hypogonadism have been identified as risk factors for bone loss and osteoporosis (Schimmer et al. 2000, Weilbaecher 2000). Osteopenia and osteoporosis are both characterized by an impaired bone mass and increased vulnerability to bone fracture. The incidence of avascular necrosis varies from 2% to 11% after HSCT (Fink et al. 1998, Socie et al. 1994, Socie et al. 1997). The hip is the affected site in more than 80% of the cases. Other locations include the knee, wrist, and ankle.

#### *2.2.2.6 Dental Late Effects*

Conditioning regimens including TBI can result in severe damage to the dental enamel organ and developing teeth among 80% of the patients (Dahllof et al. 1988, Pajari & Lanning 1995), including hypoplasia and microdontia of the crowns and thinning of the roots of permanent teeth (Uderzo et al. 1997).

#### *2.2.2.7 Endocrine Function after HSCT*

Thyroid dysfunction is one of the most frequent late complications of HSCT (43% of patients), including overt and subclinical-compensated hypothyroidism and autoimmune thyroid disease (Sklar et al. 1982). The majority of cases of overt hypothyroidism following HSCT are due to direct damage to the thyroid gland by TBI.

Growth deficiency is more evident in children who receive transplants at a younger age or have received RT (Shinagawa et al. 2001). The growth impairment is estimated to be approximately one height standard deviation score (SDS) (equivalent to 6 cm) compared with the mean genetic height (Cohen et al. 1999a, Cohen et al. 1999b, Shinagawa et al. 2001). On the other hand, children who are conditioned without TBI usually grow normally. Posttransplantation factors such as GVHD and treatment with steroids may have an influence on growth failure in childhood.

#### *2.2.2.8 Testicular Failure*

Testicular failure is a common (70% of patients) long-term consequence of HSCT (Anserini et al. 2002). The major cause of gonadal damage leading to hypergonadotropic-hypogonadism is conditioning with TBI. The testicular germinal epithelium where spermatogenesis occurs is more vulnerable to radiation and chemotherapy than the Leydig cells where testosterone secretion occurs. Therefore, while spermatogenesis is reduced or absent, testosterone levels are usually normal. The probability for recovery of spermatogenesis after HSCT is associated with the type of conditioning therapy, the age of the patient, the time interval since transplantation, and the absence of chronic GVHD (Anserini et al. 2002, Rovo et al. 2006).

**Table 2.** Evidence of ovarian damage and reproductive potential after HSCT and cancer treatment in childhood.

| Author and Year | Patients, n | Follow-up time | Cohort | Study Findings |
|---|---|---|---|---|
| Armenian et al, 2011 | 7,207/145 | 5 years | Pediatric auto/alloHSCT | Relative risk of OF 9.3/39.3 in HSCT survivors vs. only conventionally treated/controls. |
| Bakker et al, 2000 | 19 | 8 years | Pediatric alloHSCT | (6/10) 60% of those prepubertal at HSCT had spont puberty and menarche. |
| Borgmann-Staudt et al, 2012 | 138 | 6 years | Pediatric alloHSCT | Older age and Bu risk factors for OF. |
| Bresters et al, 2014 | 109 | 7 years | Pediatric HSCT | OF associated with older age at HSCT and Bu conditioning. |
| Sanders et al, 1988 | 43/144 | >1 year | Pediatric and adult alloHSCT, SAA/leukemia | After Cy ovary recovers in those younger at HSCT and some old survivors but after TBI only few young at HSCT recovered. |
| Sanders et al, 1996 | 708 | 3 years | Pediatric and adult alloHSCT | 16% recovered ovarian function and 5% became pregnant among postpubertal women. |
| Sanders et al, 2011 | 63 | 22 years | Pediatric alloHSCT, SAA | Normal puberty, menarche, fertility. |

Bu, busulfan; Cy, cyclophosphamide; HSCT, hematopoietic stem cell transplantation; HRT, hormone replacement therapy; OF, ovarian failure; SAA, severe acute anemia; TBI, total body irradiation

#### 2.2.2.9 Premature Menopause and Ovarian Failure

The oocytes are very sensitive to HSCT therapy. The age at transplantation is of major importance. The younger the age, the better the chances for gonadal recovery (Table 2). Ovarian failure in postpubertal girls is usually irreversible, whereas in prepubertal girls, there is a possibility for a subsequent spontaneous recovery and achievement of spontaneous menarche (Borgmann-Staudt et al. 2012, Spinelli et al. 1994, Sanders et al. 1988, Bresters et al. 2014). The physiological decline in the ovarian reserve explains the age-dependent sensitivity (Faddy et al. 1992).

The risk for ovarian failure depends also on the conditioning regimen used (Table 2). TBI is gonadotoxic and increases the risk of ovarian failure (Sanders et al. 1988). The fractionation of TBI reduces the risk of ovarian failure (Sanders et al. 1988). Busulfan as conditioning regimen associates with a high risk of ovarian failure and infertility (Borgmann-Staudt et al. 2012, Sanders et al. 1996, Bresters et al. 2014). Most of the females conditioned with TBI or busulfan will need hormonal replacement therapy for the induction of puberty and maintenance of menstrual cycles. Instead, the majority of girls conditioned with cyclophosphamide for SAA progress normally through puberty and experience menarche and have normal gonadotropins, have normal pregnancies and give birth to healthy offspring (Socie et al. 2003, Sanders et al. 1996, Sanders et al. 2011). This is contrary to the pregnancy rate among other female survivors, which is less than 2% (Socie et al. 2003).

**Table 3.** Evidence of Cardiovascular Late Effects after Cancer Treatment and/or HSCT in childhood.

| Author and Year | Patients, n | Follow-up time | Cohort | Study Findings |
|---|---|---|---|---|
| Alehan et al, 2012 | 72 | 9 years | Pediatric HL, chemo and/or chest RT | Mild systolic and diastolic dysfunction. |
| Armstrong et al, 2013 | 10,724 | 5 years | CCS | Cardiovascular risk factors potentiate cancer therapy-associated risk for cardiac events. |
| Bowers et al, 2006 | 37/63 | 5 years | CCS, leukemia/brain tumors | Increased risk to stroke after CRT >30 Gy. |
| Brouwer et al, 2013 | 277 | 18 years | CCS, anthracyclines and/or chest RT/TBI | Increased carotid and femoral IMT associated to RT. |
| Dengel et al, 2014 | 319 | 5 years | CCS | Increased carotid artery stiffness, premature atherosclerosis and cardiovascular disease. |
| Gillis et al, 2007 | 31 | 5 years | CCS, HR NBL, autoHSCT+ intraoperative RT | Aortic stenosis, hypertension, vascular stenosis, renal artery stenosis, middle aortic syndrome. |
| Kero et al, 2014 | 16,769 | 5 years | CCS | Cardiovascular diseases are second highest non-malignancy-related causes of mortality. |
| Küpeli et al, 2010 | 119 | 2 years | CCS, HL | Increased risk to coronary artery disease after >20 Gy chest RT. |
| Meacham et al, 2010 | 8,599 | >5 years | CCS longitudinal study | Older age, TBI/abdominal and chest RT are risk factors for hypertension, dyslipidemia, diabetes and microalbuminuria. |
| Meeske et al, 2009 | 30 | >10 years | CCS, neck RT | Increased carotid IMT and frequency of plaques. |
| Taskinen et al, 2000 | 23 | >10 years | Pediatric alloHSCT | Increased frequency of metabolic syndrome. |
| Turanlahti et al, 2012 | 25 | 2 years | Pediatric alloHSCT | Early mechanical atherosclerotic changes of the arterial wall. |
| Wilhelmsson et al, 2015 | 204 | >10 years | Pediatric alloHSCT | Cardiac late effect in 10% (mean age of 22 years). |

CCS, childhood cancer survivor; CRT, cranial radiotherapy; HL, Hodgkin lymphoma; HR NBL, high-risk neuroblastoma; HSCT, hematopoietic stem cell transplantation; IMT, intima-media thickness; RT, radiotherapy; TBI, total body irradiation

#### 2.2.2.10 Cardiovascular and Metabolic Late Effects

Cardiovascular diseases are emerging late effects after allogeneic HSCT in childhood, leading to considerable morbidity and mortality (Armenian & Bhatia 2008) (Table 3). Irradiation and allogeneic HSCT, particularly if GVHD is present, have been shown to increase the risk for early vascular disease and poor cardiac function (Rovo et al. 2012). Cardiovascular diseases are in many cases related to extended atherosclerosis, contributed by cardiovascular risk factors in adult survivors(Tichelli et al. 2007, Tichelli et al. 2008, Armenian et al. 2010, Majhail et al. 2009, Griffith et al. 2010). The few reports from survivors transplanted in childhood show similar findings (Taskinen et al. 2000, Taskinen et al. 2007).

Increased risk of classical metabolic syndrome, including dyslipidemia, central obesity, increased systolic blood pressure (BP), diabetes and physical inactivity, has been reported among pediatric long-term cancer or HSCT survivors (Heikens et al. 2000, Meacham et al. 2010, Taskinen et al. 2000). Early mechanical changes have been described in carotid arterial wall after pediatric allogeneic HSCT (Turanlahti et al. 2013). Vascular complications including aortic and renal artery stenosis and middle aortic syndrome have been reported among HR NBL survivors (Gillis et al. 2007). In a retrospective report of long-term health outcomes 10% of adult survivors after pediatric allogeneic HSCT had cardiac late effects and many suffered from metabolic disorders including diabetes type II (9%), dyslipidemia (7%), and hypertension (7%) (Wilhelmsson et al. 2015). Two of the survivors had died at a young adult age due to acute myocardial infarction and stroke. Both had received TBI-based conditioning (Wilhelmsson et al. 2015).

Increased carotid artery intima-media thickness (IMT) (Bots et al. 1997) and systemic endothelial dysfunction detected by flow-mediated dilatation (FMD) are early signs of atherosclerosis (Neunteufl et al. 1997). Increased carotid artery IMT, endothelial dysfunction, increased arterial stiffness, decreased arterial compliance, and presence of arterial plaques predict subclinical cardiovascular disease (Neunteufl et al. 1997, Burke et al. 1995, O'Leary et al. 1999, Vlachopoulos et al. 2010).

Radiation-related capillary endothelial injury associates with luminal obstruction, fibrin formation, and platelet thrombi leading to ischemia, myocardial cell apoptosis, and fibrosis (Adams & Lipshultz 2005). Radiation-induced subclinical atherosclerosis, carotid artery disease, carotid artery IMT thickening and arterial stenosis have been reported among adult cancer patients (Silverberg et al. 1978, Feehs et al. 1991, Dorresteijn et al. 2005, Shariat et al. 2008, Gianicolo et al. 2010, Carmody et al. 1999). Increased IMT and increased risk for stroke associate with RT (Dengel et al. 2014, Brouwer et al. 2013, Meeske et al. 2009, Bowers et al. 2006, Mueller et al. 2013, Bowers et al. 2005, Kupeli et al. 2010). The risk for stroke is increased with cranial radiotherapy (CRT) exceeding >30 Gy, and the risk for

coronary artery disease with chest RT doses >20 Gy (Bowers et al. 2006, Kupeli et al. 2010). Increased arterial stiffness, measured with aortic pulse wave velocity, has been reported among childhood cancer survivors after exposure to a mean dose of 212 mg/m$^2$ of anthracyclines without RT (Herceg-Cavrak et al. 2011). Another study could not confirm the independent effect of anthracyclines on arterial stiffness (King et al. 1999).

The use of anthracyclines is known to be a primary contributor to cardiotoxicity resulting from direct cardiac myocyte apoptosis and leading to decreased contractility in the long term. The risk for heart failure is further increased if childhood cancer survivors have been exposed to chest irradiation, including TBI (Lipshultz et al. 2013). In international recommendations, survivors with cumulative anthracycline doses <100 mg/m$^2$ are considered at low risk for cardiomyopathy and those with doses 100 to 250 mg/m$^2$ or chest irradiation 15 Gy to 35 Gy at moderate risk (Armenian et al. 2015). The high-risk group consists of survivors with cumulative anthracycline doses >250 mg/m$^2$ or chest irradiation >35 Gy or moderate doses of both (Armenian et al. 2015). However, there is some evidence that irradiation even with doses <15 Gy increases the risk of heart failure (Mulrooney et al. 2009, van der Pal et al. 2005, Armenian et al. 2011, Green et al. 2001).

Cardiac dysfunction after childhood cancer treatment can develop after a long period of time. Usually diastolic dysfunction is seen first, followed by decreased systolic function. Early signs of cardiac dysfunction can be detected with three-dimensional echocardiography (3DE) and Tissue Doppler Imaging (TDI) (Alehan et al. 2012, Armstrong et al. 2015). Left ventricular mass (LVM) predicts cardiovascular morbidity in general population and emphasizes the importance of estimation of LVM also among childhood cancer survivors (Hunt et al. 2009, Levy et al. 1990). Hypertension is known to independently potentiate the risk for cardiac failure (Armstrong et al. 2013). Classic cardiovascular risk factors further increase LVM and the risk of heart failure (Berry et al. 2012) (Figure 3).

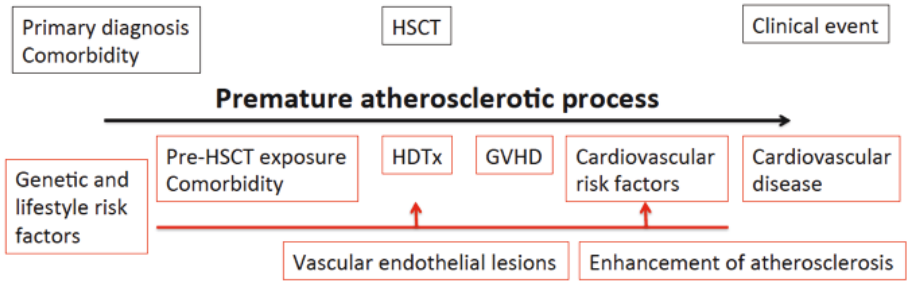


**Figure 3.** Premature atherosclerotic process described in hypothetical diagram in long-term survivors after childhood hematopoietic stem cell transplantation (HSCT). Pre-HSCT exposure, possible previous cancer therapy; HDTx, high dose therapy; GVHD, graft-versus-host disease.

