LUND UNIVERSITY

Late complications of childhood acute lymphoblastic leukaemia (ALL), with special reference to hormone secretion, cardiovascular risk and bone health

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Late complications of childhood acute lymphoblastic leukaemia (ALL), with special reference to hormone secretion, cardiovascular risk and bone health

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List of original papers

This thesis is based upon the following papers, referred to in the text by their roman numerals (I-IV):

I. **Follin C**, Thilén U, Ahrén B, Erfurth EM. Improvement in cardiac systolic function and reduced prevalence of metabolic syndrome after 2 years of GH treatment in GH deficient adult survivors of childhood acute lymphoblastic leukaemia. J Clin Endocrinol Metab 2006; 91:1872-5

II. **Follin C**, Thilén U, Österberg K, Björk J, Erfurth EM. Cardiovascular risk, cardiac function, physical activity and quality of life with and without long-term GH therapy in adult survivors of childhood acute lymphoblastic leukaemia. J Clin Endocrinol Metab 2010; 95(8):3726-35

III. **Follin C**, Link K, Wiebe T, Moëll C, Björk J, Erfurth EM. Bone loss 25 years after childhood acute lymfoblastic leukaemia: an observational study with and without growth hormone therapy. Submitted.

Abbrevations

ACTH	Adrenocorticotrophic hormone
ALL	Acute lymphoblastic leukaemia
AO	Adult onset
Apo A1	Apolipoprotein A-1
Apo B	Apolipoprotein B
BIA	Bioelectrical impedance analysis
BMAD	Bone mineral apperent density
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
BP	Blood pressure
CI	Confidence interval
CNS	Central nervous system
CO	Childhood onset
CRT	Cranial radiotherapy
CVD	Cardiovascular disease
DXA	Dual-energy X-ray absorptiometry
FSH	Follicle stimulating hormone
GH	Growth hormone
GHBP	Growth hormone binding protein
GHD	Growth hormone deficiency
GHRH	Growth hormone releasing hormone
HDL	High density lipoprotein
HPA	Hypothalamic pituitary axis
IGF-I	Insulin growth factor I
ITT	Insulin tolerance test

Introduction

Acute lymphoblastic leukaemia (ALL)

Childhood ALL is the most common malignancy of childhood and accounts for 25% of all childhood malignancies and has an annual incidence of 35-40 per 1 million children. There is a peak incidence at the age of 4 years but the disease occurs in all ages and is slightly more common in boys than in girls (Plasschaert *et al.* 2004). Based on prognostic factors, the patients were divided into three different risk groups with varying treatment intensity; standard risk, intermediate risk and high risk. The disease is characterized by the increased number of immature cells in the blood, which reduces the number of normal blood cells. Clinical manifestations include fatigue, pallor, petechiae, bleeding, and fever. In addition, leukemic spread may manifest as lymphadenopathy and hepatosplenomegaly. Other signs and symptoms of leukemia include weight loss, bone pain, and dyspnea. Signs or symptoms of central nervous system (CNS) involvement, eg, headache, nausea and vomiting, lethargy, irritability, nuchal rigidity, papilledema, are rarely observed at the time of initial diagnosis. Testicular involvement with leukemic infiltration of the testes at diagnosis is rare. However, if present, it appears as painless testicular enlargement and this patients receive radiotherapy-treatment to the testes and chemotherapy. The ALL-treatment consists of a remission-induction phase, a consolidation phase and continuation therapy to eliminate residual disease and is kept during three years. Standard treatment during 1971-1992 included prophylactic cranial radiotherapy (CRT) and combination chemotherapy such as anthracycline, with a well-known cardiotoxic effect, and methotrexate, which has been shown to decrease bone formation. Glucocorticoids, with either prednisolone or dexamethasone, which was integral in

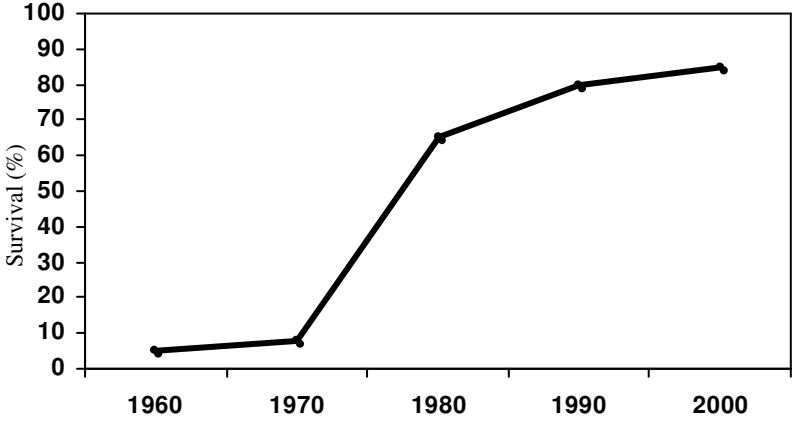
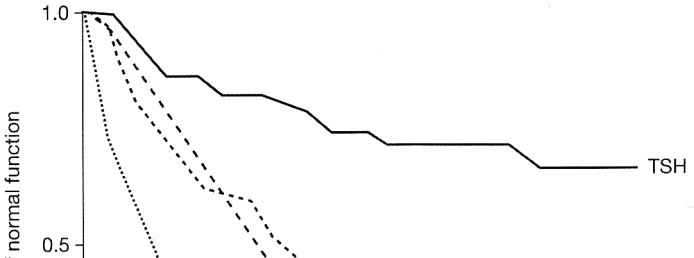


Figure 1. Survival rate of ALL survivors year 1960 - 2000

Radiation

Radiotherapy (RT) is the therapeutic use of γ-rays or X-rays to treat malignancies mediated by direct DNA damage or by the creation of free intracellular radicals. Prophylactic RT to childhood ALL patients forms together with chemotherapy the success in pediatric oncology during the 1970s and 1980s. The prevalence of RT-induced late effects has increased, due to the continuous improvement in survival of pediatric patients. Today radiobiologists divide normal tissues into acute-and late-responding tissues. In late-responding tissues such as heart and brain, the

nasopharyngeal carcinoma (Tan *et al*. 1966) was the first to describe such deficiency in patients with tumours not directly involving the hypothalamic-pituitary axis (HPA). It became apparent that the pituitary hormone deficiencies developed after many years and clinical studies suggested that the damage might be at the level of the hypothalamus (Larkins and Martin, 1973, Littley *et al*. 1990). Furthermore, histological findings in animals after RT have shown that the hypothalamus is particularly vulnerable to irradiation (Arnold, 1954). The exact mechanism of radiation damage to the hypothalamus is not known, but both vascular etiology and direct damage to the cell nuclei have been proposed. There is a remarkable difference in the incidence of anterior pituitary hormone deficiencies, with secretion of growth hormone (GH) being most frequently affected, suggests that selective hypothalamic neuronal and pituitary cell damage by direct radiation occurs. Differential radiosensitivity of HPA function has been proposed and clinical observations reveal that the GH axis is the most radiosensitive followed by the gonadotropin, adrenocorticotrophic hormone (ACTH) and thyroid stimulating hormone (TSH) axis and the HPA dysfunction worsens over time; both increased incidence and severity of hormonal deficits have been observed during extended postirradiation follow-up periods (fig. 2) (Littley *et al*. 1989).



In case of testicular relapse of ALL, the treatment consists of RT directed towards the gonads. The testicular germinal epithelium is very sensitive to damage from RT. The degree and permanency of RT-unduced testicular damage depends on the treatment field, total dose and fractionation schedule. Doses as low as 0.1-1.2 Gy damage dividing spermatogonia and disrupt cell morphology, resulting in oligozoospermia (Clifton *et al.*1983).

Growth hormone

GH was isolated from the bovine pituitary gland in 1944, and from the human pituitary in 1956. GH is secreted in an intermittent pulsatile pattern from the pituitary and is under the control of two interacting hypothalamic factors – one stimulatory, growth hormone releasing hormone (GHRH) and the other inhibitory, somatostatin – each in turn under the control of the CNS. In addition, GH secretion is regulated by negative feedback by circulating Insulin growth factor-I (IGF-I). IGF-I is a peptide bound to mainly produced in the liver and participate in the growth and function of almost every organ in the body (Ballard *et al.* 1993). The secretion of GH is augmented after onset of sleep and also under catabolic conditions of fasting and stress. Conversely, food intake and exposure to glucose, high levels of free fatty acids and obesity inhibit GH release (Williams textbook of Endocrinology). Physiologically females secrete 2 -3 fold greater amounts of GH compared with males despite maintaining similar IGF-I levels (van den Berg *et al.* 1996). GH secretion declines with age accompanied by a decrease in serum IGF-I concentration (Ho *et al.* 1987). GH acts on several organs and tissues in the body such as brain, muscle, kidney, heart and bone both directly and indirectly through production of IGF-I (Daughady and Rotwein 1989). One of the most consistent responses to GH administration is increased lipolysis and several studies have

releasing factors. Prolactin synthesis is also regulated by effects of estrogen on prolactin gene expression leading to higher prolactin levels in premenopausal women than in men. Prolactin levels rise during menarche and pregnancy. After delivery serum prolactin levels in the mother decline to the normal range if the mother does not breast-feed the child. With suckling maternal prolactin levels rise. In both men and women prolactin levels rise in response to stress (Thorner *et al.* 1977, Friesen *et al.* 1978). Prolactin acts through prolactin receptors in multiple tissues, including breast, liver, ovary, testis and prostate. In the human these receptors are stimulated by GH and prolactin with equal potency (Goodwin *et al.* 1990).

Physiological hyperprolactinemia during pregnancy and lactation and pathologic hyperprolactinemia are associated with suppression of the hypothalamic-pituitary-gonadal axis, which results in impaired gonadotropin secretion and inhibition of gonadal function (Milenkovic *et al.*1994).

Radiation induced hyperprolactinemia has been described in both sexes and in all age groups, but is most frequently encountered in the adult female (Littley *et al.* 1989, Lam *et al* 1991). A gradual decline in the elevated prolactin level may occur with time in some patients and this may reflect slowly evolving direct radiationinduced damage to the pituitary lactotroph (Littley *et al*. 1989).

The effect of hypoprolactinemia in humans has been demonstrated as incapacity of breastfeeding. Further it has been shown that PRL may play a role in a number of autoimmune processes (Reber et al. 1993) and hypo-prolactinemia is a riskfactor in children with nosocomial sepsis and multiple organ failure (Felmet et al. 2005).

Thyroid-stimulating hormone – TSH

Pituitary TSH sectretion is pulsatile with a sleep-independent nocturnal increase in

can be reversed by thyroxine administration and augmented by pharmalogical suppression of inhibitory somatostatin tone (Williams *et al*. 1985). GH has a regulatory effect on the thyroid hormone metabolism, with both a central effect, with inhibition of TSH release due to hypersecretion of somatostatin (Grunfeld et al. 1988), and a peripheral effect with an increased conversion from T_4 to T_3 (Jörgensen et al. 1994). Short term studies (1-6 months) of GH therapy to GHD patients have shown a decrease in basal T_4 and increase in T_3 and a suppression of basal TSH (Jörgenssen 1989, Jörgenssen 1994).