#### *2.2.2.11 Neuropsychological Late Effects*

Fatigue and sleeping disorders have been reported among 65% of adult patients after HSCT (McQuellon et al. 1998). Problems with short-term memory and mild cognitive impairments have been described in approximately 10% to 20% of patients within the first year after HSCT (Meyers et al. 1994). Lower intelligence quotient (IQ) levels have been identified especially among pediatric patients transplanted at a very young age, but the changes were minimal and remained stable at the 3-year follow-up (Kramer et al. 1992, Phipps et al. 2000).

#### *2.2.2.12 Secondary Malignancy*

Factors that contribute to the development of secondary malignancies after HSCT include cytotoxic and immunosuppressive therapy, GVHD or antigenic stimulation, viruses, immunodeficiency, and genetic predisposition. Incidence of secondary malignancy of 2.6% to 9.9% has been reported after childhood HSCT (Wilhelmsson et al. 2015, Bhatia et al. 1996, Danner-Koptik et al. 2013). The most common secondary malignancies after HSCT are posttransplant lymphoproliferative disorders (PTLD), hematologic malignancies (AML, MDS), and solid tumors (carcinomas, sarcomas, CNS tumors) (Deeg & Socie 1998).

#### *2.2.2.13 Late Effects Associated with Conventional Chemotherapy prior to HSCT*

The role of HSCT in treatment of childhood cancer is to consolidate the remission. Remission can be induced by conventional chemotherapy, RT and molecularly targeted agents, usually in combinations (Table 4). This conventional therapy modifies the late effects after HSCT.

Children with a history of CRT are at a higher risk of cognitive deficits after HSCT (Socie et al. 2003). Anthracyclines, such as doxorubicin, are widely used in many malignant diseases. Anthracyclines have selective toxicity to cardiomyocytes and there is a limiting cumulative dose that should not be exceeded. Asparaginases, used to treat ALL and lymphoma, cause pancreatitis, coagulopathy and thrombosis. Methotrexate, used to treat leukemias, lymphomas, and osteosarcomas, can cause renal and tubular dysfunction. Cisplatin, used to treat testicular tumors, brain tumors, osteosarcomas, and NBLs, can lead to permanent renal- and ototoxicity. Cyclophosphamide and ifosfamide, used to treat leukemias, lymphomas, NBLs, sarcomas and germ cell tumors, are nephrotoxic and increase the risk of infertility. Vincristine, used to treat ALLs, lymphomas, and solid tumors, is neurotoxic and can cause persisting peripheral neuropathy with walking difficulties.

**Table 4.** Chemotherapy Agents Used in Pediatric Oncology.

| | |
|---|---|
| **Alkylating Agents** | Cyclophosphamide, Ifosfamide, Mechlorethamine, Melphalan, Busulfan, Thiotepa, Chlorambucil, Bendamustine<br>Nitrosoureas: Carmustine, Lomustine<br>Not classical alkylating agents: Dacarbazine, Procarbatzine, Temozolomide |
| **Antimetabolites** | Cytarabine, Azacytidine, Decitabine, Gemcitabine, Cladribine, Clofarabine, Fludarabine, Nelarabine, 6-Mercaptopurine, 6-Thioguanine, Hydroxyurea, 5-Fluorouracil, Capecitabine, Methotrexate, Pemetrexed |
| **Antitumor Antibiotics** | Anthracyclines: Doxorubicin, Epirubicin, Daunorubicin, Idarubicin<br>Bleomycin, Dactinomycin, Mitomycin C, Mitoxantrone |
| **Asparaginases** | l-Asparaginase, Crisantaspase, Pegasparaginase |
| **Corticosteroids** | Dexamethasone, Prednisolone, Prednisone |
| **Platinum Compounds** | Carboplatin, Cisplatin, Oxaliplatin |
| **Retinoids** | All-trans-retinoic acid, 13-cis-retinoic acid |
| **Topoisomerase-Interactive Agents** | Etoposide, Teniposide, Irinotecan, Topotecan |
| **Tubulin-Interactive Agents** | Docetaxel, Paclitaxel, Vinblastine, Vincristine, Vinorelbine |
| **Tyrosine Kinase Inhibitors** | Imatinib, Dasatinib, Nilotinib, Ponatinib, Sunitinib, Sorafenib, Erlotinib, Gefitinib, Crizotinib, Pazopanib, Vemurafenib |
| **Proteasome Inhibitors** | Bortezomib |
| **Histone Deacetylase Inhibitors** | Vorinostat, Arsenic trioxide, Everolimus, Temsirolimus, Ruxolitinib, Vismodegib |
| **Antibodies** | Rituximab, Gemtuzumab ozogamicin, Brentuximab vedotin, Alemtuzumab, Bevacizumab |
| **Biological Response Modulator** | Aldesleukin |

## 2.3 Accelerated Aging

### 2.3.1 Aging

In the normal aging process, physiological changes occur in all organ systems. The cardiac output decreases linearly after 30 years of age, blood pressure elevates physiologically progressively after 10 years of age, and atherosclerosis clearly increases with aging. The respiratory system shows impaired gas exchange, a linear decrease in vital capacity with an increase in residual volume, and slower expiratory flow rates. The glomerular filtration rate decreases and a progressive elevation of blood glucose occurs on a multifactorial basis. Menopause is a sign of ovarian aging that typically occurs between 45 and 55 years of age. Peak bone mass is attained at 20 years of age, decreasesing physiologically thereafter (Boot et al. 2010). Osteoporosis is frequently seen after 40 years of age due to a linear decline in bone mass. Atrophy of the epidermis, stiffer dermal collagen, calcification of elastin and a decrease in the number of dermal blood vessels cause the skin to lose its tone and elasticity. Loss and atrophy of muscle cells lead to decreased lean body mass. Degenerative changes occur in many joints; this, combined with the loss of muscle mass, leads to limitations in physical activity (Boss & Seegmiller 1981).

Early appearance of the signs of aging is called premature aging. The aging process is accelerated by acute insult, such as illness, injury or major life events, affecting organs or producing inactivity. Suboptimal lifestyle choices, such as inactivity, smoking, and a diet high in fat and sugar, increase the risk of high BP and contribute to the early structural changes of the arterial wall. This leads to the stiffening of large elastic arteries and causes early vascular aging (Vatanen et al. 2015). Exposures to DNA damaging agents and genetic diseases affecting genomic stability have evolved as other causative factors for premature aging (Shay & Wright 2007).

### 2.3.2 Biomarkers of Aging

Telomeres are repetitive DNA sequences at the ends of chromosomes that are involved in the maintenance of genomic stability and DNA repair. The end-replication problem causes some telomere sequences to be lost each time a cell divides and chromosomes replicate. At some point, when cells reach maximally shortened telomere length, they enter a replicative senescence state. Tissue regeneration is reduced and this leads to tissue dysfunction and chronic conditions that are often associated with increased age such as diabetes, cancer, and cardiovascular disease (Shay & Wright 2007). Consequently, telomere shortening serves as a biomarker of cellular senescence and aging.

Dyslipidemia, inflammation, increased body mass index (BMI), hypertension, diabetes, smoking, heavy alcohol intake and physical inactivity have been associated with shortened telomeres (Weischer et al. 2012, Butt et al. 2010, Cherkas et al. 2008, Shay & Wright 2007). Abnormal telomere shortening has been reported in Wilms' tumor tissues (Stewenius et al. 2007) and buccal cell DNA samples among childhood cancer survivors who develop second malignant neoplasms (Gramatges et al. 2014). On the other hand, there is some evidence that after bariatric surgery, weight loss can increase telomere length in a 10-year follow-up (Laimer et al. 2015).

Other biomarkers of cellular senescence include sterile proinflammatory state or senescence-associated secretory phenotype, resulting in elevated levels of inflammatory markers, including C-reactive protein (CRP), interleukin-6, tumor necrosis factor (TNF), and immune cell cytokines (Tchkonia et al. 2013). It is believed that this chronic inflammation may further contribute to the development and progression of chronic conditions such as insulin resistance, diabetes, hypertension, atherosclerosis and cardiovascular disease (Cesari et al. 2003, Ness et al. 2015).

### 2.3.3 Frailty

Decreased well-being and increased levels of frailty often accompany advanced age. Although chronological and biological age correlate, individuals with the same chronological age may vary widely in terms of health and functional status. The concept of frailty attempts to explain this heterogeneity. There is a growing consensus that markers of frailty include age-associated declines in lean body mass, strength, endurance, balance, walking performance, and low activity (Ness et al. 2015). The Canadian Study of Health and Aging has approached frailty with a frailty index, measured with symptoms, signs, diseases, and disabilities as deficits up to 70 items, and an index score indicates the likelihood that frailty is present (Rockwood & Mitnitski 2007). Fried et al, have provided a standardized definition for frailty, and they have created the concept of frailty phenotype that identifies old people who are at risk of disability, falls, institutionalization, hospitalization, and premature death (Fried et al. 2001).

According to this concept, a person is prefrail if two and frail if three or more of the following criteria are fulfilled:

1) Loss of muscle mass defined by lean muscle mass below -1.5 standard deviations (SD), measured with dual-energy X-ray absorptiometry dividing by height in meters squared (Ness et al. 2013, Kelly et al. 2009).

2) Weakness defined by using different strength tests like grip strength or sit-up test (Fried et al. 2001, Ness et al. 2013).

3) Self-reported exhaustion defined by special questionnaires (Ness et al. 2013, Fried et al. 2001).

4) Slowness defined by walking or running speed below lowest $20^{th}$ percentile or less than -1.5 SD for age, sex and height values (Fried et al. 2001).

5) Low energy expenditure defined by weekly physical activity level among men with less than 383 kcal/wk and women with less than 270 kcal/wk when measured by questionnaire and converted to kilocalories per week.

Chronic disease can advance functional decline and frailty. Increased frequency of frailty phenotypes have been reported among adult childhood cancer survivors (Ness et al. 2013) and patients with chronic kidney disease (Musso et al. 2015). In the St Jude Lifetime cohort, the combined prevalence of frailty and prefrailty among childhood cancer survivors was 41% among CNS tumor, 39% among soft tissue sarcoma, 39% among other solid tumor and 30% among leukemia, lymphoma and bone tumor survivors (Ness et al. 2013). In the same study, 13% of control women and 3% of control men at the mean age of 33 years were considered frail and 32% of women and 13% of men prefrail (Ness et al. 2013). A prevalence of frailty phenotype of 11% is reported in a meta-analysis of community-dwelling persons at least 65 years old and 26% among persons older than 85 years (Collard et al. 2012). The prevalence of frailty that is similar to ≥65 years old in the control population suggests significantly accelerated aging among young adult survivors of childhood cancer.

Young adult survivors of childhood cancer report several symptoms that interfere with daily life, including exercise-induced shortness of breath (Mertens et al. 2002), fatigue (Johannsdottir et al. 2012), and reduced capacity to participate in physical activity (Jarvela et al. 2010). These symptoms may also be indicators of premature aging or frailty (Table 5).

**Table 5.** Evidence of Frailty Phenotype among Childhood Cancer Survivors.

| Author and Year | Patients, n | Follow-up time | Cohort | Study Findings |
|---|---|---|---|---|
| Chemaitilly et al, 2015 | 748 | >15 years | CCS with previous CRT | Anterior pituitary deficits GHD 46.5%, LH/FSHD 10.8%. |
| Järvelä et al, 2010 | 21 | 16 years | ALL | Poor physical activity index 30%/36% (M/F). |
| Jóhannsdóttir et al, 2012 | 584 | 4-20 years | CCS | Chronic fatigue 14%. |
| Mertens et al, 2002 | 12,390 | >5 years | CCS | Exercise-induced shortness of breath, RR 3.0. |
| Ness et al, 2013 | 1,922 | >10 years | CCS | Prevalence of pre-frail/ frail phenotype 31.5%/13.1% vs. 12.9%/2.7% (F/M). |
| Oeffinger et al, 2006 | 10,397 | 18 years | CCS | Chronic health conditions cumulative incidence 73.4%. |
| Wilhelmsson et al, 2015 | 204 | >10 years | Pediatric alloHSCT | At least one chronic health condition 84%, at least one grade 3 condition 41%. |
| Zhang et al, 2015 | 22 | 6 years | CCS | Poor Healthy Eating Index Score 52.7/100. |

ALL, survivors of acute lymphoblastic leukemia; alloHSCT, allogeneic hematopoietic stem cell transplantation; CCS, childhood cancer survivors; CRT, cranial radiotherapy; F, female; FSHD, follicle stimulating hormone deficiency; GHD, growth hormone deficiency; LHD, luteinizing hormone deficiency; M, male; RR, relative risk

# 3 Aims of the Study

The goal of this thesis was to examine ovarian function, cardiovascular health, fitness, muscle mass, and physical activity in adult and adolescent survivors after HSCT in childhood. The hypothesis of this study was that a declined function in these aging-associated measures is detected among HSCT survivors but not among the age-matched controls.

The specific aims of the present study were:

1. To evaluate long-term ovarian function after allogeneic HSCT in childhood and adolescence with retrospective case note review.

2. To investigate cardiovascular risk factors, central and peripheral arterial morphology, arterial stiffness, and endothelial function in young adult survivors of HR NBL compared to healthy age- and sex-matched controls.

3. To investigate left ventricular mass and function in conjunction with a comprehensive cardiovascular risk assessment in young adult survivors of HR NBL compared to healthy age- and sex-matched controls.

4. To estimate the prevalence of frailty and its association with cardiovascular health, inflammation, and telomere length in young adult survivors of HR NBL and age- and sex-matched controls.

# 4 Patients and Methods

## 4.1 Patients

### 4.1.1 Female Long-Term Survivors after Allogeneic HSCT in Childhood (I)

Female long-term survivors who were less than 20 years of age when receiving allogeneic HSCT at the Children's Hospital, Helsinki University Hospital, Helsinki, Finland, or Karolinska University Hospital Huddinge, Stockholm, Sweden, between 1978 and 2000, were identified through hospital records and enrolled in the study assessing long-term health outcome after childhood HSCT (Figure 4).