Growth hormone deficiency

Adult-onset GHD is usually secondary to a pituitary mass lesion and its treatment. Patients with multiple pituitary hormone deficiency are at the greatest risk for GHD (Toogood *et al.* 1994). When GHD occurs in childhood due to mass lesion, genetic defect or treatment for childhood cancer the deficiency is likely to persist into adulthood (Sklar *et al.* 1995).

Growth hormone deficiency is usually the first and is frequently the only manifestation of neuroendocrine injury following cranial irradiation. The severity and speed of onset of radiation-induced GH deficiency is dose dependent and the incidence increase with time after irradiation.

In adults the diagnosis of growth hormone deficiency is complicated by the lack of specific symptom, in contrast to the diagnosis in childhood where growth retardation is the key symptom. The symptoms of GHD include increased fat mass, in particular visceral fat, (Bengtsson *et al.* 1993), reduced muscle mass (De Boer *et al* 1992), dyslipidemia, insulinresistance, impaired cardiac performance, (Molitch *et al.* 2006) reduced bone mineral density (BMD) (Rosén *et al.* 1993), low energy levels and vitality. Decreased quality of life has been shown among

responses to mechanistically different provocative tests might be observed. A reduction in GH-dependent markers, IGF-I and IGFBP-3 is consistent with GH deficiency. However, a normal IGF-I level does nor exclude the diagnosis, and is frequently seen in patients with radiation-induced GH deficiency defined by pharmacological tests (Tillman et al. 1998). Thus, it had been thought that neither of these markers can be used as a reliable marker of radiation-induced GHD.

Growth hormone treatment

Children with GHD have been treated with GH since the late 1950s. GH was then prepared from human cadaveric pituitaries. Due to the limited supply of the hormone, GH therapy was restricted to hypopituitary children with severe growth retardation. Recombinant human GH became available in the middle of the 1980s and this made it possible to perform clinical studies in adults with GHD. These studies showed that GH therapy increased serum IGF-I and lean mass, reduced fat mass and improved quality of life (Salomon *et al.* 1989, Jörgensen *et al.* 1989). In these initial studies high doses of GH based on body weight was used, which resulted in side-effects such as fluid retention (Salomon *et al.* 1989, Jörgensen *et al.* 1989, Bengtsson *et al.* 1993). In later studies the GH dose was gradually uptitrated and based on the clinical response (serum IGF-I, body composition and well-being) which resulted in similar efficacy and much less side-effects (Drake *et al*. 1998).

Cardiovascular risk and cardiac function in hypopituitary patients

Hypopituitary patients on conventional hormone replacement but without GH

In childhood onset GH deficient subjects, GH therapy for 3-5 years have shown beneficial effects on cardiac function and CV risk factors (Thuesen *et al* 1994, Ter Maaten *et al*. 1999, Amato *et al* 1993).

Bone mineral density in hypopituitary patients

Multiple studies investigating BMD have shown that adults with severely GHD is approximately 1 SD score below the mean (Holmes *et al*. 1994). Approximately 20% of AO and 35% CO adult patients with GHD have osteoporosis. The age of onset of GHD appears to determine the severity of osteopenia. Patients younger than 30 years have the most severe ospeopenia (Molitch *et al.* 2006). The severity of GHD is correlated with the severity of osteopenia.(Lissett *et al.* 2002).
Patients with AO GHD have been shown to be both normal (Toogood *et al.* 1997) and low (Degerblad *et al.* 1995).
Adults with CO GHD, due to primarily hypothalamic-pituitary disease, have shown reduced BMD and bone mineral content (Kaufman *et al* 1992, De Boer *et al.* 1994). A low BMD has been recorded in both patients with isolated GHD and together with multiple pituitary deficiencies indicating that GHD *per se* is responsible for their osteopenia (Kaufman *et al* 1992). CO GHD patients are shorter than controls but even after correction for height BMD is reduced (De Boer *et al.* 1994).
GH stimulates both bone formation and resorption. With less than 12 months of GH therapy, BMD may not increase, but after 18-24 months of treatment most studies have shown increases in BMD (Shalet *et al.* 2003). Those patients with the most severity of bone mineral loss has the greatest improvement in response to treatment (Johannson *et al.*1996) and BMD of men respond better to GH than the BMD of woman (Drake *et al.* 2001).

osteoporosis/osteopenia and meningiomas. Anterior-pituitary deficiencies represent the most common complications of successful cancer therapy in both children and adults (Darzy *et al.* 2009). These abnormalities evolve with time after irradiation and persist into adult life and inflict a negative impact on growth, body composition, sexual function and quality of life. Therefore, regular surveillance is mandatory so that diagnosis can be made and hormone replacement initiated to minimize these adverse sequelae.

Even if CRT is no longer routinely used in Sweden, or in other countries, we estimate that about 500 survivors of ALL have been subjected to this therapy in Sweden, and with the corresponding numbers in other countries. Knowledge of the late effects associated with cancer in children continues to increase, but there is a lack of data on very long-term survivors who are now adults in the third and fourth decades of life. It is important that we improve our knowledge of the long-term impact of cancer therapy if we are to offer intervention strategies to prevent adverse late effects. Therefore, in study II-IV we aimed to evaluate very long-term effects, e.g. 25 years after ALL diagnosis, on cardiovascular risk, bone mineral density and hormone secretion after childhood ALL.

Growth hormone deficiency

GHD is the most frequent complication and usually the only overt manifestation of neuroendocrine injury in the majority of irradiated patients. Low-dose cranial irradiation (18-24 Gy) as used prophylactically in ALL usually cause isolated GH-deficiency (Shalet *et al.* 1976, Kirk *et al.* 1987, Brennan *et al.* 1998). Younger children receiving prophylactic cranial irradiation for ALL are more sensitive than older children or adults to the risk of developing GH deficiency following radiation to the HPA (Brauner *et al.* 1986, Shalet *et al.* 1976). Dissimilar growth patterns in ALL children have been reported (Kirk *et al.* 1987, Calyton *et al.* 1988)

(Bowers *et al*. 2006), and related mortality (Mertens *et al*.2001) in the years after treatment. Furthermore, the risk of obesity after ALL appears to be greatest among females diagnosed before 4 years (Oeffinger *et al*. 2003). CRT has been implicated as a potential cause of excess weight gain among survivors. The mechanism by which CRT leads to obesity is unknown, but hypothalamic damage leading to GHD and leptin insensitivity have been suggested (Brennan *et al*. 1999) and Nysom *et al*. (1999) correlated obesity to exposure to CRT and GHD. In a previous study an increased risk for the metabolic syndrome was found in those with reduced spontaneous GH secretion (Talvensaari *et al.* 1996). Further, physical activity and energy expenditure have been reported to be lower in ALL patients treated with CRT (Warner *et al.* 1998). Obesity in childhood is an important predictor of eventual development of adult-onset diabetes mellitus, hypertension, dyslipidemia and cardiovascular disease (Sinaiko *et al*. 1999).

Cardiac functions

Cardiotoxicity is a well-recognized late effect of therapy for childhood ALL and is a serious problem for long-term survivors. It may be caused by chemotherapy and/or chest irradiation. Anthracyclines are the most common class of chemotherapeutic agents associated with adverse effects on the heart. Anthracyclines were introduced in the late 1960s and early 1970s. They are highly effective against a wide range of malignancies and survival from pediatric cancers has increased since their introduction (Steinherz *et al.* 1998). Cardiotoxicity can be present as acute, early onset or late onset. The late cardiac effects are defined as complications that occur at least 5 years after completed therapy. Anthracycline cardiotoxicity can be divided in asymptomatic (subclinical) and symptomatic (clinical) cardiotoxicity. Asymptomatic cardiotoxicity is defined as various cardiac abnormalities diagnosed with different diagnostic methods in asymptomatic

Bone health

Treatment for ALL includes many known risk factors for low BMD. High doses of glucocorticosteroids and MTX, included in nearly all ALL treatment regimes, can at least temporarily affect bone formation (Robson *et al.*1998, Sala *et al*. 2007). Further, boys with radiation to the testes need adequate testosterone supplementation and ALL patients treated with cranial radiotherapy (CRT) are at risk for particularly growth hormone deficiency (GHD). GH status affects bone mineralization with GHD leading to reduced BMD (O´Halloran *et al.* 1993).

BMD has been found to be reduced in ALL children after completion of therapy (Gilsanz *et al.* 1990, Leeuw *et al.* 1990). The BMD was less in those treated with CRT than in the non-irradiated group (Gilsanz *et al.* 1990).

As patients with CO GHD are shorter (Link *et al.*2004) their bones have smaller width and will also be thinner i.e. volume corrections, using bone mineral apparent density (BMAD) is therefore preferable when comparing ALL patients with healthy controls (Katzman *et al.* 1991).

There are conflicting results regarding long-term BMD in former ALL patients as adults where six studies have shown low BMD, 4-24 years after 18-30 Gy of CRT (Gilsanz *et al.* 1990, Nussey *et al.* 1994, Nysom *et al.* 1998, Hoorweg.Nijman *et al.* 1999, Brennan *et al.* 1999, Thomas *et al.* 2008) and two studies did not show decreased BMD, 4 to 20 years after 18-24 Gy (Jarfelt *et al.* 2006, Mandel *et al.* 2004). In these studies GH status has only been taken into account in subgroups of patients and often with GH tests not always optimal to unmask radiation induced GHD.

Aims

I. To asses whether a low dose of GH for 12 and 24 months to young adult survivors of ALL with childhood onset GHD resulted in improvement in cardiovascular risk factors and cardiac measurements and function.

II. To evaluate 5 years with and 8 years without GH therapy on cardiovascular risk, cardiac function, physical performance and quality of life in adult ALL survivors with GHD.

III. To evaluate bonemineral density and markers of bone turnoverover in ALL survivors with GHD. Another aim was to investigate the effect of 5 years GH therapy on BMD in a subgroup of GH deficient ALL patients and to compare them to an untreated GH deficient ALL group after 8 years.

IV. To study the prevalence of PRL and TSH insufficiency in adults with childhood onset ALL. Further, to study the effect of GH therapy on PRL secretion and thyroid hormone secretion in GHD ALL patients.