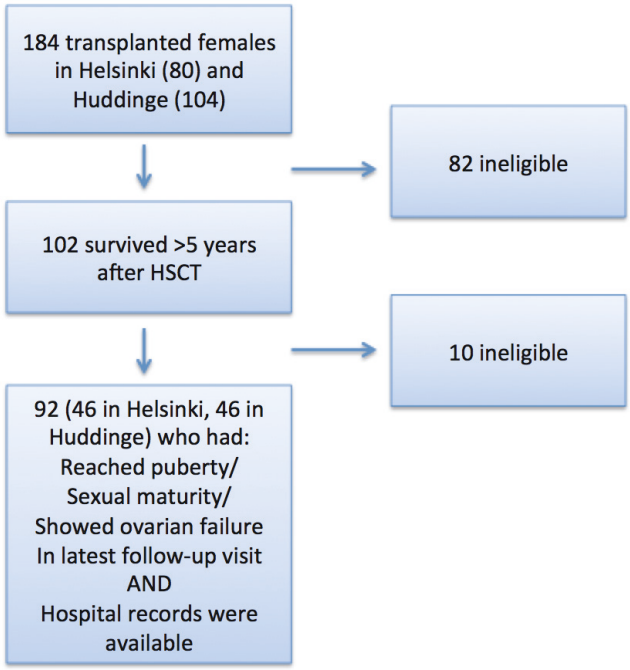


**Figure 4.** Patient selection process in Helsinki-Huddinge retrospective study. HSCT, hematopoietic stem cell transplantation.

#### *4.1.1.1 Treatment Characteristics*

Leukemia patients were treated according to the Nordic protocols for ALL (since 1982) and AML (since 1984) (Gustafsson et al. 2000, Lie et al. 2005). Conditioning treatments were changed over time and protocols differed between Helsinki and Huddinge (Table 6).

During the years 1978-1995, leukemia patients at the Karolinska University Hospital Huddinge, received cyclophosphamide (120mg/kg) combined with sTBI 10 Gy as the standard conditioning. In 1993 fTBI 12 Gy was introduced. Since 1996, the patients transplanted with AML have been conditioned with cyclophosphamide (120mg/kg) combined with busulfan (16mg/kg). Patients with SAA were conditioned with cyclophosphamide (200mg/kg). Patients with congenital metabolic defects were conditioned with busulfan (16mg/kg), usually with cyclophosphamide (200mg/kg).

During the years 1978-1983, patients treated at the Helsinki University Hospital received sTBI 10-12 Gy. Since 1984 patients have received fTBI 10-12 Gy together with cyclophosphamide (120mg/kg), cytarabine (36mg/$m^2$), melphalan (210mg/$m^2$) or busulfan (16mg/kg). Patients with SAA were conditioned with cyclophosphamide (200mg/kg) with or without fTBI 10 Gy or total lymph node irradiation (TNI) 6 Gy.

**Table 6.** Patient characteristics of female long-term survivors after pediatric HSCT.

| Characteristics | Study patients n=92<br>n (%) |
|---|---|
| **Diagnosis group** | |
| ALL | 33 (36) |
| AML | 24 (26) |
| SAA | 13 (14) |
| Others | 22 (24) |
| **CRT for leukemia** | |
| No | 45 |
| Yes | 12 |
| **Remission status of leukemia** | |
| CR 1 | 28 |
| CR 2-4 | 29 |
| **Conditioning** | |
| sTBI+Cy | 29 (32) |
| fTBI+Cy | 22 (24) |
| fTBI+Cy+ETO | 1 (1) |
| fTBI+cytarabine | 16 (17) |
| fTBI+melphalan | 3 (3) |
| Bu | 2 (2) |
| Bu+Cy | 8 (9) |
| Cy only | 10 (11) |
| Cy+TNI | 1 (1) |

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; Bu, busulfan; CR, complete remission; CRT, cranial radiotherapy; Cy, cyclophosphamide; ETO, etoposide; fTBI, fractioned total body irradiation; HSCT, hematopoietic stem cell transplantation; SAA, severe aplastic anemia; sTBI, single fraction total body irradiation; TBI, total body irradiation; TNI, total nodal irradiation

### 4.1.2 Long-Term Survivors of HR NBL and Healthy Controls (II-IV)

A national cohort of long-term HR NBL survivors was enrolled in this follow-up study (Figure 5). One patient with high-risk retinoblastoma treated in accordance with the HR NBL protocol was also included. All patients over 16 years of age at the time of the study were included. The patients were born in 1996 or earlier and were treated at any of the five University Hospitals in Finland between 1980 and 2000.

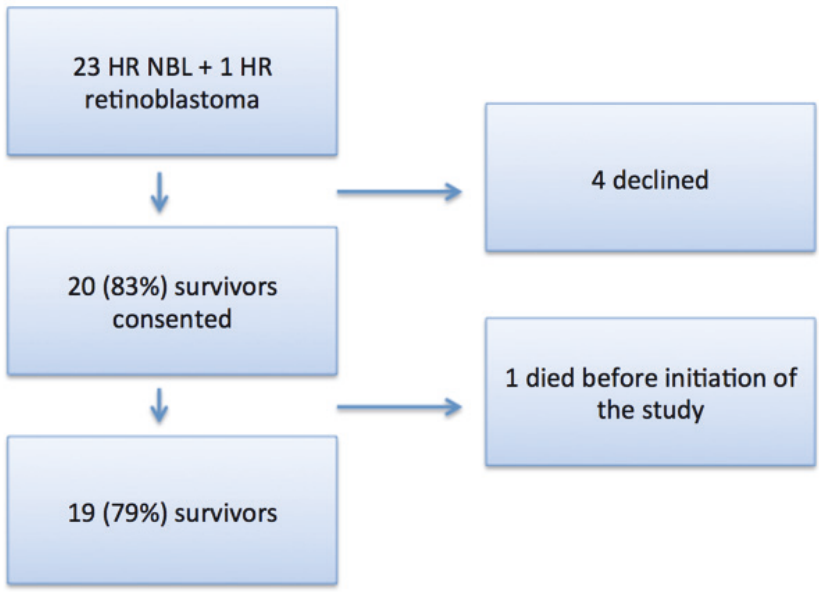


**Figure 5.** Study recruitment process in high-risk neuroblastoma (HR NBL) clinical study. HR, high-risk.

Twenty healthy individually age- and sex-matched controls were recruited as controls among medical students and teenage children of hospital employees.

#### *4.1.2.1 Treatment Characteristics*

HR NBL diagnosis was based on the INSS classification system (Brodeur et al. 1993, Brodeur et al. 1988) and was also in accordance with the newer INRG system (Cohn et al. 2009) (Table 1). Conventional chemotherapy included combinations of cyclophosphamide, dacarbazine, vincristine, etoposide, cisplatin, doxorubicin, ifosfamide, or carboplatin and was administered to make the primary operation easier, to eradicate metastatic disease, and to clean bone marrow to obtain an autologous stem cell graft (Saarinen-Pihkala et al. 2012, Saarinen et al. 1996). The

mean cumulative doxorubicin exposure was 88 mg/m$^2$ (range 0-210) and the mean cumulative cisplatin exposure 387 mg/m$^2$ (range 180-820).

All patients were consolidated with HDTx and autologous HSCT. Patients with bone metastases, MYCN amplification and poor treatment responses were stratified into fTBI-based HDTx 10-12 Gy. Single HDTx included PEM-TBI (cisplatin, etoposide and melphalan, and TBI of 10-12 Gy in five to six fractions), CEM-TBI (carboplatin, etoposide and melphalan, and TBI of 10 Gy in five fractions), L-PAM (melphalan) or ETO/Topo-Carbo-TT (etoposide or topotecan, carboplatin and thiotepa) (Saarinen-Pihkala et al. 2012). Two patients received tandem HDTx and both were consolidated with L-PAM in the first HDTx; in the second HDTx one received ETO-Carbo-TT without TBI and the other with TBI of 10 Gy (Saarinen-Pihkala et al. 2012).

After recovery, local RT was administered to the primary and bulky metastatic sites to all but one survivor and consisted of orbital area with 20 Gy, mandibular area with 32 Gy, occipital area with 20 Gy, left lung with 12.6 Gy, left kidney, lumbosacral area with 18 Gy, mediastinum and clavicular area with 24 Gy, abdomen with 7.2 Gy or retroperitoneal area with 12.6-45 Gy (Saarinen-Pihkala et al. 2012).

## 4.2 Methods

### 4.2.1 Analysis of Ovarian Function in the Retrospective Study (I)

The follow-up data were collected from medical records by one PhD student (V.A.) in Finland and one (W.M.) in Sweden. The follow-up time comprised the time from HSCT to the latest documented follow-up visit. Occurrence of spontaneous puberty based on breast development, age at menarche, the use of estrogen substitution for non-contraceptive reasons, and pregnancies were collected from the follow-up records if available. Information about pubertal or postpubertal serum follicle stimulating hormone (FSH) levels prior to initiating the estrogen substitution, or any postpubertal value for those without estrogen substitution, were collected.

Serum FSH values 1-12 IU/L were considered within the normal reference range and serum values >25 IU/L at menopausal level. Premature menopause was documented by increased levels of FSH and failure to accomplish pubertal maturation or menstruation among girls who showed some ovarian activity after HSCT. Increased levels of FSH documented ovarian failure with no ovarian activity after HSCT.

### 4.2.2 Clinical Examination (II-IV)

The recruitment letter was sent to all HR NBL survivors in Finland. The age-matched controls were contacted personally. After consenting, the study participants

were invited to fill in a questionnaire, and clinical examinations including interview were performed during the winter 2011-2012. Clinical data on the cancer treatment and medical history were collected from the medical records. Cumulative doses of anthracycline and cisplatin were calculated from medical records. If cumulative doses were not found, estimated doses from the treatment protocol were used.

Height and weight were measured and BMI [weight (kg)/height $(m)^2$] and body surface area (BSA) ($m^2$) from Mostellers' formula [square root of height (cm)*weight (kg)/3600] were calculated. Very high frequency ultrasound of the arteries, 3DE and TDI ultrasounds, FMD test, 24-hour ambulatory blood pressure measurement (ABPM), body composition measured with dual-energy X-ray absorptiometry, and physical performance tests were performed. A detailed history of health, smoking and physical activity limitations, and ongoing endocrine replacement therapies and anti-hypertensive medications were assessed by a questionnaire. Clinical examination day was planned and organized by a PhD student (V.A.).

#### *4.2.2.1 Biochemistry (II-IV)*

Laboratory analyses were determined from venous blood samples taken in the morning of the examination day following overnight fasting (minimum 11 hours), using standard techniques in the laboratory of Helsinki University Central Hospital. Glucose and lipid metabolism analyses were performed using standardized methods for blood glucose, glycosylated hemoglobin A1c (GHbA1c), total cholesterol, high- and low-density lipoproteins (HDL, LDL) and triglycerides. Cardiac biomarker N-terminal pro-B-type Natriuretic Peptide (pro-BNP), inflammation markers high-sensitivity C-reactive protein (hsCRP) and TNF-alpha, insulin-like growth factor 1 (IGF-1), renal function parameters cystatin C, uric acid and urine albumin/creatinine ratio were assessed using standardized methods in Studies II-IV.

#### *4.2.2.2 Ambulatory Blood Pressure (II-IV)*

Right arm systolic and diastolic BPs were measured during imaging ultrasound examinations in the supine position at rest with appropriate-sized cuffs with the GE Carescape Dinamap V100 device and the average of three BP measurements was used in vascular calculations.

ABPM was performed with an automated monitor (Schiller BR-102 Plus, Schiller AG, Baar, Switzerland) using oscillometric measurement as backup to ensure the accuracy of auscultator measurement results. ABPMs were obtained for at least 20 hours. The ABPM device was programmed to measure BP every 30 minutes between 7 am and 10 pm and hourly thereafter until 7 am the following morning.

The subjects kept a diary based on which daytime and nighttime periods were defined, as well as what kind of activity occurred, for example doing sports, watching a horror movie, walking or anything which can affect BP. Twenty-four-hour mean total, daytime and nighttime systolic and diastolic BPs, mean heart rate and the proportions of systolic and diastolic measurements exceeding limits were assessed in accordance with the European recommendations (Lurbe et al. 2009, O'Brien et al. 1991). BP load was calculated by dividing the count of measurements exceeding the 95$^{th}$ percentile cut-off values by the count of measurements during the study period. Nocturnal dipping was denoted as the difference between average daytime and nighttime BPs.

### 4.2.3 Arterial Morphology and Stiffness (II)

#### *4.2.3.1 Arterial Morphology*

B-mode ultrasound recordings of bilateral common carotid, brachial, radial and femoral arteries were obtained with very-high-resolution ultrasound (VHRU) using the Vevo 770 ultrasound system (Visualsonics, Toronto, Canada) with mechanical 25 MHz (RMV710B), 35MHz (RMV712) and 55 MHz (RMV708) transducers. The highest ultrasound frequency able to image the vessel was used with 55 MHz applied for the radial and 25/35 MHz for the brachial, femoral and common carotid arteries. All images were obtained and measurements made later offline by one senior pediatric cardiologist (S.T.) blinded to the study group and clinical characteristics.

The mean of three electronic caliper measurements per artery was calculated and the mean of right and left assessments was used in the final analyses as no differences between right and left were observed. IMT from the carotid artery, and IMT and intima-media-adventitia thicknesses (IMAT) from all other arteries were measured together with the lumen diameter (LD) at end-diastole (Figure 6). A double line appearance in the proximity of the lumen-intima interface was observed when imaging the radial artery with 55 MHz (assessed in all subjects) and in the common femoral artery using 35 MHz (not accessible in all subjects) and was interpreted as a thickened intima layer.