Subjects

Patients

Paper I- IV
A consecutive series of 58 patients treated for childhood ALL with chemotherapy and CRT during 1971-1992 at the Children's Hospital Lund, Sweden, and at least 18 years of age were invited to participate in a study in year 2000. At that date 14 patients were excluded for various reasons: 7 patients declined to participate due to lack of time or fear of more hospital visits, 2 were treated for severe epilepsy with uncontrolled seizures, one was pregnant, one was breastfeeding, one had recently been operated for a brain tumor, one was on treatment with GH and one had emigrated. Thus, the final cohort included 44 GH deficient ALL survivors and 16 (8 women) of them were randomly chosen for GH therapy. Only one of these patients was previously treated with GH from age 12 to 14 years. Further, a subgroup of 13 ALL patients (4 women) were not receiving GH therapy, but had regular contact with a doctor or a nurse during the following 8 years. Two of these patients were previously treated with GH, from age 10 to 14 years. The remaining 15 ALL patients were not included at baseline in this protocol, due to the following reasons; pregnancy (n=3), declined GH therapy (n=8), not GHD (n=1), declined participating in the study (n=3).
Initially, the children were treated according to the protocols of the Swedish Child Leukaemia Group and since 1981 according to the common protocols in the five Nordic countries (Gustafsson). At baseline investigations, all patients had been off all kind of chemotherapy for a median 16.7 years (6.3-23.6). For patients characteristics and medical history see table 1.

Table 1. Patients' characteristics, medical history and GH status in 44 former ALL patients with and without GH treatment. Data are presented as median and range (min-max).

	5 years of GH therapy (n=16)	8 years of GHD (n=13)	Excluded patients (n=15)
Men/women (n)	8/8	9/4	6/9
Age at baseline (yr)	25 (22-32)	25 (19-32)	25 (20-32)
Height (cm)	161 (149-181)	170 (148-186)	162 (150-192)
Weight (kg)	66 (44-127)	79 (53-121)	68 (50-98)
Age at diagnosis (years)	3.9 (1-17)	4.2 (2-9)	6.3 (2-13)
Years since CRT (years)	21 (8-27)	19 (9-27)	19 (9-27)
Target dose CRT (Gy)	24 (18-24)	24 (24-25)	24 (18-30)
Cumulative anthracycline dose (mg/m^2)	120 (80-540)	120 (40-540)	120 (60-540)
MTX dose it (mg/ m^2)	60 (12-144)	72 (12-204)	114 (60-324)
Standard/intermediate/high-risk group (n)	11/0/5	10/0/3	8/1/6
Spinal radiation (n)	2	2	1
Testes-radiation (n)	5	5	2
Peak GH during ITT (µg/L)	0.4 (0.4-2.4)	0.2 (0.04-4.6*)	0.4 (0.2-3.8)
IGF-1 (µg/L)	124 (75-264)	142 (81-329)	132 (78-256)
GH during childhood	1	2	1
Oral contraception	2	1	2

* One patient responded with 4.6 µg/L but had < 1 during GHRH-Arginine-test and IGF-I – 3.0 SD.

Control subjects

Paper I-IV

Methods

Study designs

Paper I was of open design and CV risk factors and Doppler echocardiography examinations were performed in 18 ALL survivors with GHD, previously treated with cranial radiation and chemotherapy, before and after 12 and 24 months of GH therapy in a low dose (median 0,5 mg/day). Body composition was estimated with BIA and DEXA. Doppler echocardiography was performed with measurements of cardiac dimensions as well as systolic and diastolic function. Levels of serum insulin, blood glucose, leptin, HDL- and LDL cholesterol, Apo A1, Apo B and triglycerides were assessed. The degree of physical execise was investigated with questionnaires.

Paper II was of open design and ALL patients with GHD and controls matched for age, gender, residence and smoking were investigated with CV risk factors and Doppler echocardiography examinations at baseline, and after 5 years (n=16) with a low GH dose (0.5 mg/day), and without GH therapy for 8 years (n=13). Body composition was estimated with DEXA. Doppler echocardiography was performed with measurements of cardiac dimensions as well as systolic and diastolic function. Levels of serum insulin, blood glucose, HDL- and LDL cholesterol, Apo A1 and Apo B were assessed. The degree of physical exercise and quality of life was investigated with questionnaires.

Paper III was a cross-sectional study of BMD and BMAD of the femoral neck, lumbar spine 2–4 and body composition, assessed with DXA (Lunar Expert XL and Lunar Prodigy, Lunar Co., Madison, WI, USA). Markers of bone turnover

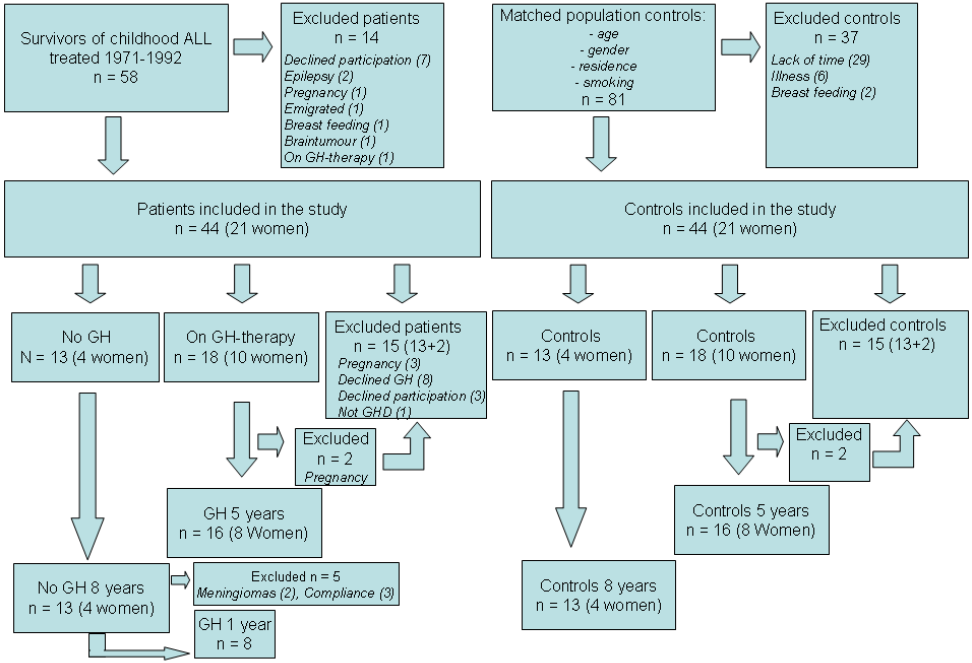


Figure 3. Flow chart of excluded and included former ALL patients and matched population controls.

Blood pressure and heart rate

In **study I** and **II** blood pressure was measured in the right arm in the supine position after 10 minutes rest. Two measurements were made and mean value was calculated. Heart rate in **study I** and **II** was obtained from ECG recordings with measurements of the R-R interval (heart rate = 60*1000/R-R interval in ms).

Waist hip ratio, BMI and BIA

In **study I** and **II** waist circumference was measured at the midpoint between the lower rib margin and the iliac crest and hip circumference at level of trochanters, enabling the calculation of the waist hip/hip ratio (WHR). Body mass index (BMI) in **study I-III** was calculated as bodyweight (kg) divided by height (metres) squared. Body composition in **study I** was assessed by bioelectric impedance analysing using the BIA 101-S technique (RJL-Systems, Detroit, MI, USA). Data are presented as percentage and kg fat and fatfreee mass. Body composition in **study II** and **III** was assessed with DXA (Lunar Expert XL, Lunar Madison, USA).

Criteria for metabolic syndrome

Metabolic syndrome was defined according to the International Diabetes Federation (IDF) guidelines (IDF consensus), which is the European guideline, with central obesity (waist ≥ 94 cm in men and ≥ 80 cm in women) as a key issue in association with two or more of the following: 1) elevated triglycerides (TG); 2) elevated fasting plasma glucose; 3) raised blood pressure; 4) reduced high-density-lipoprotein cholesterol (HDL-C).

Test procedures

TRH-test
The test was performed on days 2-6 of the menstrual cycle and started at 10 am after an overnight fast. Patients and controls received a bolus of 200 µg of TRH iv. Blood was taken for PRL, TSH and free T_4 analyses at 15 minutes before and 0, 10, 20, 30, 60 and 90 minutes after the TRH-injection.

Biochemical assays
Bood samples were drawn in the morning after fasting since midnight and were stored at – 80 ˚C until analysis.

GH
Serum GH was analyzed by an immunofluorometric method (Wallace Oy, Turku, Finland). The detection level for serum GH was 0.01 µg/L. The intra and interassay CV´s were 5% and 3 %, respectively, at a level of 1.5 µg/L.

IGF-I
Serum IGF-I was measured by a chemiluminescent immunoassay (Immulite 2500, Siemens, Munich, Germany). The normal range was 117-329 µg/L in subjects aged 24-30 year. The imprecion (CV%) was 5.5% at the level of 74 µg/L and 5% at the level of 210 µg/L.

Thyroid hormones
Serum TSH, free T4, free T3 and cortisol were analysed with an immunofluorometric technique (Auto Delfia, Wallac, Oy, Turku, Finland). The intra-assay CVs for serum free T4 and serum free T3 were <4.1% and 8.0%, respectively, and inter-assay CVs were 4.1% and 8%, respectively. The reference ranges for serum free T4 and serum free T3 were 9-22 pmol/L and 3.1-6.9 pmol/L,

Blood glucose
Venous blood glucose was analysed with Hemocue Blood Glucose Analyser (Hemocue AB, Ängelholm, Sweden). The instrument was controlled daily using a standard microcuvette and weekly using a hemolysate (Eutrol, Wageningen, The Netherlands) with known glucose concentration.

Insulin
Serum insulin was measured with a competitive radioimmunoassay with intra- and interassay CVs of 7.1%.

Leptin
Serum leptin was analysed with a double-antibody radioimmunoassay using rabit antihuman leptin antibodies, ^{125} I labeled human leptin tracer and human leptin as standard (Linco Res., St Charles, Mo USA). Interassays CV is 1.9% at low levels (<5 ng/ml) and 3.2%at high levels (10-15 ng/ml). Intraassay CVe used the SPSS version 15.0 for the statistical analysis is 1.5% at low levels and 3% at high levels. The limit of detection is 0.5 ng/ml.

Total-, LDL, and HDL-cholesterol, Apo A, Apo B and triglycerides
Fasting total-, LDL, and HDL-cholesterol, Apo A, Apo B and triglycerides levels were measured by standard procedures.

Questionnaires

Physical exercise
The degree of physical exercise during spare time and working time was assessed by self-rating questionnaire in which patients and controls classified their physical activity according to a four-grade scale.

Statistics

In ***paper I-IV*** data are presented as median and range. The level of significance was set at $p \leq 0.05$ in all four papers.

In ***paper I-IV***, comparisons between patients and controls and intraindividual comparisons of data before and after GH treatment were calculated with the Wilcoxon signed rank test for matched pairs. Univariate correlations were assessed using Spearman`s rank order correlation test.

To assess the differences between the GH treated and the non-GH treated group in ***paper II***, we calculated the differences in CV risk factors between 5 respectively 8 years and baseline and then tested the differences using Mann-Whitney U-test with two independent groups, i.e. GH-treated *vs* non-GH treated. Exact calculations of p-values were used to handle ties correctly in the non-parametric methods.

We used the SPSS version 15.0 for the statistical analysis.