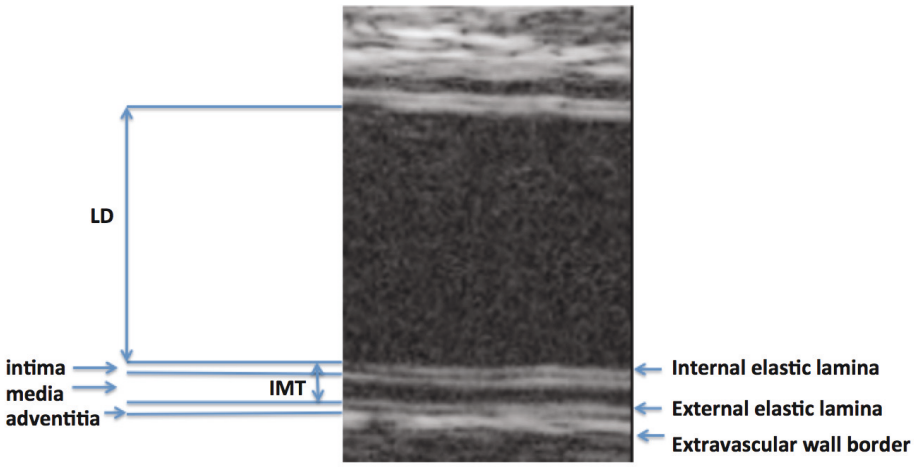


**Figure 6.** Image of the radial artery obtained with 55 MHz in a HR NBL patient. The arrows mark the leading edges of the dimensions measured. LD, lumen dimension; IMT, intima-media thickness.

All arterial sites were screened for the presence of arterial plaques (Figure 7). The presence of a local arterial plaque was defined as a structure that encroaches into the arterial lumen at least 0.5 mm or 50% of the surrounding IMT or demonstrates an IMT of 1.5 mm or more (Touboul et al. 2012).

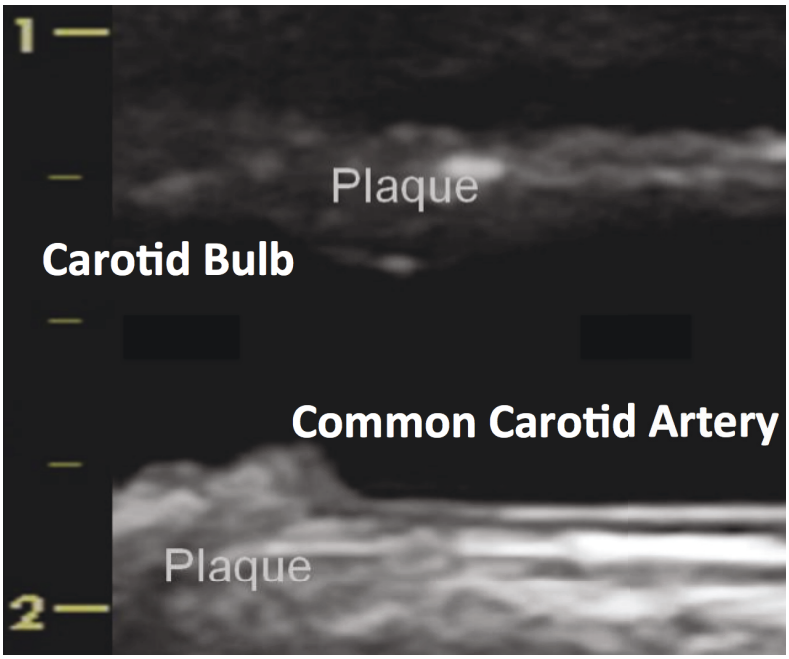


**Figure 7.** Image of the carotid artery obtained with 12 MHz in a total body irradiation treated high-risk neuroblastoma survivor, displaying plaques encroaching into vessel lumen from the near and far walls of the artery at the bifurcation of the common carotid artery and extending distally to the carotid bulb.

#### *4.2.3.2 Arterial Stiffness*

Common carotid peak-systolic and end-diastolic LDs were measured with 25 MHz with simultaneously obtained electrocardiogram (ECG) and the average of three ultrasound measurements was used in vascular stiffness calculations. Stiffness index was calculated from carotid measures as Ln (systolic BP/diastolic BP)/[(peak-systolic LD- end-diastolic LD)/end-diastolic LD)]. Carotid compliance was calculated from carotid measures as [(peak-systolic LD- end-diastolic LD)/end-diastolic LD]/(systolic BP- diastolic BP).

#### *4.2.3.3 Flow-Mediated-Dilatation*

Brachial artery FMD ultrasound studies were performed by an operator experienced in this technique (O.T.H.) with Vivid 7 ultrasound (Vivid 7; GE Vingmed AS) using the 12L vascular probe (GE Medical Systems). Patients were examined after 20 min of rest in a quiet, temperature-controlled and darkened room. Right brachial artery diameter was tracked using semi-automatic border detection software both at rest

and during reactive hyperemia induced by inflation of a pneumatic tourniquet placed around the forearm to a pressure of 300 mmHg for four minutes (Figure 8), as previously done with children (Tounian et al. 2001, Aggoun et al. 2004). Repeat ultrasound records of the brachial artery were obtained at a fixed distance from an anatomic marker at rest and continuously 30 to 210 seconds after cuff release.

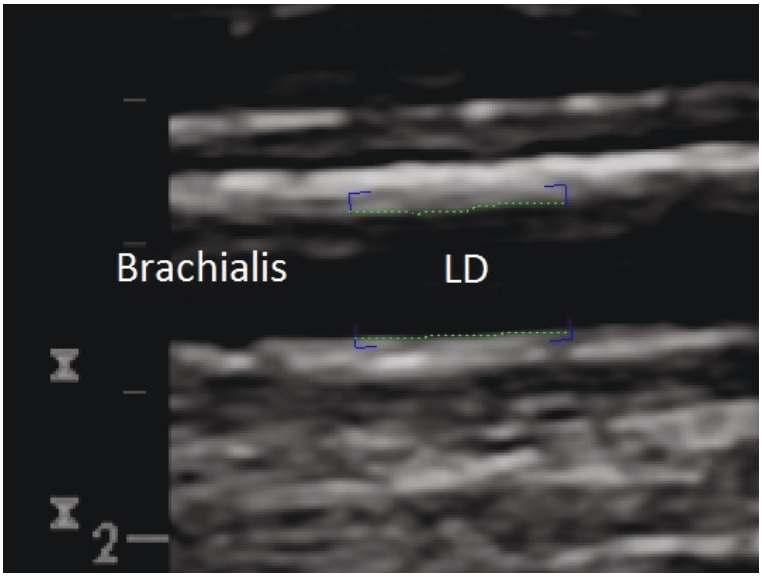


**Figure 8.** Flow-mediated dilatation analysis in a high-risk neuroblastoma survivor. Lumen diameter (LD) tracked using semi-automatic border detection software from brachialis artery.

All ultrasound scans were analyzed offline by the same observer (V.A.) blinded to the subjects' details. Datasets were analyzed with a semi-automatic image analysis software package (AMS II, version 1.1331, Chalmers University of Technology, Gothenburg, Sweden). The recognition of each diameter measurement was based on the direction and timing of the electrocardiography wave at end-diastole. The mean values of base and FMD diameters were calculated from three measurements.

$$FMD\% = \frac{BAD_{\text{hyperemia}} - BAD_{\text{at rest}}}{BAD_{\text{at rest}}} X100$$

Abbreviations: BAD= brachial artery diameter; FMD= flow-mediated-dilatation

### 4.2.4 Three-Dimensional Echocardiography and Tissue Doppler Imaging (III)

LVM and left ventricular (LV) function were examined in the morning prior to blood sampling. One senior pediatric cardiologist (O.T.H) performed cardiac imaging and 3DE analyses from standard views in accordance with the American Society of Echocardiography and the European Association of Cardiovascular Imaging Expert Consensus (Plana et al. 2014). TDI analyses of systolic and diastolic functions were performed retrospectively by one observer (V.A.).

#### *4.2.4.1 Assessment of 3DE Left Ventricular Volumes and Mass*

All studies were performed using the Philips IE33 ultrasound system (Philips, Andover, MA, USA) with an X5-1 matrix-array transducer. 3DE datasets were analyzed using commercial software (Qlab v8, Philips Medical Systems, Andover, MA, USA). The 3DE LVM was calculated at both end-diastole and end-systole. 3DE measurements were made by subtracting the endocardial volume from the epicardial volume and multiplying by 1.05 to correct for the density of cardiac muscle (Figure 9). As the agreement between systolic and diastolic LVM was good, the repeatable systolic LVM was selected as a final outcome variable (Ojala et al. 2015). LVM was normalized to height (in meters) to the power of 2.7 (LVM index, LVMI) and expressed as $g/m^{2.7}$ (de Simone et al. 1992). Survivors' LVMI was compared to the study controls, and values exceeding 2SDs were consider as LV hypertrophy.

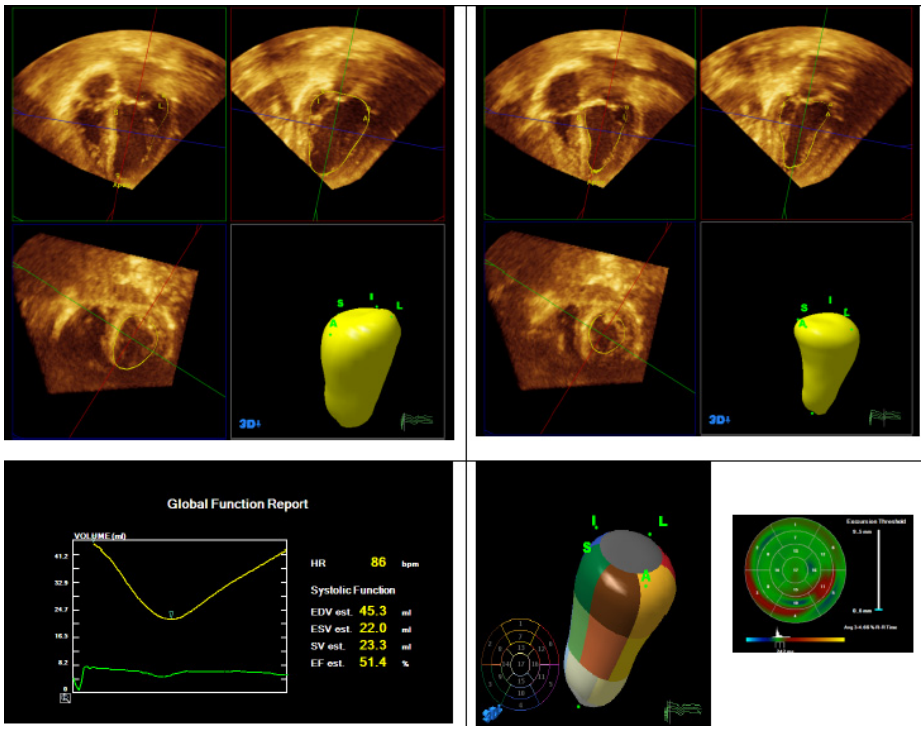


**Figure 9.** The 3DE left ventricular mass measurements performed by subtracting the endocardial volume from the epicardial volume at both end-diastole and end-systole. 3DE end-systolic (ESV) and end-diastolic volumes (EDV), systolic function with ejection fraction (EF), and 16-segment synchrony dataset analyses in a high-risk neuroblastoma survivor.

#### *4.2.4.2 Assessment of Cardiac Function and Dyssynchrony*

Ejection fraction (EF), stroke volume and 16-segment dyssynchrony index (16 SDI) were calculated according to standard formulas from the 3DE endocardial datasets of LV volumes at end-diastole and end-systole (Figure 9). TDI was performed from the apical four-chamber view with Pulse Wave Doppler (PW). The peak systolic (TDI S') and early diastolic (TDI E') tissue velocities were measured as a mean of three cardiac cycles directly on the spectral display (Figure 10). The recognition of each peak velocity was based on the direction and timing of the wave on ECG. Systolic functional evaluation was based on 3DE EF, 3DE stroke volume (SV) and TDI S' (Plana et al. 2014). The early LV inflow diastolic velocity (PW E) was measured with PW (Figure 10). The calculation of E/E' was used as an estimation of cardiac diastolic function (Plana et al. 2014).

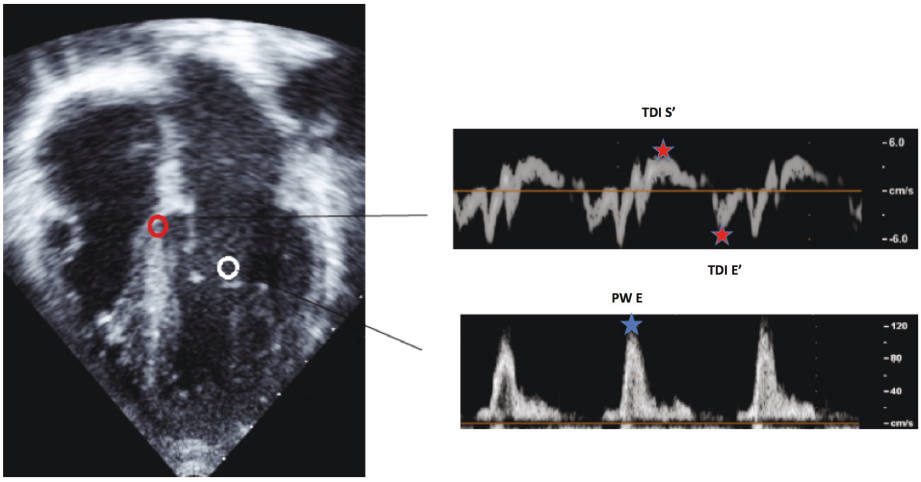


**Figure 10.** Example of analysis of peak velocities measured by Tissue Doppler Imaging (TDI) in a high-risk neuroblastoma survivor. The peak systolic (TDI S') and diastolic (TDI E') velocities are indicated. The early left ventricular inflow diastolic velocity (PW E) was measured with Pulse Wave Doppler (PW).

### 4.2.5 Tests for Physical Performance (III-IV)

Muscular endurance, strength and speed were investigated by different tests: leg-lift, repeated squatting, sit-up, back extension and shuttle-run. One physiotherapist (K.M.) performed the tests. Age- and sex-spesific control values from our previous study were used (Hovi et al. 2010). A designated personal muscle sum score was determined by calculating the mean SDS value for the five muscle tests. The SDS values of patients were used in the analyses. In addition, the study subjects were interviewed and a detailed history of activity levels was recorded in accordance with the National Health and Nutrition Examination Study.

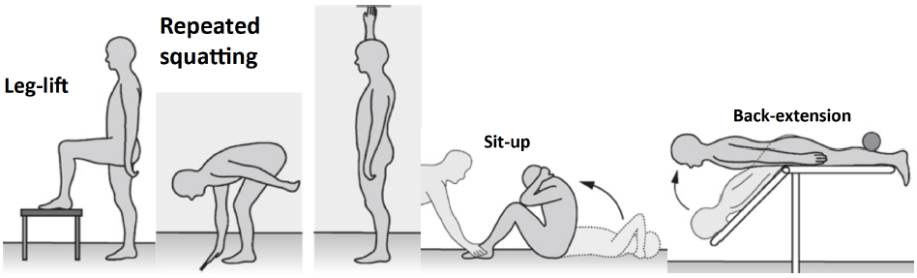


**Figure 11.** Physical performance tests.

The tests used, described more precisely in our previous report (Hovi et al. 2010), are described briefly below (Figure 11):

1. Leg-Lift Test: This test measures the speed endurance of lower extremities, especially the hip flexors. The subject stands in front of a bench and is asked to lift both feet alternately onto the bench as quickly as possible for 30 seconds.

2. Repeated Squatting Test: This test measures the dynamic endurance of lower extremities. The subject is asked to squat and stand up as many times as possible for 30 seconds.

3. Sit-Up Test: This test measures the dynamic muscular endurance of the trunk flexors. The subject, lying supine with the knees flexed and ankles supported, is asked to rise to a sitting position as many times as possible for 30 seconds.

4. Back-Extension Test: This test measures the dynamic endurance of the trunk extensors. The subject lies prone on a bench with the ankles supported and the trunk from the sacral spine upward unsupported when in the horizontal position and is asked to lower and lift the trunk as many times as possible for 30 seconds.

5. Shuttle-Run Test: The test measures acceleration, maximal speed, and speed differentiation. The subject makes a 10 x 5 m shuttle run as fast as possible.

### 4.2.6 Frailty Phenotype and Telomere Length Analysis (IV)

Participants were defined as pre-frail if they fulfilled two and frail if they fulfilled three or more of the following criteria as originally defined by Fried et al. (Fried et al. 2001) (Table 7).

**Table 7.** Frailty phenotype.

| Characteristics of Frailty | Outcome |
|---|---|
| Low lean muscle mass | <-1.5SD lean mass (kg)/ height ($m^2$) |
| Low energy expenditure | Men <383 kcal/wk, Women <270 kcal/wk |
| Slowness | Shuttle run test <-1.5SD |
| Weakness | Sit up test <-1.5SD |
| Exhaustion | Not assessed in this study |

SD, standard deviations

Body composition, as total body and trunk fat mass (kg), and total body lean mass (kg) and total body and trunk fat percentage, were measured with dual-energy X-ray absorptiometry (DXA; Hologic® Discovery A, Bedford, MA, software version

12.3:3). Low lean muscle mass was classified as less than -1.5SD for race-, age- and sex-specific values from the National Health and Nutrition Examination Study (Kelly et al. 2009). In accordance with the National Health and Nutrition Examination Study, detailed information of activities from physical performance interview was converted to kilocalories per week (kcal/wk) (*Centers for Disease Control and Prevention: National Health and Nutrition Examination Survey Physical Activity and Physical Fitness: PAQ 2007*). Shuttle-run and sit-up test results were evaluated as SD values comparing to the age- and sex-specific values from our previous study (Hovi et al. 2010).

#### *4.2.6.1 Telomere Length Analysis*

Frozen blood samples, which were collected in the morning of the examination day, were used for telomere length analysis in Karolinska Institute, Sweden, by two experienced operators (H.M. and H.T.). Genomic DNA was isolated using phenol/chloroform and telomere length was assessed by real-time PCR as described (Cawthon 2002).

### 4.2.7 Statistical Methods

Data are presented as mean±SD (range). Mann-Whitney U test (for continuous variables), Fisher's exact test and $\chi^2$ test (for categorical variables) were conducted to test differences between two groups. Association between variables was analyzed with Spearman's correlation.

In Study I, odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using bi- and multivariate logistic regression analyses. Categorical predictors used in logistic bi- and multivariate regression analysis were the dummy variables (0/1) prepubertal at HSCT, leukemia diagnosis, SAA diagnosis, TBI and sTBI or fTBI. The continuous variables were age at HSCT, follow-up time, serum level of FSH, and for the leukemia group only, remission status at HSCT and CRT.

In Study II, simple and multiple linear regression analyses were used to study associations with vascular parameters and cardiovascular risk factors, treatments and BSA between survivors and controls or TBI- and non-TBI survivors. Age and gender were not adjusted for, due to matching survivor and control groups.

In Study III, LVMI and LVMI/end-systolic volume (ESV) were used in simple and multiple linear regression analyses as dependent variables and survivor/control, triglycerides and mean 24-hour systolic BP (24SYS) as independent variables.

In Study IV, all data are presented as median/mean±SD (range). The phenotypes of frailty, pre-frailty and non-frailty were the primary outcomes. The parameters that

correlated significantly with telomere length were further added to simple and multiple linear regression analysis; as independent variables we used GHbA1c, hsCRP, and IGF-1.

A p-value of <0.05 was considered statistically significant. Analyses were performed using the IBM SPSS statistical software package (version 22). All statistical analyses in Studies II-IV were made by V.A. and in Study I by a statistician (J.B.) at Karolinska Institute.

### 4.2.8 Ethical Considerations

The Research Ethics Committee of Helsinki University Hospital (Study I-IV) and Karolinska University Hospital Huddinge (Study I) approved the studies. All participating patients and controls, or a parent in case of minors, signed a written informed consent form, in accordance with the Declaration of Helsinki (Studies II-IV).

# 5 Results

## 5.1 Ovarian Failure and Premature Menopause (I)

Treatment characteristics of long-term female survivors after allogeneic HSCT in the Helsinki and Huddinge study are presented in Table 6 and Table 8. Patients were divided into three groups according to their pubertal status at HSCT. In the prepubertal group (n=70) pubertal status was Tanner 1, in midpubertal group (n=12) Tanner 2-3, and in postpubertal group (n=8) Tanner 4-5 at the time of HSCT. In Figure 12, the natural history of ovarian function during longitudinal follow-up after HSCT is described.

**Table 8.** Characteristics of female long-term survivors after allogeneic HSCT.

| Characteristics (years) | Study patients n=92<br>Mean (range) |
|---|---|
| Age at HSCT | 9 (1-19) |
| Age at last visit | 22 (9-41) |
| Follow-up time | 13 (6-27) |

HSCT, hematopoietic stem cell transplantation

Of the 70 girls who were prepubertal at HSCT, 30 showed no ovarian activity after HSCT. Forty prepubertal girls initiated spontaneous puberty, but 14 of them entered into premature menopause requiring estrogen substitution at a mean (±SD) age of 17±3.2 years (range 13-25) at 11±4.4 years (6-21) after HSCT (Figure 12). In addition, three midpubertal girls temporarily recovered their ovarian function after HSCT (Figure 12).

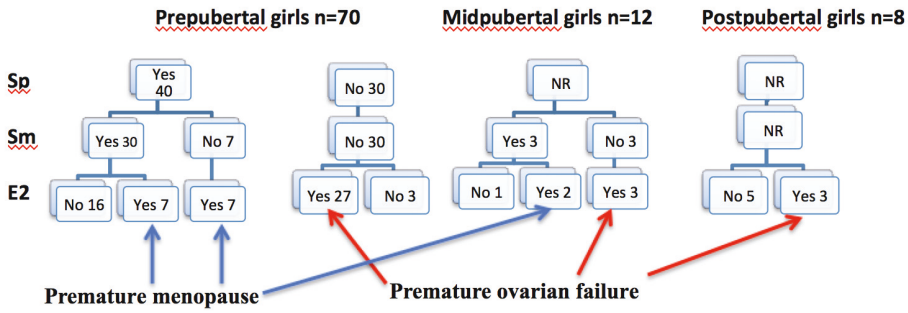


**Figure 12.** Spontaneous onset of puberty (Sp) and menarche (Sm) and need of estrogen substitution (E2) at the latest follow-up visit defined by the pubertal status at the hematopoietic stem cell transplantation. NR, not relevant.

Patients were classified into three groups according to the treatment they received before HSCT. The first group, leukemia group, consisted of AML (n=24) and ALL (n=33) patients. The second group consisted of patients with SAA (n=13) and the third group, others, was a heterogeneous group of patients with non-malignant disorders or malignant diseases without cytotoxic therapy given before HSCT conditioning. The incidence of spontaneous puberty and menarche and the use of estrogen substitution at the latest follow-up visit are presented in Table 9.

**Table 9.** Incidence of spontaneous puberty and menarche and use of estrogen substitution at the latest follow-up visit with total numbers of patients included in the evaluations in each group.

| | **Spontaneous puberty 57%(n=40)** | | **Spontaneous menarche 45%(n=33)** | | **No estrogen substitution 34%(n=28)** | |
|---|---|---|---|---|---|---|
| | **% (Total n)** | **P** | **% (Total n)** | **P** | **% (Total n)** | **P** |
| **Primary diagnosis (n=92)** | | | | | | |
| Leukemia (n=57) | 49(45) | | 33(45) | <0.01 | 30(54) | <0.05 |
| SAA (n=13) | 75(8) | | 80(10) | | 67(11) | |
| **Cranial radiotherapy (n=57)** | | | | | | |
| CRT- (n=45) | 57(35) | | 43(35) | <0.01 | 36(42) | |
| CRT+ (n=12) | 20(10) | | 0(10) | | 8(12) | |
| **Remission status of leukemia patients (n=57)** | | | | | | |
| CR 1 (n=28) | 61(23) | | 52(25) | <0.005 | 37(27) | |
| CR 2-4 (n=29) | 36(22) | | 10(20) | | 22(27) | |
| **Conditioning regimens** | | | | | | |
| TBI (n=71) | 53(53) | <0.05* | 35(54) | <0.001* | 29(68) | <0.001* |
| Busulfan (n=10) | 50(10) | <0.05* | 50(10) | <0.05* | 33(8) | <0.01* |
| Cy only (n=10) | 100(7) | | 100(8) | | 100(6) | |
| sTBI (n=29) | 52(21) | | 18(22) | <0.05 | 15(27) | |
| fTBI (n=42) | 53(32) | | 47(32) | | 39(41) | |

CR, complete remission; CRT, cranial radiotherapy; Cy, cyclophosphamide; fTBI, fractionated total body irradiation; HSCT, hematopoietic stem cell transplantation; SAA, severe aplastic anemia; sTBI, single fraction total body irradiation; TBI, total body irradiation

* compared with Cy.

Patients with SAA showed higher incidence of spontaneous menarche ($p<0.01$) and they did not need estrogen substitution at their latest follow-up visit ($p<0.05$) when compared with the patients with leukemia. The girls who received only cyclophosphamide as conditioning experienced spontaneous puberty and menarche

and there was no need for estrogen replacement therapy. Compared to those with sTBI, the girls who received fTBI experienced more often ($p<0.05$) spontaneous menarche. Leukemia survivors transplanted in the second or later remission and those who received CRT had lower incidence of spontaneous menarche than those transplanted in the first remission ($p<0.005$) and those with no CRT ($p<0.01$).

Serum FSH levels were measured at the mean (±SD) age of 15±3.7 years (range 8-30) and 7±4.9 years (0.1-21) after HSCT (Table 10). Leukemia patients had higher FSH levels than patients with SAA ($p<0.05$). Survivors with TBI or busulfan-based conditioning had higher levels of FSH compared to those with conditioning with only cyclophosphamide.

**Table 10.** Serum FSH levels, measured 7 years after transplantation at a mean age of 15 years, from the pubertal or postpubertal period.

| | | FSH levels (IU/l) | | |
|---|---|---|---|---|
| | **n=76** | **Mean±SD** | **Range** | **P** |
| **Primary diagnosis** | | | | |
| Leukemia | 48 | 43±36.5 | 1-160 | <0.05* |
| SAA | 8 | 15±12.4 | 1-33 | |
| Others | 20 | 36±42.9 | 2-143 | |
| **Conditioning** | | | | |
| TBI | 59 | 39±35.5 | 1-160 | <0.05** |
| Busulfan | 10 | 57±48.3 | 3-143 | <0.01** |
| Cyclophosphamide | 7 | 7±8.0 | 1-25 | |

HSCT, hematopoietic stem cell transplantation; SAA, severe aplastic anemia; TBI, total body irradiation

* compared with SAA and ** compared with cyclophosphamide.

Of the female survivors, ten (11%) women had given birth to 12 children and had had a total of 14 pregnancies at the mean (SD) age of 22±6.3 years (range 9-41). Four of the women with offspring had a diagnosis of leukemia and had received fTBI. Six of the mothers had a non-malignant diagnosis. One had received sTBI with ovarian shielding, three had received busulfan-based conditioning, and two cyclophosphamide-based conditioning.

In bivariate logistic analysis, younger age at HSCT predicted spontaneous puberty (OR 1.2, 1.0-1.4, p=0.015). The SAA diagnosis (OR 6.1, 1.3-31.0, p=0.030), no TBI-based conditioning (OR 5.2, 1.6-16.5, p=0.006), no leukemia diagnosis (OR 3.6, 1.3-9.7, p=0.011) and lower FSH serum level (OR 1.03, 1.01-1.06, p=0.002) predicted spontaneous menarche. The predictors for the need of estrogen substitution at the latest follow-up visit were sTBI (OR 4.3, 1.3-14.1, p=0.016) and no SAA diagnosis (OR 0.2, 0.1-0.9, p=0.033).

## 5.2 Cardiovascular Risk Factors and Blood Pressure (II-IV)

Clinical characteristics of the survivors of HR NBL and the age- and sex-matched healthy controls are presented in Table 11 (Study II- IV). Nineteen survivors and their sex- and age-matched healthy controls constituted the study population. Ten of the 19 survivors received TBI-based HDTx 10-12 Gy.