Results

Paper I

Lipoproteins, leptin, and anthropometric measures

After 12 months of GH treatment, serum leptin (p=0.002) and leptin/kg FM (p=0.01) were significantly lower. When stratified for gender serum leptin in both men and women were significantly lower after 12 months of therapy (p=0.03 and p=0.02, respectively). At 12 and 24 months of GH replacement a significant decrease in plasma glucose levels was recorded.
After 12 months of GH therapy, percentage FM had decreased significantly (p=0.002), and percentage MM and LM had increased significantly (p=0.004 and p=0.002, respectively). After 24 months of GH treatment LM was still significantly increased (p=0.005). GH treatment caused a decrease in hip (p=0.04) and waist (p=0.02) circumferences during the first 12 months, and at 24 months hip (p=0.06) and waist circumferences (p=0.1) had decreased further. In detail, 55% of the patients and 6% of the controls had impaired fasting plasma glucose at baseline. After 12 and 24 months of GH treatment none of the patients had an increase in plasma glucose (table 2).

The metabolic syndrome

When using the International Diabetes federation definition of the metabolic syndrome, which consider central obesity as a key issue, together with at least 2 of 4 possible risk factors (raised TG, reduced HDL-cholesterol, raised blood pressure,

Table 2. Biochemical and body composition characteristics in 18 former acute lymphoblastic leukemia patients at baseline, at 12 months and 24 months of GH treatment and in 18 sex and age matched controls at baseline.

	Controls baseline (n=18) Median range	Patients baseline (n=18) Median range	Patients 12 months GH (n=18) Median range	Patients 24 months GH (n=13) Median range	p[a]	p[b]	p[c]
S-Insulin (mIE/L)	5 (2-10)	6 (2-25)	5 (2-23)	6 (3-40)	0.2	0.2	>0.3
P-glucose (mmol/L)	5.0 (4.2-5.6)	5.5 (3.3-6.8)	4.6 (2.9-5.4)	4.7 (4.2-5.5)	>0.3	0.04	0.02
S-Cholest. (mmol/L)	4.1 (3-5.8)	4.4 (3.3-6.6)	4.7 (3.7-6.8)	4.5 (3.4-6.3)	0.1	0.3	>0.3
S-HDL (mmol/L)	1.4 (7-21)	1.3 (0.7-1.8)	1.3 (0.6-1.8)	1.4 (1.0-2.0)	>0.3	>0.3	0.3
S-LDL (mmol/L)	2.3 (1.5-4.2)	3.1 (1.5-4.8)	2.8 (1.7-4.5)	3.0 (2.2-4.8)	0.03	>0.3	>0.3
S-Triglyc. (mmol/L)	0.8 (0.3-3.2)	0.9 (0.4-2.3)	1.1 (0.4-2.7)	1.0 (0.4-2.7)	>0.3	0.04	0.04
S-Leptin (mg/ml)	6 (1-26)	21 (4-39)	13 (2-25)	14 (3-32)	0.06	0.002	0.3
Leptin/kg /fat mass	0.4 (0.04-2)	0.9 (0.3-1.4)	0.7 (0.2-1.3)	0.8 (0.2-1.2)	0.05	0.01	0.09
S-IGF-1 (µg/L)	194(107-279)	139 (81-289)	230 (82-311)	236(169-337)	0.05	0.002	0.002
Weight (kg)	66 (52-92)	66 (44-127)	67 (45-128)	66 (46-84)	>0.3	0.4	0.04
Fatmass (%)	26 (15-43)	35 (26-45)	31 (17-43)	31 (17-44)	0.03	0.002	0.2

Doppler echocardiography and physical exercise

Twenty-four months of GH treatment gave a small increase in LV-mass index and improved systolic function, measured as FS (p=0.03) and EF (p=0.03)(Table3).
There were no significant differences in physical exercise during spare time or working time between the patients at baseline and after the two GH treatment periods (all p-values >0.3).

Table 3. Doppler echocardiography data in 13 former acute lymphoblastic leukemia patients at baseline and at 24 months of GH treatment and in 13 sex and age matched controls at baseline.

	Controls baseline Median (range) n=13	**Patients baseline** Median (range) n=13	**Patients 24 months GH** Median (range) n=13	p^a	p^b
Heart rate at rest (beats/min)	69 (58-102)	69 (61-89)	65 (58-96)	>0.3	0.3
Body surface area (m^2)	1.71 (1.54-2.21)	1.61 (1.33-2.02)	1.65 (1.37-1.98)	0.005	0.19
Cardiac measurements corrected for BSA					
LV mass index (g/m^2)	89 (49-117)	72 (62-116)	76 (57-123)	0.08	0.06
LA area (cm^2/m)	8.2 (6.0-11.5)	8.6 (5.5-12,2)	8.3 (4.6-12.3)	0.5	0.8
LV area (cm^2/m^2)	15.2 (13.1-19.5)	14.1 (11.3-17.6)	16.4 (13.4-20.7)	0.2	0.15
LVIDd (mm/m^2)	26.8 (24.1-31.3)	28.1 (23.1-35.3)	27.3 (25-34)	0.2	0.2
Left ventricular systolic function					
Fractional shortening (%)	38(30-48)	36 (22-46)	40 (29-48)	0.2	0.03
Ejection fraction (%)	77 (66-86)	74 (52-85)	78 (65-87)	0.2	0.03
Left ventricular diastolic					

Paper II

Comparisons before and after 5 years of GH therapy in ALL patients and to controls before and after 5 years

CV risk factor and body composition

Patients had significantly higher LDL-C levels at baseline compared to controls, but with no significant change after 5 years of GH therapy (table 4). In comparison to baseline, levels of glucose and the ApoB/ApoA1 ratio decreased significantly, and the HDL-C levels increased significantly after GH therapy and with no significant differences in these levels between patients and controls after 5 years (table 4).

BMI, weight, fat mass and lean mass increased significantly after 5 years of GH therapy and with no differences in these levels in comparison to controls after 5 years (table 4).

Six patients and 1 control subject had MetS at baseline and after 5 years of GH therapy 1 patient and 2 controls had MetS.

There were no significant differences in systolic or diastolic blood pressure in comparison to controls before or after 5 years.

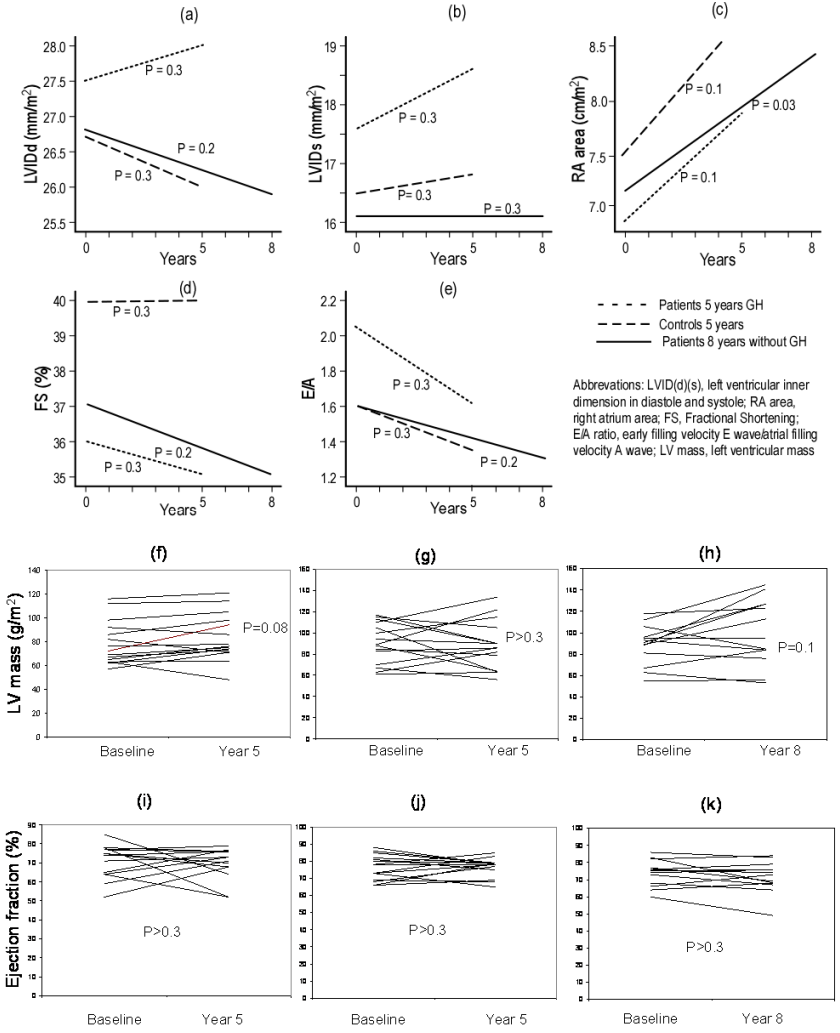
Echocardiography measurements

At baseline, LV mass and RV area, were significantly smaller among patients compared with controls (P=0.05, P=0.04, respectively). After 5 years of GH therapy LV- and RV area increased significantly (P=0.02, P=0.05, respectively),

4e) but with no significant differences in either of the diastolic measurements in comparison to controls after 5 years (both; P≥0.1).

Table 4. Biochemical and body composition characteristics in 16 former ALL patients (8 women) at baseline and after 5 years of GH therapy and in 16 sex- and age- matched controls at baseline and after 5 years. Data are presented as median and range (min-max).

	Patients at baseline	**Controls at baseline**	**Patients after 5 years GH therapy**	**Controls after 5 years**	**P[a]**	**P[b]**	**P**
Age (years)	25 (19-31)	25 (19-31)	30 (24-36)	30 (24-36)	>0.3	>0.3	>0.3
S-Insulin (mIE/L)	5.9 (2-28)	4.7 (2.4-15.7)	8 (2-35)	3 (2-15)	>0.3	0.3	0.06
P-glucose (mmol/L)	5.5 (3.3-6.8)	5.0 (4.55-6.2)	4.8 (3.9-5.9)	4.8 (3.8-5.5)	>0.3	0.03	>0.3
S-HDL (mmol/L)	1.3 (0.7-1.8)	1.3 (0.7-1.9)	1.4 (0.6-2.2)	1.4 (0.8-2.2)	>0.3	0.03	>0.3
S-LDL (mmol/L)	3.0 (1.5-4.8)	2.2 (1.5-4.9)	3.0 (2.3-5.1)	2.5 (1.6-4.8)	0.04	0.3	0.1
ApoB/A1	0.7 (0.3-1.4)	0.5 (0.2-1.2)	0.6 (0.4-1.2)	0.5 (0.3-1.4)	0.08	0.006	0.3
BMI (kg/m^2)	23 (20-37)	22 (18-35)	26 (20-40)	23 (19-40)	>0.3	0.003	0.2
Weight (kg)	66 (44-127)	69 (52-109)	69 (47-135)	74 (53-126)	0.3	0.008	>0.3
Waist (m)	0.82 (0.7-1.2)	0.80 (0.7-1.1)	0.80 (0.8-1.2)	0.79 (0.7-1.2)	>0.3	0.2	0.2
Waist/hip	0.92 (0.8-1.2)	0.84 (0.8-	0.92 (0.8-1.1)	0.88 (0.8-1.1)	0.04	0.3	0.1


(a)
LVIDd (mm/m²)
28.0
27.5
27.0
26.5
26.0
25.5
P = 0.3
P = 0.2
P = 0.3
0
5
8
Years
(b)
LVIDs (mm/m²)
19
18
17
16
P = 0.3
P= 0.3
P = 0.3
0
5
8
Years
(c)
RA area (cm/m²)
8.5
8.0
7.5
7.0
P = 0.1
P = 0.03
P = 0.1
0
5
8
Years
(d)
FS (%)
40
39
38
37
36
35
P = 0.3
P = 0.2
P = 0.3
0
5
8
Years
(e)
E/A
2.2
2.0
1.8
1.6
1.4
1.2
P = 0.3
P = 0.3
P = 0.2
0
5
8
Years
Patients 5 years GH
Controls 5 years
Patients 8 years without GH
Abbrevations: LVID(d)(s), left ventricular inner dimension in diastole and systole; RA area, right atrium area; FS, Fractional Shortening; E/A ratio, early filling velocity E wave/atrial filling velocity A wave; LV mass, left ventricular mass
(f)
LV mass (g/m²)
P=0.08
Baseline
Year 5
(g)
P>0.3
Baseline
Year 5
(h)
P=0.1
Baseline
Year 8
(i)
Ejection fraction (%)
P>0.3
Baseline
Year 5
(j)
P>0.3
Baseline
Year 5
(k)
P>0.3
Baseline
Year 8

Comparisons before and after 8 years without GH therapy in former ALL patients and to controls before and after 8 years

CV risk factors and body composition

Significantly higher levels of serum insulin and plasma glucose, but also significantly lower levels of HDL-C were recorded among patients at baseline in comparison to controls (table 5). Significant differences in serum insulin and HDL-C shown at baseline were sustained in the patients in comparison to controls after 8 years (table 5).

Table 5. Biochemical and body composition characteristics in 13 former ALL patients (4 women) at baseline and
8 years after confirmed growth hormone deficiency (GHD) and in 13 age- and sex-matched controls at baseline and after 8 years. Data are presented as median and range (min-max).

	Patients at baseline	**Controls at baseline**	**Patients after 8 years of GHD**	**Controls after 8 years**	**P[a]**	**P[b]**	**P[c]**
Age (years)	25 (19-31)	25 (19-31)	33 (27-39)	33 (27-39)	>0.3	>0.3	>0.3
S- Insulin (mIE/L)	7 (2-32)	6 (2-10)	8 (4-24)	4 (3-8)	0.01	0.2	0.03
P-glucose (mmol/L)	4.9 (3.0-6.2)	4.2 (3.6-4.7)	5.2 (4.3-8.8)	5.1 (4.6-5.7)	0.02	0.02	>0.3
S-HDL (mmol/L)	1.2 (0.5-1.8)	1.5 (1.0-2.3)	1.1 (0.5-1.2)	1.3 (0.9-1.8)	0.00 4	0.2	0.03
S-LDL (mmol/L)	2.8 (1.1-4.8)	2.3 (1.2-4.9)	2.5 (2.1-5.0)	2.7 (1.8 4.5)	>0.3	0.08	0.3
ApoB/ApoA1	0.6 (0.3-1.4)	0.5 (0.2-0.8)	0.6 (0.5-1.1)	0.4 (0.3-1.2)	0.08	0.3	0.08

At baseline, significantly higher BMI, waist circumference and fat mass were recorded among the patients compared with controls (table 5). After 8 years of GHD, BMI, weight, waist circumference, and fat mass increased significantly, but lean mass decreased significantly compared to baseline levels. Only the significant difference in BMI between the patients and controls was sustained after 8 years (table 5).

Three patients and 1 control subject had MetS at baseline and after 8 years, 6 patients and 2 controls had MetS.

There were no significant differences in systolic or diastolic blood pressure in comparison to controls before or after 8 years.

Echocardiography measurements (not performed in controls after 8 years)

At baseline, no significant differences in LV mass, LA area, LV area, LVID d and RA area (all; $P \geq 0.1$), were recorded in patients compared with controls, except for RV area which was significantly smaller ($P=0.02$). After 8 years of GHD, in comparison to baseline levels, no significant differences in LV mass (Fig. 4 h), LA area ($P=0.08$) and LVIDd (Fig. 4a) were seen. LV area ($P=0.002$), RV area ($P=0.005$) and RA area (Fig. 4c) increased significantly among patients after 8 years in comparison to baseline levels.

Systolic function: No significant differences were recorded in EF and FS between patients and controls at baseline (both; $P \geq 0.1$), or in comparison between baseline and 8 years of GHD (Fig. 4k,4d).

Diastolic function: Patients had a significantly lower E/A ratio ($P=0.02$) and E deceleration time ($P=0.01$) compared to controls at baseline, but without differences between baseline compared to 8 years among patients (both; $P \geq 0.1$).

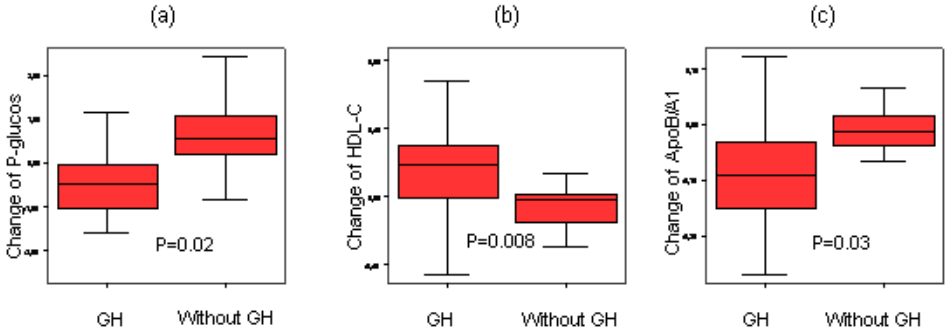


Figure 5. Differences in the change of CV risk factors between 16 GH- and 13 non-GH- treated ALL patients. Data are presented as median (range=min-max).

Paper III

Anthropometric and hormone assessments in 44 patients in comparison with matched controls at baseline

Height was significantly reduced by 13 cm in the male patients and by 9 cm among the female patients (Table 5). BMI and FM were significantly increased in ALL women. LM was significantly decreased in both genders (Table 5).
Serum levels of IGF-I were significantly lower in both genders, to - 1.6 SD in ALL women and to - 1.25 SD in ALL men. The serum TSH levels were without significant differences between ALL women and men in comparison to controls (1.9 vs 2.0 mU/L, and 2.1 vs 1.9 mU/L, respectively; >0.3). Serum free T4 was

were compared to those males with GHD and testosterone substitution (n= 10) the BMD measurements were without significant difference.
No significant differences in the serum levels of osteocalcin and crossLaps (Table 6) or in calcium, albumin, phosphate, or PTH levels were recorded between patients and controls in either gender.

Table 6. Anthropometric measurements, body composition, bone mineral density (BMD), bone mineral apparent density (BMAD), z-scores using DXA and serum hormone levels at baseline in 21 former ALL women and 23 former ALL men and matched controls

	Patients Women Median (range) N=21	**Controls Women Median (range) N=21**	**P**	**Patients Men Median (range) N=23**	**Controls Men Median (range) N=23**	**P**
Age (yr)	26 (22-33)	26 (22-33)	--	25 (20-32)	25 (20-32)	--
Height (cm)	158 (145-180)	167 (159-174)	0.001	172 (160-192)	185 (168-200)	0.002
Height (SDS)	-1.6 (-2.0- -1.1)	0.0 (-0.5-0.7)	0.001	-1.25 (-2.0- -0.49)	0.2 (-0.7-0.5)	0.002
BMI (kg/m^2)	26 (20-33)	21 (18-34)	0.02	24 (19.2-37)	23 (20-34)	0.1
Weight (kg)	68 (44-81)	61 (52-94)	>0.3	79 (53-127)	80 (65-109)	>0.3
Fatmass (kg)	28 (12-42)	16 (11-46)	<0.001	19.6 (38-60)	13.6 (70-35)	0.07
Fatmass (%)	42 (28-53)	30 (21-49)	0.001	28 (7-47)	17 (8-39)	0.01
Leanmass (kg)	33 (28-45)	38 (34-48)	0.01	40 (32-57)	47 (38-62)	0.02
Leanmass (%)	43 (28-53)	64 (46-73)	0.001	67 (49-84)	76 (57-83)	0.01
IGF-I (µg(L)	137 81-289)	196 (107-338)	0.03	159 (110-329)	191 (110-341)	0.04
BMAD fem neck (g/cm^3)	0.2 (0.2-0.3)	0.2 (0.2-0.3)	>0.3	0.2 (0.1-0.3)	0.2 (0.2-0.3)	>0.3
BMD fem neck (g/cm^2)	1.0 (0.8-1.2)	1.0 (0.8-1.3)	0.3	1.1 (0.8-1.3)	1.1 (0.9-1.4)	>0.3

History of previous fractures, daily calcium intake and physical exercise in 44 ALL patients in comparison with 44 matched population controls at baseline

No significant difference in the number of previous fractures or in present calcium intake was recorded in patients compared with controls of both genders (P>0.3).
Neither was there any difference in the degree of physical exercise during leisure time between patients and controls of both genders (P>0.3). However, during working time both genders reported significantly higher degree of physical exercise compared to controls (P=0.04).

Correlations between disease-related factors to Z-scores and BMD among the 44 ALL patients at baseline

The cumulative dose of MTX (p.o) was negatively correlated to the Z-scores at L2-L4 (r = - 0.31, P=0.03) (Fig. 6), but without significant correlation to BMD at L2-L4 (r= - 0.22, P=0.2).
The cumulative dose of corticosteroids, level of serum IGF-I, maximum GH response to GHRH-Arginine, dose of CRT, and time since diagnosis were without significant correlations to BMD or to Z-scores at L2-L4 and fem.neck (all; P>0.3)

Comparisons before and after 5 years of GH therapy in ALL patients and to the same matched population controls at baseline and after 5 years

No significant differences in BMD at any site or to the Z-Score at L2-L4 were recorded in former ALL patients of both genders before compared to after 5 years of GH therapy, and with no differences in these measures in comparison to controls (table 7)**.** The Z-score at femoral neck decreased, but not significantly after 5 years of GH therapy, and became significantly lower in ALL females, but not in males (fig. 7).
Serum crossLaps levels in the whole patient group increased significantly after GH therapy, and became significantly higher than controls (Table 7). No significant change in serum levels of osteocalcin was recorded after 5 years and these levels remained similar to controls after 5 years. A significant increase in serum IGF-I levels were recorded from - 2.5 SD to + 0.05 SD (P=0.03) and without differences in these levels compared to controls after 5 years. The IGF-I levels in males increased to + 0.05 SD and in females to - 0.7 SD. Serum levels of thyroid hormones, testosterone and estradiol were without difference compared to controls after 5 years (all; P>0.3).