**Table 11.** Clinical characteristics among the survivors of HR NBL and the controls.

| | Controls (n=20) Mean±SD | Survivors (n=19) Mean±SD | P | Survivors Non-TBI (n=9) Mean±SD | Survivors TBI (n=10) Mean±SD | P |
|---|---|---|---|---|---|---|
| Age at study (years) | 22.4±4.5 | 22.7±4.9 | 0.966 | 23.1±6.0 | 22.3±3.8 | 0.683 |
| Sex (M/F) | 9/11 | 8/11 | 1.000 | 5/4 | 3/7 | 0.370 |
| Follow-up time (years) | - | 20.1±4.8 | - | 21.1±6.1 | 19.2±3.4 | 0.288 |
| Age at diagnosis (years) | - | 1.9±1.1 | - | 1.2±0.6 | 2.6±0.9 | 0.006 |
| Age at first HSCT (years) | - | 2.5±1.0 | - | 2.0±0.6 | 3.1±1.0 | 0.018 |
| Age at second HSCT (years) | - | 2.3±1.0 | - | 1.5 (n=1) | 3.0 (n=1) | 0.317 |
| DOXO ($mg/m^2$) | - | 88±68 | - | 45±76 | 126±29 | 0.010 |
| Cisplatin ($mg/m^2$) | - | 387±203 | - | 500±240 | 285±83 | 0.059 |

DOXO, cumulative doxorubicin dose; HSCT, hematopoietic stem cell transplantation; TBI, total body irradiation

Cardiovascular risk profiles and biochemical markers of frailty of the survivors and controls are described in (Table 12). The survivors' height, weight and BSA were significantly smaller compared to the controls. Smoking was more common in the survivor group. Total cholesterol and triglycerides were significantly higher in the survivor group.

**Table 12.** Cardiovascular risk factors and markers of inflammation among the survivors of HR NBL and the controls (Study II-IV).

| | Controls (n=20) | Survivors (n=19) | |
|---|---|---|---|
| | Mean±SD | Mean±SD | P |
| Height (cm) | 174±10 | 157±11 | <0.001 |
| Weight (kg) | 71.7±13.9 | 54.1±17.6 | 0.002 |
| BSA ($m^2$) | 1.9±0.2 | 1.5±0.3 | <0.001 |
| BMI ($kg/m^2$) | 23.6±4.1 | 21.4±4.5 | 0.092 |
| Smoking (yes/no) | 0/20 | 4/15 | 0.047 |
| fP-Cholesterol (mmol/L) | 4.1±0.7 | 4.5±0.8 | 0.044 |
| fP-HDL (mmol/L) | 1.5±0.3 | 1.6±0.4 | 0.844 |
| fP-Triglycerides (mmol/L) | 0.9±0.4 | 1.3±0.6 | 0.017 |
| fP-Glucose (mmol/L) | 5.1±0.3 | 5.3±0.5 | 0.129 |
| fP-GHbA1c (%) | 5.1±0.2 | 5.5±1.1 | 0.100 |
| Pro-BNP (ng/L) | 41± 30 | 128± 206 | 0.004 |
| S-hsCRP (mg/L) | 2.6±7.8 | 3.5±3.2 | 0.002 |
| S-IGF-1 (nmol/L) | 40±19 | 28±12 | 0.032 |

BMI, body mass index; BSA, body surface area; GHbA1c, glycosylated hemoglobin A1c; IGF-1, insulin-like growth factor 1; Pro-BNP, N-terminal pro-brain natriuretic peptide; hsCRP, high-sensitivity C-reactive protein; TBI, Total body irradiation; TNF, tumor necrosis factor

No difference in glucose metabolism was observed between the survivors and the controls. We did not measure waist circumference so we were not able to evaluate all the criteria of metabolic syndrome in the survivor group, but the tendencies of other factors are presented in Table 13. One survivor was using treatment for diabetes and metabolic syndrome.

**Table 13.** Risk factors for metabolic syndrome in the survivors of HR NBL.

| **Metabolic syndrome criteria*** | **Survivors n=19 n (%)** |
|---|---|
| SBP ≥ 130 or/ and DBP ≥ 85 or BP medication | 9 (47) |
| Triglycerides ≥ 1.7 mmol/L | 2 (11) |
| HDL < 1.0 mmol/L men, <1.3 mmol/L women | 3 (16) |
| Fasting glucose ≥ 5.6 mmol/L or medication | 2 (11) |
| Waist circumference (men/ women, > 100/90 cm) | Not assessed |
| Two ≥ criteria | 5 (26) |
| Three ≥ criteria | 1 (5) |

The presence of three or more risk factors is required to fulfill the metabolic syndrome criteria in all five categories

BP, blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; SBP, systolic blood pressure

* According to harmonization study and joint statement (Alberti et al. 2009).

The mean 24h systolic (24SYS%) (49% vs. 19%) and diastolic (24DIA%) (34% vs. 17%) BP loads were significantly higher ($p<0.001$, $p=0.040$) in the survivors compared to the controls. Daytime systolic and diastolic loads and nighttime systolic load were also significantly higher among the survivors (Table 14).

**Table 14.** Twenty-four-hour ambulatory blood pressure profiles in the survivors of HR NBL and the controls.

| Blood pressure | Controls (n=20) | Survivors (n=19) | |
|---|---|---|---|
| **Mean 24h BP** | **Mean±SD** | **Mean±SD** | **P** |
| Systolic BP (mmHg) | 122±10 | 129±14 | 0.083 |
| Diastolic BP (mmHg) | 70±6 | 75±12 | 0.237 |
| Systolic load (%) | 19±17 | 49±29 | <0.001 |
| Diastolic load (%) | 17±15 | 34±30 | 0.040 |
| Patients with systolic load ≥25% (n) | 3 | 13 | 0.001 |
| Patients with diastolic load ≥25% (n) | 4 | 8 | 0.176 |
| **Mean daytime BP** | | | |
| Systolic BP (mmHg) | 126±10 | 133±15 | 0.164 |
| Diastolic BP (mmHg) | 74±6 | 79±12 | 0.118 |
| Systolic load (%) | 19±17 | 48±30 | 0.001 |
| Diastolic load (%) | 17±15 | 35±30 | 0.034 |
| Patients with systolic load ≥25% (n) | 3 | 14 | <0.001 |
| Patients with diastolic load ≥25% (n) | 5 | 10 | 0.105 |
| **Mean night-time BP** | | | |
| Systolic BP (mmHg) | 111±10 | 122±16 | 0.019 |
| Diastolic BP (mmHg) | 61±5 | 66±12 | 0.278 |
| Systolic load (%) | 19±19 | 57±37 | 0.001 |
| Diastolic load (%) | 18±20 | 34±38 | 0.267 |
| Patients with systolic load ≥25% (n) | 7 | 14 | 0.010 |
| Patients with diastolic load ≥25% (n) | 5 | 8 | 0.320 |

BP, blood pressure

## 5.3 Arterial Intima Thickening and Plaque Formation (II)

Arterial measurements are presented in Table 15. The survivors had smaller brachial and femoral lumen diameters when compared to the control group. Carotid IMT was increased among the survivors. Local plaques were found in three survivors treated with TBI in the carotid bulb or in the common femoral artery. A double line appearance at the lumen-intima interface in the radial artery was displayed in five survivors and at the common femoral artery in two. The TBI-treated survivors displayed smaller arterial LDs in brachial (p=0.004), radial (p=0.004) and femoral (p=0.018) arteries and increased carotid artery IMT (p=0.004) when compared to the non-TBI survivors.

**Table 15.** Arterial measurement in the HR NBL survivors and the controls.

| | Control n=20 Mean±SD | Survivor n=19 Mean±SD | P |
|---|---|---|---|
| **Arterial lumen diameters** | | | |
| BALDD (mm) | 7.27±0.96 | 5.82±0.72 | <0.001 |
| FALDD (mm) | 3.21±0.52 | 2.80±0.49 | 0.017 |
| **Arterial morphology** | | | |
| CCAIMT (mm) | 0.41±0.04 | 0.47±0.09 | 0.035 |
| PLAQUE (yes/no) | 0/20 | 3/16 | 0.106 |
| RAIDL (yes/no) | 0/20 | 5/14 | 0.020 |
| FAIDL (yes/no) | 0/20 | 2/17 | 0.231 |
| **Carotid artery stiffness** | | | |
| CACOMP (%/10mmHg) | 3.35±0.68 | 2.65±0.62 | 0.002 |
| CASTIF | 3.50±0.73 | 4.13±0.69 | 0.013 |

CACOMP, Common carotid artery compliance; CASTIF, Common carotid artery stiffness index; CCAIMT, Common carotid artery intima-media thickness; BALDD, Brachial artery lumen diameter in end-diastole; FAIDL, Femoral artery intima double layer; FALDD, Femoral artery lumen diameter in end-diastole; PLAQUE, Arterial plaque; RAIDL, Radial artery intima double layer

Carotid artery compliance was decreased and carotid artery stiffness increased among the survivors when compared to the controls.

To evaluate associations between vascular parameters and cardiovascular risk factors and treatments, and to adjust differences in BSA and cardiovascular risk factors between the survivors and the controls and the TBI and non-TBI groups, simple and multiple linear regressions were performed. TBI treatment and low BSA were identified as independent predictors of small arterial lumen diameter and increased carotid IMT among the survivors. Triglycerides and 24SYS were independently associated with carotid IMT. The cumulative doxorubicin dose was not significantly associated with vascular parameters in multiple linear regression analyses. Triglycerides were associated with carotid compliance in simple linear regression analysis.

In the FMD analyses, we found no significant differences between the survivors and the controls (10.2% vs. 9.6%) or between TBI and non-TBI survivors (13% vs. 8%).

## 5.4 Left Ventricular Hypertrophy and Cardiac Dysfunction (III)

### *5.4.1 Left Ventricular Mass*

3DE LVMI was significantly higher in the survivor group when compared to the controls (Table 16). When it was adjusted to the ESV the difference was more evident. Three (16%) HR NBL survivors presented LV hypertrophy >2SD when LVMI was compared to the control group. End-diastolic volume (EDV) was significantly decreased in the subgroup of survivors who had received TBI when compared to those without TBI (41.1mL vs. 48.5mL, p=0.004).

**Table 16.** 3DE LVM and volume analyses in HR NBL survivors and controls.

| | **Controls (n=20) Mean±SD** | **Survivors (n=19) Mean±SD** | **P** | **Survivors Non-TBI (n=9) Mean±SD** | **Survivors TBI (n=10) Mean±SD** | **P** |
|---|---|---|---|---|---|---|
| 3D ESV/$m^2$ (mL) | 19.4±6.3 | 18.3±3.9 | 0.431 | 19.0±4.4 | 17.7±3.6 | 0.568 |
| 3D EDV/$m^2$ (mL) | 50.6±15.2 | 44.6±6.6 | 0.092 | 48.5±3.9 | 41.1±6.8 | 0.004 |
| 3D LVMI ($g/m^{2.7}$) | 28.1±4.7 | 33.9±9.7 | 0.038 | 34.3±10.5 | 33.6±9.4 | 0.935 |
| 3D LVMI/ESV ($g/m^{2.7}mL$) | 0.9±0.5 | 1.3±0.7 | 0.006 | 1.2±0.5 | 1.5±0.8 | 0.369 |

EDV, end-diastolic volume; ESV, end-systolic volume; LVMI, left ventricular mass index; TBI, total body irradiation

LVMI correlated positively with 24SYS (r=0.465, p=0.003) and with serum triglycerides (r=0.444, p=0.005) when adjusted to ESV. 24SYS (p=0.024) and being a HR NBL survivor (p=0.020) were shown to be predictors of increased LVMI in simple linear regression analyses, but no independent predictive effect was found when they were entered in multiple linear regression analysis.

### *5.4.2 Left Ventricular Function*

Abnormal 3DE LV EF <50% (Armstrong et al. 2015) as a sign of systolic dysfunction was observed in 10.5% of the patients and abnormal diastolic function in 15.8% (TDI E/E'>15) (Lipshultz et al. 2013). All of the controls had normal systolic and diastolic functional measurements. Cardiac function results are described in Table 17. Systolic function was significantly decreased (TDI S') among the survivors compared to the controls (7.5 cm/s vs. 9.3 cm/s, p=0.006) and the TBI-

treated survivors had the lowest function when compared to the non-TBI group (6.8 cm/s vs. 8.2 cm/s, p=0.041). The same result was found in the diastolic function (TDI E/E', Table 17). No difference was detected in 3D synchronization indexes between the survivors and the controls (Table 17). LVMI correlated with both decreased systolic (TDI S', r=-0.335, p=0.037) and diastolic function (TDI E/E', r=0.440, p=0.005).

**Table 17.** Left ventricular function in HR NBL survivors and controls.

| | Controls (n=20) Mean±SD | Survivors (n=19) Mean±SD | P | Survivors Non-TBI (n=9) Mean±SD | Survivors TBI (n=10) Mean±SD | P |
|---|---|---|---|---|---|---|
| **Systolic function** | | | | | | |
| 3DE EF (%) | 61.8±6.9 | 58.7±7.5 | 0.191 | 60.8±7.7 | 56.7±7.1 | 0.253 |
| 3DE SV/$m^2$ ($mL/m^2$) | 31.2±10.1 | 26.3±5.6 | 0.092 | 29.5±4.4 | 23.4±5.1 | 0.018 |
| TDI S' (cm/s) | 9.3±2.0 | 7.5±1.4 | 0.006 | 8.2±1.2 | 6.8±1.4 | 0.041 |
| **Diastolic function** | | | | | | |
| TDI E' (cm/s) | 13.2±2.1 | 10.9±2.0 | 0.003 | 11.9±1.4 | 10.0±2.1 | 0.041 |
| TDI E/E' | 8.0±1.8 | 11.2±3.8 | 0.003 | 9.0±1.8 | 13.2±4.1 | 0.022 |
| **Synchronization** | | | | | | |
| 3DE 16DSI | 1.9±1.1 | 2.5±1.7 | 0.346 | 2.7±2.3 | 2.2±0.9 | 0.870 |

16SDI, 16 segments Systolic Dyssynchrony Index; 3DE, 3 dimensional echocardiography; E, Mitral peak velocity in early diastole; E', Early diastolic peak mitral annular velocity; EF, Ejection fraction; S', Peak myocardial sustained systolic velocity; SV, Stroke volume; TBI, Total body irradiation; TDI, Tissue Doppler Imaging

Cardiac analysis of one HR NBL patient is presented in Figure 9 and Figure 10. The analysis showed decreased systolic (3D EF 51%, TDI S' 4.6 cm/s) and diastolic function (TDI E/E' 19.6). No dyssynchrony (16 SDI 2%) was detected. The heart was hypertrophic (LVMI 36 $g/m^{2.7}$, 1.63SD) due to the hypertension. The patient's physical performance was poor and less than half a year after the study the patient died. Autopsy revealed pancreatitis as cause of death. The patient also had pathological coronary artery disease.