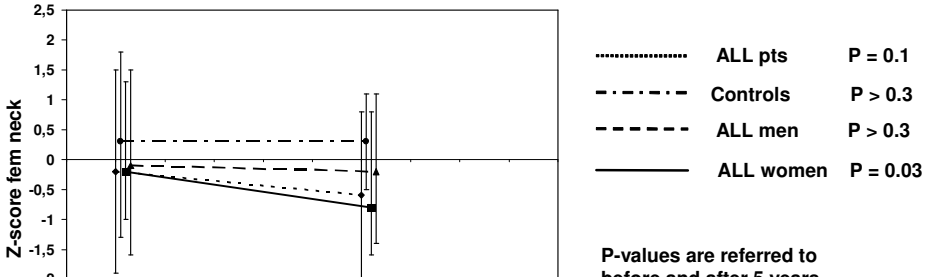


Table 7. Bone mineral density (BMD), bone mineral apparent density (BMAD) and biochemical assays in former ALL patients at baseline and after 5 years of GH treatment and control subjects at baseline and after 5 years

	Patients baseline Median (range) N=15	**Controls baseline Median (range) N=15**	**Patients after 5 yrs GH treatment N=15**	**Controls after 5 yrs Median (range) N=15**	**P[a]**	**P[b]**	**P[c]**
Age (yr)	25 (22-32)	25 (22-32)	31 (27-38)	31 (27-38)	-	-	-
BMD Femoral neck (g/cm^2)	1.0 (0.8-1.3)	1.0(0.9-1.3)	0.9 (0.8-1.2)	1.1 (0.9-1.3)	0.2	0.3	>0.3
Z-score Femoral neck (SDS)	-0.2 (-1.6-1.5)	0.3 (-0.9-2.0)	-0.6 (-2.3-1.1)	0.3 (-0.9-2.0)	0.2	0.1	>0.3
BMD L2-L4 (g/cm^2)	1.1 (1.0-1.5)	1.2 (1.1-1.5)	1.1 (1.0-1.3)	1.2 (1.0-1.5)	0.2	>0.3	>0.3
Z-score L2-L4 (SDS)	-0.4 (-1.6-2.1)	0.1 (-1.5-2.1)	-0.3 (-2.1-1.3)	-0.2 (-2.3-1.8)	>0.3	>0.3	>0.3
CrossLaps (µg/L)	436(211-937)	518(274-1001)	453 (152-1559)	417 (26-711)	>0.3	0.04	0.02
Osteocalcin (ng/L)	30 (21-58)	30 (17-51)	27 (18-106)	25.5 (11-428)	>0.3	>0.3	>0.3
IGF-I ((µg/L)	124 (75-264)	139 (99-250)	176 (110-301)	135 (73-253)	0.05	0.03	0.09

P[a] Patients' baseline *vs* controls at baseline
P[b] Patients' baseline *vs* 5 yrs of GH treatment
P[c] Patients' 5 yrs of GH treatment *vs* controls 5 yrs after baseline

Comparisons before and after 8 years without GH therapy in former ALL patients and to the same matched population controls at baseline and after 8 years

8 years (Table 8). Levels of thyroid hormones, estradiol and testosterone remained at similar levels as controls after 8 years (all; P > 0.3).

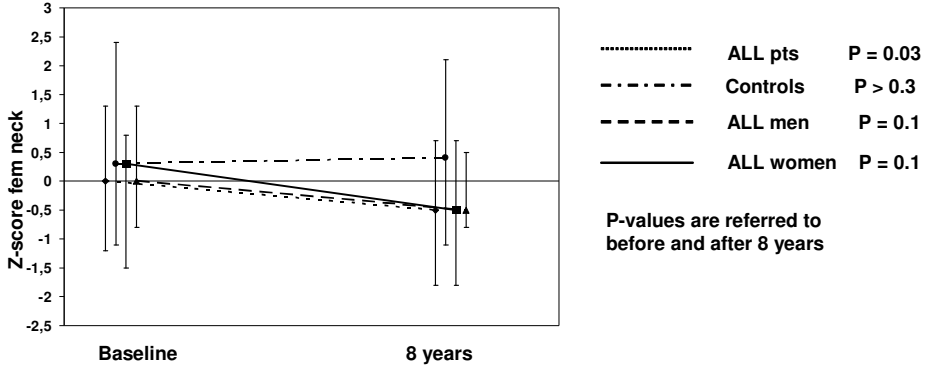


Figure 8. BMD Z-score at femoral neck in 13 ALL patients before and after 8 years without GH therapy and in 13 controls before and after 8 years.

Table 8. Bone mineral density (BMD), bone mineral apparent density (BMAD) and biochemical assays in 13 former ALL patients (4 women) at baseline and after 8 years after confirmed GHD and control subjects at baseline and after 8 years

	Patients baseline Median (range) N=13	**Controls baseline Median (range) N=13**	**Patients after 8 yrs Median (range) N=13**	**Controls after 8 yrs Median (range) N=13**	**P[a]**	**P[b]**	**P[c]**
Age (yr)	25 (19-32)	25 (19-32)	33 (27-40)	33 (27-40)	-	-	-
BMD femoral neck	1.1 (0.8-1.3)	1.1 (0.9-1.3)	1.0 (0.8-1.3)	1.1 (0.9-1.3)	>0.3	0.2	0.2

Paper IV

Comparisons of PRL and thyroid hormone levels at baseline

Basal PRL levels (Table 9) and PRL AUC (Fig. 9) were significantly lower in patients compared to matched controls of both gender.
Significantly higher serum free T_4 levels, but within the normal range, were recorded among ALL females, but not in ALL males compared to controls, but with no differences in basal TSH levels and free T_4 or T_3 levels compared to controls (Table 9). No significant differences in testosterone levels in men and estradiol levels in women were recorded between patients and controls (Table 9). Serum IGF-I levels were significantly reduced in ALL patients compared to matched controls of both gender.

Table 9. Serum hormone levels in 21 former ALL women and 23 former ALL men and matched controls.

	Patients Women Median (range) N=21	**Controls Women Median (range) N=21**	**P**	**Patients Men Median (range) N=23**	**Controls Men Median (range) N=23**	**P**
Age (yr)	25 (22-32)	26 (22-32)	---	25 (19-32)	25 (19-32)	---
BMI (kg/m^2)	26 (20-33)	21 (18-34)	0.02	24 (19-37)	23 (20-35)	0.1
Insulin (mIU/liter)	6.4 (2-31.5)	6 (2.4-9.7)	0.1	6.6 (1.9-27.9)	4.5 (2.3-15.7)	0.004
Prolactin (µg/liter)	7 (4-25)	18 (7-24)	<0.001	8 (2-18)	13 (7-31)	<0.001
IGF-I (µg/liter)	137 81-289)	196 (107-338)	0.03	159 (110-329)	191 (110-341)	0.04
TSH (mU/liter)	1.9 (0.6-4.0)	2.0 (0.7-5.3)	>0.3	2.1 (0.9-4.4)	1.9 (0.8-3.8)	0.2
free T4 (pmol/liter)	14 (11-17)	12 (10-16)	0.04	14 (12-20)	14 (12-20)	>0.3

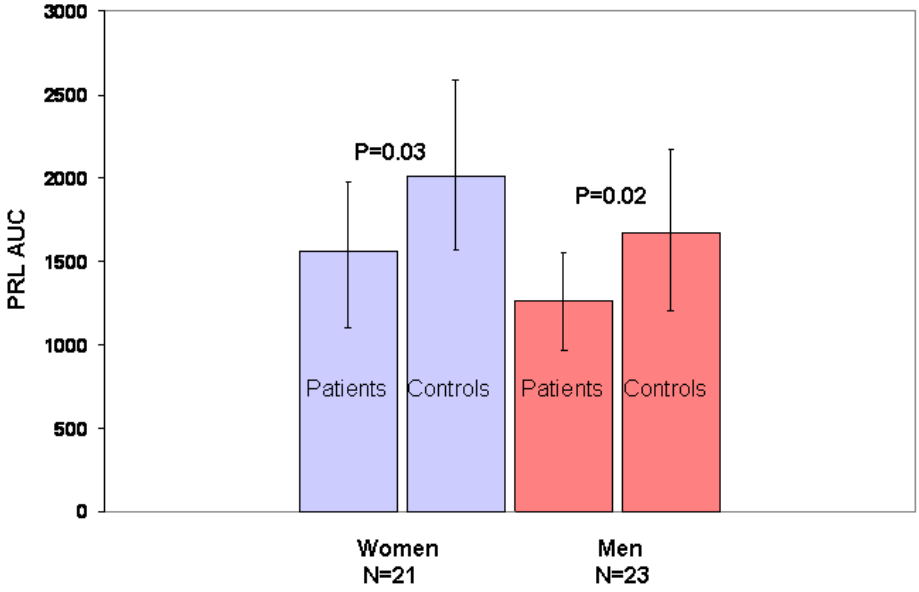
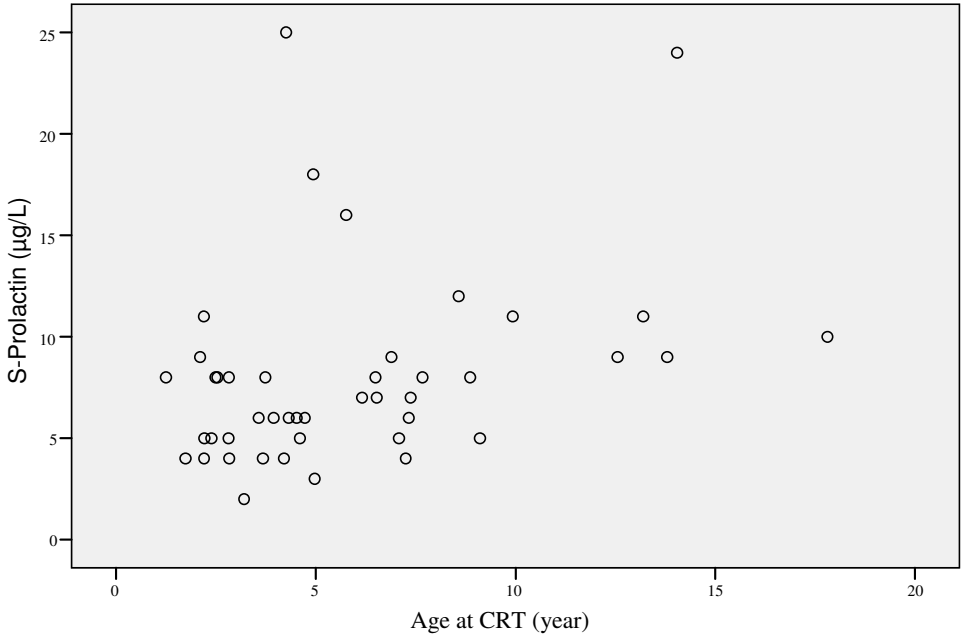


Figure 9. Prolactin area under the curve during GHRH-Arginine test in 21 former ALL women and 23 former ALL men and matched controls. Data presented as median and min-max.