### *5.4.3 Physical Performance*

The survivors of HR NBL showed poor physical performance when compared to the controls. Survivors performed significantly poorer in all but the leg-lift test (p=0.058) (Figure 13).

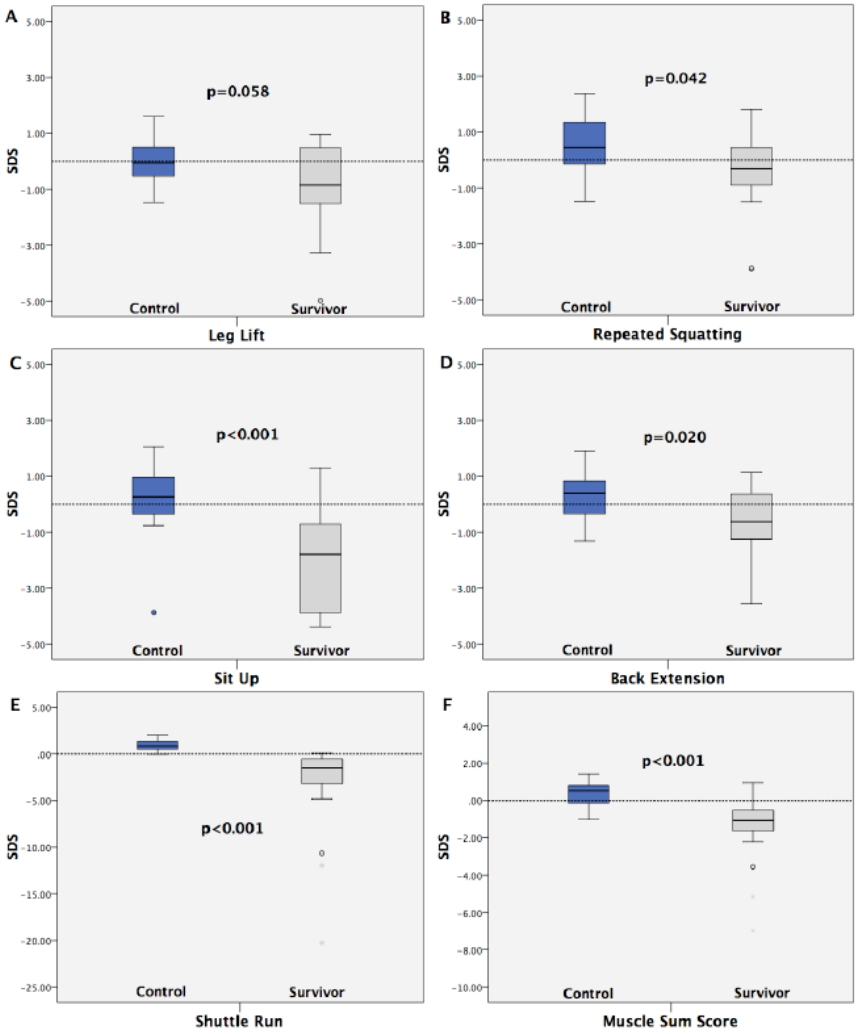


**Figure 13.** Physical performance tests: (A) leg lift, (B) repeated squatting, (C) sit-up, (D) back extension, (E) shuttle run and (F) muscle sum score compared between controls and HR NBL survivors.

## 5.5 Accelerated Aging and Frailty Phenotype (IV)

Clinical characteristics of the survivors of HR NBL and the controls are described in Table 11 and body composition, life-style habits and physical condition in Table 18. The survivors had significantly lower total body lean mass, higher fat mass percentage and higher trunk fat percentage when compared to the controls. No difference in BMI was observed but BSA was smaller in the survivor group. The survivors reported lower frequency, intensity and duration of physical activities when converted to kilocalories expended per week. They were slower in the shuttle run test and the number of sit-ups was lower when compared to the controls.

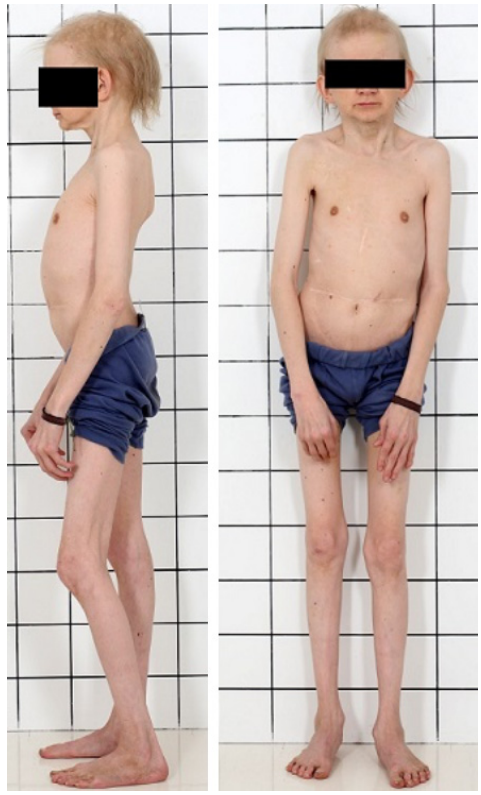

**Figure 14.** Twenty-seven-year-old high-risk neuroblastoma survivor who met four criteria of frailty phenotype.

**Table 18.** Characteristics of body composition, life-style habits, physical condition and telomere length in the HR NBL survivors and the controls.

| Characteristic | Survivors | | Controls | | |
|---|---|---|---|---|---|
| | **Mean±SD** | **Range** | **Mean±SD** | **Range** | **P** |
| **Anthropometric measurement** | | | | | |
| Lean mass (kg) | 36.2±12.1 | 16.8-70.2 | 53.8±12.3 | 33.7-78.3 | <0.001 |
| Lean mass/height$^2$ (kg/m$^2$) | 14.3±2.9 | 9.6-22.4 | 17.6±2.7 | 12.2-24.0 | <0.001 |
| Fat mass (kg) | 16.5±7.1 | 3.7-29.8 | 15.8±6.6 | 6.5-29.5 | 0.667 |
| Fat mass % | 27.6±8.9 | 3.8-39.7 | 21.9±8.0 | 10.3-36.3 | 0.030 |
| Trunk fat mass % | 24.6±8.8 | 3.6-39.4 | 17.5±8.0 | 3.6-33.5 | 0.015 |
| **Life-style habits and physical condition** | | | | | |
| Total kcal expended per week | 496±595 | 0-2338 | 1592±1337 | 0-5468 | <0.001 |
| Shuttle run (time in seconds spent for 10x5m) | 30.5±10.6 | 21.0-60.1 | 20.6±1.7 | 17.3-24.1 | <0.001 |
| Sit-ups (per 30 second) | 10±8 | 0-23 | 20±6 | 2-28 | <0.001 |
| Telomere length (AU) | 0.67±0.38 | 0.24-1.73 | 1.00±0.65 | 0.25-3.08 | 0.044 |

AU, arbitrary unit

Prevalence of the frailty phenotype was 9/19 and pre-frailty phenotype 2/19 among the survivors. None of the age- and sex-matched healthy controls presented a pre-frailty or frailty phenotype. Relations of frailty phenotypes with demographic characteristics, lifestyle habits, self-reported chronic conditions, medical treatments, physical health limitations, and markers of frail health are presented in Table 19.

**Table 19.** Characteristics by frailty phenotype in 19 survivors and 20 controls.

| Characteristic | Non-frail n=28 | | Frail or pre-frail n=11 | | |
|---|---|---|---|---|---|
| | n | % | n | % | P |
| Current smoker | 1 | 3.6 | 3 | 27.3 | 0.060 |
| Exposure to TBI | 3 | 10.7 | 7 | 63.6 | 0.002 |
| Estrogen/testosterone substitution | 3 | 10.7 | 9 | 81.8 | <0.001 |
| **Physical health limitations** | | | | | |
| Vigorous activities (lifting heavy objects, running, strenuous sports) | 4 | 14.3 | 9 | 81.8 | <0.001 |
| Moderate activities (pushing a vacuum cleaner, moving a table, carrying groceries) | 0 | 0 | 3 | 27.3 | 0.018 |
| Walking a few hundred meters | 0 | 0 | 3 | 27.3 | 0.018 |
| **Potential markers of frail health** | **Median** | **Range** | **Median** | **Range** | **P** |
| BSA ($m^2$) | 1.8 | 1.3-2.3 | 1.4 | 0.9-2.0 | <0.001 |
| Telomere length (AU) | 0.81 | 0.24-3.08 | 0.46 | 0.24- 1.25 | 0.077 |
| S-hsCRP (mg/L) | 0.71 | 0.11-34.46 | 3.76 | 0.26- 11.39 | 0.005 |
| 24h mean heart rate (beats /min) | 74 | 52-99 | 82 | 61-90 | 0.038 |
| BALDD (mm) | 3.04 | 2.22-4.34 | 2.55 | 2.21-3.29 | 0.011 |
| FALDD (mm) | 6.73 | 5.11-9.21 | 5.79 | 4.61-7.40 | 0.014 |
| CAIMT (mm) | 0.43 | 0.34-0.55 | 0.47 | 0.35-0.72 | 0.083 |
| Radial artery intima double layer, n | 1 | | 4 | | 0.017 |
| Arterial plaque, n | 1 | | 2 | | 0.187 |
| U-Albumin/creatinine ratio (mg/mmol) | 0.3 | 0.1-24.1 | 1.6 | 0.5-104.5 | 0.001 |
| S-IGF-1 (nmol/L) | 38 | 12-90 | 24 | 14-44 | 0.029 |

AU, arbitrary unit; BALDD, brachial artery lumen diameter in end-diastole; BSA, body surface area; CAIMT, common carotid artery intima-media thickness; FALDD, femoral artery lumen diameter in end-diastole; hsCRP, high sensitivity C-reactive protein; IGF-1, insulin-like growth factor 1; TBI, total body irradiation

Frailty phenotype was associated with increased frequency of physical activity limitations such as inability to walk a few hundred meters. Frailty/pre-frailty phenotype was associated with an increased resting heart rate, decreased femoral and brachial artery LDs and the presence of intima double-line layer, but not with LVM or LV function.

Telomere length correlated significantly with GHbA1c (r=0.331, p=0.043), IGF-1 (r=0.361, p=0.026) and hsCRP (r=-0.385, p=0.017). Simple and multiple linear regression analyses identified low IGF-1 (p=0.010) and low GHbA1c (p=0.007) as independent predictors of decreased telomere length.

The survivors presented significantly higher levels of hsCRP when compared to the controls and the frailty/pre-frailty phenotype compared to the non-frailty phenotype. Increased hsCRP correlated with several adverse cardiovascular risk factors including triglycerides (r=0.516, p=0.001), subclinical atherosclerotic findings (carotid IMT, r=0.511, p=0.001), and systolic (TDI S', r=-0.0358, p=0.027) and diastolic function (TDI E/E', r=0.387, p=0.016) of LV. HsCRP correlated also with increased fat mass (fat mass %, r=0.403, p=0.012), decreased lean mass (lean mass/height$^2$, r=-0.324, p=0.047), decreased total kcal expended per week (r=-0.565, p<0.001) and decreased performance in physical tests such as shuttle run test (r=0.494, p=0.002) and sit-ups (r=-0.441, p=0.006).

# 6 Discussion

## 6.1 Early Ovarian Aging and Premature Menopause after Pediatric Allogeneic HSCT (I)

In the present study, declining ovarian function was associated with older age at HSCT. More than half of the survivors showed no ovarian activity after HSCT, suggesting that the entire follicle pool was depleted at the time of HSCT. In the previous studies, very young survivors with larger ovarian reserves have had better changes to at least temporarily recover their ovarian function after HSCT. The present observations are in line with these studies (Sanders et al. 1988, Spinelli et al. 1994, Borgmann-Staudt et al. 2012).

At their latest follow-up visit, 41 of the young women who were prepubertal, 11 of those 12 who were midpubertal and three of the eight who were postpubertal at the time of HSCT needed estrogen replacement therapy. Most of these survivors showed no ovarian activity after HCST. After HSCT, 14 prepubertal girls and two midpubertal girls temporarily recovered their ovarian function but entered premature menopause. The identification of these survivors with residual ovarian function is a major challenge because there might be a short-term benefit from fertility preservation techniques (Das et al. 2012, Socie et al. 2003).

Compared to survivors conditioned with cyclophosphamide only, female survivors conditioned with TBI, especially sTBI, or busulfan were at higher risk for premature menopause. Leukemia survivors who received CRT for anti-leukemia therapy before HSCT or were transplanted after disease relapse were at increased risk for permanent ovarian failure. From previous studies it is known that >30 Gy CRT is damaging to the hypothalamic pituitary region resulting in gonadotropin-releasing hormone deficiency (Cohen et al. 2008). In our study, female survivors who had received CRT at doses of 14-24 Gy had high menopausal serum levels of FSH and presented no signs of hypogonadotrophic hypogonadism.

At a mean age of 22 years, ten women had become pregnant. One survivor became pregnant after ovarian shielding, which confirms that this method during TBI potentially protects the ovary from irradiation damage (Nakagawa et al. 2006).

Female survivors transplanted at a young age with allogeneic HSCT are at high risk for early ovarian aging. Older age at HSCT, TBI- and/or busulfan-based conditioning increase the risk considerably. Girls need to be followed up regularly so that a possible window of ovarian recovery can be detected.

## 6.2 Early Vascular Aging after Pediatric HSCT (II)

Increased stiffness of the arteries has been reported as a hallmark of early vascular aging (Nilsson 2014). The long-term survivors of HR NBL presented increased arterial stiffness, intima thickening, and frequent plaque formation at a mean age of 22.7 years. The prevalence of plaques (19%) was similar to that in the general population for males over 40 and women over 50 (Joakimsen et al. 1999). All these findings are indicative of significant early vascular aging after therapy for HR NBL.

TBI was an independent predictor for the development of atherosclerotic vascular changes after therapy for HR NBL. In adults, obstruction of the arterial lumen (Carmody et al. 1999, Sutton et al. 2009), radiation dose-dependent IMT thickening (Feehs et al. 1991, Dorresteijn et al. 2005, Shariat et al. 2008, Gianicolo et al. 2010) and advanced accelerated carotid artery disease have been reported after local RT (Silverberg et al. 1978). Childhood cancer survivors, especially pediatric CNS tumor survivors and survivors of Hodgkin's disease, have shown increased carotid and femoral IMT (Dengel et al. 2014, Brouwer et al. 2013, Meeske et al. 2009), coronary artery disease (Kupeli et al. 2010) and increased occurrence of stroke after irradiation therapy (Bowers et al. 2006, Mueller et al. 2013, Bowers et al. 2005).

Previous studies with pediatric CNS tumor survivors have reported a radiation dose above 30 Gy as a threshold for an increased occurrence of stroke (Bowers et al. 2006). Radiation doses of more than 20 Gy have been associated with the development of coronary artery disease among survivors with Hodgkin lymphoma (Kupeli et al. 2010). Early mechanical changes such as decreased distensibility of the carotid arterial wall have been described after TBI-based conditioning for pediatric HSCT with doses of 10-14 Gy after a minimum of two years' follow-up time (Turanlahti et al. 2013). The present study shows that even lower irradiation exposures of 10-12 Gy as part of HSCT conditioning are able to induce vascular damage that can be detected after a long-term follow-up of 20 years.

The previous studies have shown that subclinical atherosclerotic changes in the carotid artery reflect more generalized atherosclerotic pathology (Neunteufl et al. 1997). In our study, common carotid, brachial, radial and femoral arteries were examined with highest 25/35/55 MHz frequency transducers since ultrasound penetrance limits the use of the highest frequencies in the non-invasive assessment of the more deeply located proximal arteries. We were able to detect a double line appearance in the proximity of the lumen-intima interface in five of the survivors. The double line appearance was interpreted as intima thickening. All survivors with plaques also had double line appearance.

The significantly increased cardiovascular risk profile in the HR NBL survivors is likely to play a minor role in the vascular findings observed as no difference in

cardiovascular risk factors was observed between TBI and non-TBI survivors. However, systolic BP emerged as an independent predictor for increased carotid IMT. The identification of cardiovascular risk factors together with the adverse effects of irradiation stresses the importance of monitoring these emerging subclinical organ dysfunctions that may impact the early vascular aging observed among long-term childhood cancer survivors.

## 6.3 Hypertension and Left Ventricular Hypertrophy after Pediatric HSCT (III)

The adult and adolescent very long-term survivors of HR NBL presented increased LVM, which was associated with decreased systolic and diastolic LV function, increased cardiac biomarkers and poor physical performance. Systolic BP was associated with LVM, suggesting a role in the development of LV hypertrophy in long-term survivors.

The use of anthracyclines is known to be a contributing factor, and a combination of anthracyclines and chest irradiation increases the risk of developing congestive heart failure (Lipshultz et al. 2013). In previous studies, there has been only a little evidence of increased risk of heart failure with chest irradiation with doses below 15 Gy (Armenian et al. 2015). In the present study, the survivors treated with TBI 10-12 Gy and moderate dose of anthracyclines (100-250mg/$m^2$) (Armenian et al. 2015) presented the most remarkable changes in cardiac function. The TBI-treated survivors showed smaller EDV, increased LVM and increased TDI E/E', suggesting restrictive physiology. Diastolic dysfunction has been associated with radiation-induced cardiotoxicity in previous studies (Alehan et al. 2012). Increased serum pro-BNP levels and heart rate among survivors supported the findings of cardiac dysfunction in the present study. Increased serum pro-BNP is used as a biomarker for the diagnosis of symptomatic heart failure (Braunwald 2008). The observed increased heart rate is known to be a risk factor for sudden death and an indicator of autonomic dysfunction (King et al. 2006).

The survivors had higher plasma levels of triglycerides and cholesterol, higher incidence of smoking, higher BP and poorer physical performance than the control group. Most of these cardiovascular risk factors are known to increase when people enter middle age. Early intervention may reduce the risk of premature cardiac disease. Cardiovascular risk factors should be routinely screened during the follow-ups of the survivors.

## 6.4 Frailty after Pediatric HSCT (IV)

Adult and adolescent long-term survivors of HR NBL showed a high frequency of frailty phenotype when compared to their age-matched controls, suggesting accelerated aging. Frailty phenotype associated with poor cardiovascular health.

The prevalence of frailty phenotype in the survivors of HR NBL was 47.4% while combined prevalence of frailty and pre-frailty was 57.9%. In previous studies, a 26.1% prevalence of frailty has been reported among community-dwelling elderly persons over 85 years of age (Collard et al. 2012). In the present study, the survivors at a mean age of 22 years had higher prevalence of frailty than elderly persons over 85 years (Collard et al. 2012). This suggests significantly advanced aging. Combined frailty phenotype in the present survivor cohort was also higher when compared to results from the St Jude Lifetime Cohort Study among survivors of CNS tumors (41.2%), soft tissue sarcomas (39.4%), other solid tumors (38.7%) and leukemia, lymphoma and bone tumors (30%) (Ness et al. 2013).

In the present study, telomere length was significantly shorter among the survivors compared to the age- and sex-matched healthy controls. Telomere shortening accelerates in replicative proliferative tissues when tissue is exposed to inflammation, oxidative stress, radiation or toxins, leading to cellular senescence and the process of aging (Shay & Wright 2007). Telomere shortening has also been associated with chronic somatic diseases of aging (Shay & Wright 2007). In our study, we could not associate telomere length with frailty phenotype. Instead, we found that low IGF-1 was an independent predictor of shorter telomere length. Short telomere length that associates with poor growth hormone status (Chemaitilly et al. 2015) may be partly explained by exposure to CRT. Low GHbA1c was also an independent predictor of shortened telomere length. Low GHbA1c is known to be a marker of increased mortality among diabetic patients and a marker of poor nutrition in frail persons without diabetes (Abdelhafiz & Sinclair 2015). Childhood cancer survivors have hormonal impairments, poor nutritional intake and poor physical activity, all of which can together lead to lower lean mass, physical weakness, low activity levels and slowness. Association of poor nutritional status with frail health may explain the connection between low GHbA1C and short telomere length.

The frailty phenotype in our study identified HR NBL survivors at risk for poor cardiovascular health. The frailty phenotype was associated with increased resting heart rate, which is known to be an independent risk factor for sudden death in adults (Kannel et al. 1987). Frailty was also associated with decreased femoral and brachial artery diameters in end-diastole and the appearance of intima double-line layer, suggesting subclinical atherosclerosis.

The frail survivors presented an increased prevalence of hypogonadism requiring hormonal replacement therapy and lower levels of IGF-1. These results are in line with a previous study where exposure to CRT and abdominal/pelvic irradiation associated with frail health (Ness et al. 2013). Anterior pituitary deficits, including growth hormone deficiency, are common after CRT with doses exceeding 22 Gy (Chemaitilly et al. 2015). High-risk gonadal toxicity is known to associate with HDTx and conditioning with TBI (Vatanen et al. 2013, Wilhelmsson et al. 2014). In this study, 52.7% of frail/pre-frail survivors had received TBI 10-12 Gy, and three had received 20-32 Gy doses to the cranial area. In line with a previous study, gonadal deficiency was not limited to survivors exposed to TBI (Chemaitilly et al. 2015). Accordingly, more than 80% of the frail survivors presented gonadal deficiency in this study, and 63% of them were exposed to TBI.

In this study, hsCRP correlated with frailty phenotype and was associated with increased percentage of fat mass, decreased lean mass, decreased total kcal expended per week, decreased performance in physical performance tests, several cardiovascular risk factors and measures of subclinical atherosclerosis and decreased LV function. Our findings are well in line with previous studies where sterile inflammation and elevated circulating CRP associated with early arterial changes (Jarvisalo et al. 2002), physical disability (Adriaensen et al. 2014), low lean mass and obesity (Cesari et al. 2005, Wang et al. 2011). Our findings support the hypothesis that inflammation plays a role in the pathogenesis of early vascular aging (Jarvisalo et al. 2002).

## 6.5 Strengths and Limitations of the Study

**(Study I)** The study with two hospital cohorts of female long-term survivors of pediatric allogeneic HSCT is limited by the young age of survivors and the lack of possibility to evaluate the wish to become pregnant. This information would have been needed to analyze the precise incidence of fertility after HSCT. The small number of leukemia survivors treated without TBI also limited us to evaluate the effects of previous CRT and relapsed disease on ovarian function in this patient group.

The major strength of this study was the longitudinal follow-up into puberty and adulthood of a well-defined cohort of girls and young women after pediatric allogeneic HSCT that made it possible to identify girls with temporary recovery of ovarian function.

**(Study II-IV)** Studies of a national cohort of HR NBL survivors were limited by the small sample size, while 79% of the national cohort was enrolled. The small sample size is mainly attributable to the poor prognosis of HR NBL patients in the previous decades.

The small sample size may have precluded statistically significant differences between groups in endothelial function and might also pose some limitations in the interpretation of the multiple linear regression analyses. Conventional treatment and specific chemotherapeutic agents varied over time; as a result, we failed to link cardiovascular changes to specific chemotherapeutics. The TBI-conditioned survivors also received higher cumulative doses of doxorubicin than the non-TBI survivors. The cumulative doses among the survivors were, however, low (Armenian et al. 2015) (91 mg/m$^2$ (0-210)) and are unlikely to explain the vascular differences between the TBI and the non-TBI survivors. Previous reports have found associations between endothelial dysfunction and anthracyclines (Herceg-Cavrak et al. 2011) but some have failed to show significant associations between anthracyclines and atherosclerosis when anthracyclines were combined with RT (King et al. 1999).

Major strengths of the study include the population-based design, extended follow-up from early childhood to young adulthood, comprehensive cardiovascular assessment with novel vascular ultrasound technology VHRU and novel 3D echocardiography, and the comprehensive set of metabolic cardiovascular risk and physiologic factors, telomere length and inflammatory markers linked to the frailty and pre-frailty phenotypes.

## 6.6 Future Considerations

**(Study I)** The identification of the patient group who temporarily recover their ovarian function after HSCT is a major challenge in the future in terms of fertility counseling and fertility preservation after transplantation. The period of recovery can be identified only with regular follow-up.

**(Study II-IV)** Future studies will need to address cardiovascular risk factors, early vascular aging, LVM and LV function and frailty among other patient groups exposed to TBI and other conditioning therapies in larger cohorts. It is also important to further evaluate the possible additive adverse effect of previous cancer therapy prior to HSCT on these parameters.

These results advocate a more comprehensive long-term follow-up with detection and intervention for cardiovascular risk factors in survivors after pediatric HSCT. Increased LVM and early vascular aging, as well as the observed systolic and diastolic cardiac dysfunction emphasizes the importance of assessing cardiovascular parameters in the clinical follow-up in order to facilitate early detection of subclinical disease and interventions to reduce the impact of common cardiovascular risk factors in the long term. Since lifestyle choices can influence cardiovascular health, frailty status and early vascular aging, a healthy lifestyle, non-smoking, low-fat diet and physical activity should be advocated among all survivors after HSCT. Survivors should be encouraged to rehearse speed and muscle strength in order to maintain their muscle mass to prevent the progression of premature aging and frailty.

# 7 Conclusions

The main conclusions of this thesis are:

### *Study I*

Girls and young women conditioned with TBI or busulfan regimens prior to allogeneic HSCT in childhood are at high risk of premature ovarian aging including permanent ovarian failure and premature menopause. The use of CRT for anti-leukemia therapy before HSCT further decreases the possibility of spontaneous menarche.

### *Study II*

The adolescent and young adult long-term survivors of HR NBL treated in early childhood with HSCT including TBI show signs of early vascular aging. Arterial plaques were found in three survivors treated with TBI and thickened intima layer in four TBI-treated and one non-TBI-treated survivor. Carotid IMT and stiffness were increased and compliance decreased among the survivors. Brachial and femoral lumen diameters were smaller among the survivors. The survivors displayed higher levels of plasma cholesterol and triglycerides, and smoking was more common compared to the control group.

### *Study III*

The HR NBL survivors treated with HSCT in early childhood show signs of increased LVM when compared to the age- and sex-matched healthy controls. The survivors' systolic and diastolic LV function was decreased. The increase in cardiac biomarkers and poor physical performance support these findings. Taken together, the increased LVM and decreased EDV suggest possible myocardial fibrosis-related restriction among the TBI-treated survivors. There was association with 24SYS and LVM suggesting an important role of BP in the development of LV hypertrophy and long-term cardiovascular health in these patients.

### *Study IV*

Frailty phenotype, including decreased muscle mass, slowness, weakness, exhaustion and low physical activity, is prevalent after HSCT and suggests significantly accelerated aging. The frailty phenotype identifies survivors at risk of poor cardiovascular health. Chronic inflammation correlates with frailty phenotype, increased percentage of fat mass, decreased lean mass, decreased total kcal expended per week, decreased performance in physical performance tests and cardiovascular health. The survivors of HR NBL treated with HSCT in early childhood have shorter telomere length when compared to the healthy age- and sex-matched controls.

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# Original Publications