Correlations with PRL at baseline


S-Prolactin (µg/L)
25
20
15
10
5
0
0
5
10
15
20
Age at CRT (year)

significant differences in these levels in comparison to controls after 5 years of GH therapy ($P < 0.3$) (data not shown).
No differences in testosterone or estradiol levels were recorded among patients after 5 years (data not shown).

Comparisons in basal PRL, thyroid hormone levels, sex steroids, and serum IGF-I before and after 8 years with GHD in 13 former ALL patients

The basal PRL levels decreased further and significantly after 8 years [9 µg/l (3-21) vs 6 µg/l (3-16), $P = 0.03$]. There was no significant difference in basal TSH levels (2.1 mU/l vs 2.1 mU/l, $P > 0.3$), but free T4 levels had increased significantly (13 pmol/l vs 15 pmol/l, $P = 0.01$) and free T3 levels decreased significantly (6.1 pmol/l vs 5.2 pmol/l, $P = 0.03$), but with no significant differences in these levels compared to controls after 8 years ($P < 0.3$) (data not shown).
There were no significant differences in serum testosterone and estradiol levels after 8 years in ALL patients (data not shown). Serum IGF-I decreased from - 2.0 SD to – 3.2 SD, corresponding to a decrease by 30%.

Baseline and TRH stimulated TSH and PRL AUCs in 13 GH deficient ALL patients, and correlations between basal PRL or PRL AUC and serum IGF-I

Compared with controls the subgroup of 13 patients had significantly lower PRL AUC after TRH ($P=0.001$, Fig. 11a).
There were significantly positive correlations between both basal serum PRL and

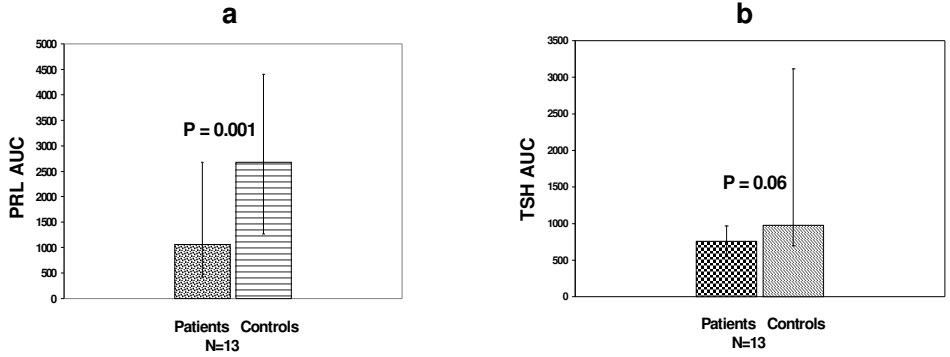


Figure 11. **a)** Prolactin (Prl AUC) and **b)** TSH (TSH AUC) area under the curve during TRH test in 13 former ALL patients (4 women) and 13 matched controls. Data are presented as median and min-max.

Effects of 1 year of GH therapy on TRH stimulated TSH and PRL AUCs in 8 GHD ALL patients (GH-group II)

The TRH stimulated PRL AUC [1975 (652-2677) vs 1837 (692-2350), $P > 0.3$] and TSH AUC (755 vs 607, $P > 0.3$) did not change significantly after one year of GH therapy.

Discussion

GH therapy in adult ALL survivors

Previously, positive effects of GH therapy on cardiovascular risk factors and bone mineral density among non-ALL GH-deficient patients has been demonstrated. But it remains to be confirmed among adult ALL survivors. The increase in cardiovascular risk factors and prevalence of metabolic syndrome (Link *et al.* 2004) in this young cohort of ALL is of great concern. This prospective, observational study is the first one that has determined the short- and longterm effects of GH therapy in adult survivors of ALL with confirmed GHD. Thus, our intention in this thesis was to start treating a small subgroup of ALL patients as it was not without controversy in year 2000 to treat with a drug with a potential mutagenic effect, as GH therapy in childhood cancer survivors showed a relative risk of a second neoplasia (Sklar *et al.* 2002). In an extended analysis of 32 months of GH therapy, the risk for a second neoplasm was attributable to meningiomas and when this tumor was excluded the risk for a second neoplasm was not significantly increased, even though a true biological effect of GH on the progression of the meningiomas could not be excluded (Ergun-Longmire *et al.* 2006). Another intention was to use a moderate GH dose, aiming at a serum IGF-I level of ± 0 SDS, as antracycline-treated childhood cancer survivors have exhibited significant cardiac deterioration during GH therapy (Lipshultz *et al.* 1989). In addition, it is important to use a low dose of GH in order to avoid negative effects on glucose metabolism, as GH treatment to GHD patients have been reported to detoriate insulinsensitivity (Fowelin *et al.* 1993).

The clustering of metabolic risk factors is well recognized but the value of classifying the Mets as a predictor of cardiovascular risk is unclear (Sattar *et al.* 2008). Thus, it has been shown that the Mets is associated with a double increased risk for cardiovascular events (Garmi *et al.* 2007). So far, most studies of former ALL patients have focused on one or more components of the Mets and reports of the syndrome as a whole is limited (Talvensaari *et al.* 1996). Mets is associated with signs of early atherosclerosis and may represent the connection to the increased long-term risk of cardiovascular disease. In addition, information of treatment options are lacking, as there are no special guidelines for treatment of Mets in survivors of cancer. The increased prevalence of Mets which was reduced with GH therapy in *paper I and II* is an important finding, and might be a way to reduce cardiovascular events in the future. In the general population the risk for the MetS is in part explained by reduced physical activity. CRT-treated ALL children have a higher risk of obesity and reduced physical activity compared to a group of chemotherapy-treated patients (Mayer *et al.* 2000) and are more likely to report inactivity during leisure-time than the general population (Florin *et al.* 2007). In contrast, we recorded increased physical activity during work-time in the untreated ALL group (*paper II*). GH therapy may increase the spontaneous physical activity, due to improved health-related quality of life, leading to improved CV risk factors.

The reduction in plasma glucose after one and five years of GH therapy (*paper I , II*) is surprising as previous studies in non-ALL GH-deficient patients have shown increased blood glucose and reduced insulin sensitivity (Nörrelund *et al.* 2000, Maison *et al.* 2004) and fasting glucose increases with age in normal subjects (Eliasson *et al.* 2002). A possible explanation is the moderate GH dose, used in the present studies, which tend not to deteriorate insulin sensitivity in GHD patients

therapy in anthracycline-treated ALL survivors. Lipshultz *et al.* (2005) recorded an increased LV wall thickening, but no effect on the LV dysfunction after 4 years of GH therapy in ALL children. The patients in *paper II* had a significantly lower LV-function compared to controls after 5 years, at least presented as group data. For each individual we observed a variety of cardiac outcomes as deterioration, no deterioration or even improvement. GH therapy in non-ALL patients have also shown marked short term improvement in cardiac function (Cittadini *et al.* 1994, Cuocolo *et al.* 1995). Colao *et al* (1995) found an increased EF in CO GHD adults after 12 months of GH therapy, but this improvement remained abnormal compared to matched controls. Other treatments, as angiotensin-converting enzyme inhibitors (Enalapril®) has been used in ALL children, between 6 and 10 years of age, but again with a transient effect on LV dysfunction (Lipshultz *et al.* 2002).
Instead of GH therapy other cardio protective drugs as stattins is an option, but in this young age side effects and fertility issues are a concern. Only GH therapy has the potential to increase lean mass and aerobic exercise capacity, together with favourable metabolic effects (Molitch *et al.* 2006).

Bone mineral density in ALL survivors

Paper II shows that former ALL patients have, 20 years since CRT treatment, no significant difference in BMD, BMAD or expressed as Z-scores at femoral neck or L2-L4 in comparison to matched population controls. But after an additional 8 years, a subgroup of the ALL patients were found to have a decrease in both BMD at L2-L4, and particularly Z-scores at femoral neck. It is well-known that low BMD is related to increased fracture risk (Melton *et al.* 1993) and a decrease in BMD may increase long-term morbidity by increasing the risk of fracture at a

GHD is probably a state of low bone turnover (Bravenboer *et al.* 1996) and a reduction in markers of bone turnover was also seen after 8 years in ALL patients, which coheres with the finding that patients with the most profound GHD also experienced the most depressed bone markers (Coalo *et al*. 1999).
Paper III is the longest follow up of bone mass and metabolism in irradiated ALL patients and it is also the first to include proper controls and to control for variables as, physical activity, calcium intake and all other hormones of importance for bone health.
Failure to reach an optimal peak bone mass (PBM) may increase the risk of osteoporosis and fracture as an older adult, although this is unproven. Children with GHD might need GH therapy during the transition to adulthood to reach their PBM and prevent osteoporosis and it has been shown that ALL survivors who had received GH therapy during childhood had a BMD not different from the control group (Nussey *et al*. 1994). The ALL patients in paper III are GH-naïve and have probably changed from partial GHD to more severely GHD later in life. Thus, there are several reasons for the decrease in Z-scores during GH therapy shown in paper III; no recent GH therapy, too low GH dose (females) and a start of GH therapy at 25 years might also be somewhat late to improve BMD and PBM. However, besides GHD, other unknown reasons are possible. The present patient population had only dropped to Z-scores at -0.6 SD, which it not low, and does not need immediate intervention, but it occurred during 8 years of follow up when PBM is attained. But interference with attainment of PBM is perhaps the most important determinant of lifelong skeletal health, which means that continuous surveillance is important.

A further causative factor to low BMD is reduced physical activity, shown among ALL children (Warner *et al*. 1998). Link *et al.* (2004) have shown that adult ALL patients experience no difference in the degree of physical exercise during spare

ALL disease and treatment, one option is to use the QCT to investigate cortical and trabecular bone as two different entities.
It is reasonable to believe that the consequence of the ALL disease itself and its treatment, particularly MTX therapy, together with the aggravating state of GHD, during a sensitive period of bone formation, have caused the decrease in Z-scores, at 31 to 33 years of age. Besides treatment of GHD and hypogonadism, intervention to avoid smoking and alcohol and professional advice concerning nutrition and physical activity is of pertinent importance. Further studies are needed to fully understand the reason for the decrease in Z-scores and reduced markers of bone formation. Whether higher GH doses particularly in women, will normalize Z-scores needs further investigation.

Prolactin and TSH

GH deficiency and preccocious puberty are the most common central endocrinopathies observed in ALL survivors. ALL treated with conventional CRT doses do not usually develop other central endocrinopathies such as adrenal insufficiency, gonadotropin insufficiency or central hypothyroidism, as these are associated with higher doses.
An association between PRL insufficiency and CRT-treatment in adult patients with GHD and without evidence of multiple hormone deficiency has not been shown previously. In rats exposed to CRT, GH and PRL were shown to be the most sensitive of all pituitary hormones, and GH and PRL levels decreased dramatically with time and dose after irradiation followed by TSH as the next most sensitive hormone (Robinson *et al*. 2001).
In paper IV we show for the first time that CRT treated long-term survivors of ALL are PRL insufficient 20 years after diagnosis, and we report a progressive

PRL deficiency among the patients treated with CRT after surgery for a pituitary adenoma

Recently it was reported that former ALL patients experienced insufficient breast-feeding, but with no information about serum PRL levels or of exact GH status, but they postulated that GHD may bee a causal factor (Johnston et al. 2007). In our own clinic we experienced that out of 7 pregnant ALL women with GHD, only one could breast feed.

Good clinical data concerning the effect of PRL deficiency are lacking, and the role of this hormone, except for the role of lactation in woman, remains uncertain. PRL is a multifunctional pituitary hormone and PRL receptors are expressed in nearly all organs. Some reports in humans show that PRL may play a role in a number of autoimmune processes (Reber *et al.* 1993) and hypo-prolactinemia is a riskfactor in children with nosocomial sepsis and multiple organ failure (Felmet *et al*. 2005). Further, PRL seem to be a metabolic hormone and regulates enzymes and transporters that are associated with glucose and lipid metabolism (Ben-Jonathan *et al.*2006). In addition, it has been shown that PRL inhibits adiponectin production in human adipose tissue and in mice (Nilsson *et al*. 2005), which indicates its potential involvement in the manifestation of insulin resistance. This is in accordance with the negative correlations between PRL AUC after GHRH-Arginine and basal insulin and BMI, shown in paper IV. Also Weaver *et al.* (1991) showed a relation between insulin resistance/secretion and impaired PRL and GH secretion in obese women. Furthermore, in subjects with sexual dysfunction PRL deficiency was associated with the metabolic syndrome and anxiety symptoms (Corona *et al*. 2009).

After 28 years since diagnosis there was evidence of a decrease in TRH stimulated TSH stimulation, but without any clear changes in basal TSH or free thyroid hormone levels, indicating normal thyroid function. GH has a regulatory effect on several hormonal systems e.g. the thyroid hormone metabolism, with both a

might explain the incapacity of breast feeding. We found a further decrease in basal PRL levels after another 5 and 8 years irrespective of GH therapy. In addition, we found a lower AUC TSH after TRH stimulation compared with controls, reflecting a CRT-induced progressive damage. Since radiation-induced pituitary damage is both dose-and time dependent, the ALL patients should be monitored clinically for symptoms of these disorders and tested.

Conclusions and perspectives for the future

It is now more than 20 years since recombinant GH became available, which made it possible to study the effect of GH therapy also in adult life. Since then there have been numerous studies showing that GH therapy affects body composition, bone mass and cardiovascular risk factors in patients with GHD of other causes than ALL. We are the first to treat a homogenous group of adult ALL survivors with GH and to study the effect on CV risk factors, BMD and other pituitary hormone deficiencies than GHD. In addition, there are no other published observational studies with respect to the effect of GH in other adult cancer survivors.

Both short - and long-term GH therapy was found to decrease the prevalence of the metabolic syndrome among ALL survivors, while in non-GH-treated ALL patients with GHD the prevalence increased. The improvement of cardiac systolic function after 2 years of GH was not maintained after 5 years of GH therapy. On the other hand, we found no clear deterioration which has been shown after treatment with anthracycline as this drug has a progressive negative effect on the heart. In this young cohort with an increased risk of adverse late complications which may result in future cardiovascular events, we found GH therapy beneficial and safe throughout a rather long period of time. The effect of GH therapy on endpoints such as cardiovascular morbidity and mortality have to be investigated in the future, as cardiovascular events are the leading non-malignant cause of

ALL survivors have PRL insufficiency 20 years after treatment, with a further decrease in basal PRL levels after another 5 to 8 years. This explains the very high percentage of ALL women, who had suffered from insufficient breast feeding. The results demonstrate a CRT-induced progressive effect on lactotroph function. This irreversible and progressive nature of radiation-induced pituitary hormone deficiencies high-lights the importance, that ALL survivors are regularly tested to ensure appropriate diagnosis and timely hormone replacement therapy. In a report from the childhood cancer survivor study, young adult survivors of ALL were more than three times as likely as sibling controls to have a chronic endocrine condition (Mody *et al.* 2008). Because of the fact that many treatment-related late complications may not become clinically apparent until the survivor gets older, long-term surveillance is required and an imperative concern is that patients are not lost in the transition from pediatric to adult clinics.

Conclusions

- 24 months of GH replacement showed improvement in cardiac systolic function and reduced prevalence of metabolic syndrome in adult GHD survivors of CO ALL. This suggests that two years of GH treatment reverses some but not all cardiovascular risk factors in former CO ALL patients with GHD.

- Five years with a low GH dose reduces the prevalence of MetS, together with favourable effect in plasma glucose and lipid levels, but does not result in benefits or in deterioration of cardiac function among anthracycline-treated former ALL patients. This indicates a reduced risk of future cardiovascular events in this young population while the cardiac function needs long-term attention and other treatment strategies.

- On average 25 years since diagnosis GH deficient ALL patients experienced a significant decrease in Z-scores at femoral neck and L2-L4, which means a future premature risk for osteoporosis. Whether higher GH doses, particularly in women, will improve Z-scores needs further investigation.

- ALL survivors treated with CRT were PRL insufficient 20 years after diagnosis, which explains the incapacity of breast feeding. During follow

Populärvetenskaplig sammanfattning

Endokrinologi kommer från grekiskan och betyder läran om de inre sekretoriska systemen. Hormoner är substanser som hämmar eller stimulerar cellernas olika arbeten. Hypofysen, som styr hormonproduktionen i kroppen, är ca 15 x 10 x 6 mm, väger 500-900 mg och är placerad strax under hjärnan. Två tredjedelar av hypofysen utgörs av framloben och en tredjedel av bakloben. Tillväxthormon (GH) insöndras stötvis från hypofysen och utgör den största andelen av hormonproducerande celler i framloben. Insöndringen är högst på natten och de högsta nivåerna ses under puberteten och sjunker därefter linjärt från 20 års ålder till livets slut. GH-produktionen är ca 3 ggr större hos kvinnor i fertil ålder än hos män. GH är nödvändigt för normal längdtillväxt under barndomen.

Orsaken till hypofyssvikt är vanligtvis godartade tumörer i hypofys och dess behandling. Strålbehandling på grund av maligniteter i barndomen är en allt vanligare orsak till GH-brist. De celler som först brukar svikta är GH-celler följt av luteiniserande hormon, follikelstimulerande hormon (LH och FSH, styr könshormonproduktion), tyroideastimulerande hormon (TSH, styr ämnesomsättningen), adenocorticotropt hormon (ACTH, styr kortisolproduktionen) och prolaktin (har betydelse för bröstutveckling och amning). GH-brist hos vuxna ger ospecifika symtom som ökad fettansättning, minskad muskelmassa, tunn krackelerad hud, nedsatt livskvalité, trötthet. På 50- och 60-talet utvanns GH ur mänskliga hypofyser och behandling gavs enbart för att öka barns längdtillväxt. Sedan mitten på 80-talet framställs GH syntetiskt och idag behandlar man även vuxna.

Denna avhandling baseras på en grupp unga vuxna som behandlats för akut lymfatisk leukemi (ALL) i barndomen med strålbehandling mot skalle och cytostatika och som har verifierad GH-brist. Målet med studierna var att

länder. Det är därför viktigt att studera seneffekter efter ALL och utvärdera behandlingsmöjligheter för dem som är överlevare.

Hjärt-kärlsjukdomar är den största icke-maligna dödsorsaken bland patienter som överlevt cancer i barndomen. I de två första arbetena undersökte vi om GH har någon effekt på riskfaktorer för hjärt-kärlsjukdomar hos ALL patienter som har GH-brist. Blodprover för kontroll av blodsocker, insulin, leptin och olika sorters kolesterol utfördes Vi mätte hur mycket fett och fettfri massa patienterna hade samt midja-höftmått. Hjärtfunktionen undersöktes med ultra-ljud. Andelen patienter som hade det metabola syndromet noterades. Varje patient jämfördes med friska matchade kontrollpersoner från den allmänna befolkningen både före och efter 5 år. GH-behandlingen utvärderades efter 2 och 5 år. De patienter som varit GH-behandlade i 5 år jämfördes dessutom med en grupp ALL patienter som haft GH-brist i 8 år men som inte fått GH-behandling.

Efter 2 år förbättrades en del riskfaktorer samt andelen patienter som hade det metabola syndromet minskade. Hjärtats systoliska funktion förbättrades. Efter 5 år hade andelen patienter som hade det metabola syndromet minskat, medan det ökat bland de som haft GH-brist i 8 år. Hjärtfunktionen förbättrades inte hos dem som fått GH, men den blev ej heller sämre hos de icke-GH-behandlade gruppen.

Vår slutsats är att den minskade prevalensen av metabola syndromet tillsammans med förbättrade blodsocker-nivåer och blodfetter kan leda till mindre risk för framtida hjärtkärlsjukdomar. När det gäller hjärtfunktionen så behövs regelbunden kontroll och eventuell annan behandling.

I delarbete III var ett mål att undersöka benmineralmängd med hjälp av DXA (lågdos röntgen av skelettet) och jämföra resultatet med friska kontroller. Ett annat mål med studien var att undersöka effekten av GH-behandling efter 5 år hos ALL och jämföra med friska kontroller samt en grupp som ej varit GH-behandlade på samma sätt som i studie II. Patienterna hade normalt BMD jämfört med friska kontroller vid inklusionen. Kvinnorna i den GH-behandlade gruppen och

Vi fann att ALL hade signifikant lägre basala och stimulerade prolaktinnivåer jämfört med kontrollerna. Bland de 13 patienterna fann vi också signifikant lägre stimulerade prolaktinnivåer jämfört med kontroller. Det fanns en tendens till lägre TSH hos ALL jämfört med kontrollerna, men det nådde inte signifikans. Prolaktinnivåerna sjönk hos ALL oavsett om de var GH-behandlade eller ej.
ALL har 20 år efter diagnos prolaktinsvikt, vilket kan förklara deras oförmåga att amma. Efter ytterligare 5 till 8 år sjönk prolaktinnivåerna ytterligare, vilket pekar på en progressiv strålningseffekt på hypofysen.
GH-behandling av ALL är säker och har en positiv effekt på riskfaktorer för hjärt-kärlsjukdom. Vi fann ingen säker positiv effekt på hjärtfunktion, benmineralmängd eller prolaktinnivåer.

Acknowledgements

I have ambivalent feelings in expressing my sincere gratitude in English (why hide behind a language you don't really master?). I think the Swedish language is well suited for giving well deserved thanks to all these people who have been important to me during my time as a PhD-student. Therefore I will continue writing this part in Swedish. If you don`t understand this part, don´t worry, the Swedish language is not a big deal anyway.

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