Endocrine Effects of the Treatment for Acute Lymphoblastic Leukemia and Hodgkin's Lymphoma in Childhood

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Endocrine Effects of the Treatment for Acute Lymphoblastic Leukemia and Hodgkin's Lymphoma in Childhood

Endocriene effecten van de behandeling voor acute lymfatische leukemie en Hodgkin lymfoom op de kinderleeftijd

Proefschrift

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Voor Monique
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Contents

Chapter 1

General Introduction

General Introduction

1.1 Acute Lymphoblastic Leukemia (ALL)

One quarter of all cases of pediatric malignancies is acute Lymphoblastic leukemia (ALL). Per year approximately 4 in 100.000 children are diagnosed with ALL. The disease has a peak incidence between the third and sixth year of life. Predisposing factors for ALL are Down syndrome, Fanconi anemia, Bloom syndrome, ataxia teleangiectasia and some immune disorders [1-3], but these represent a very small minority of cases.

In the last decades survival of childhood cancer improved significantly. Because of this, the side effects of treatment, both during and after therapy become increasingly important. The 5-year survival rates of acute Lymphoblastic leukemia (ALL) increased to 80% in the last years [3-6].

Since the mid-seventies, in the Netherlands children with ALL are treated uniformly, according to national protocols of the Dutch Childhood Oncology Group (DCOG). Early protocols were prednisolone based and used cranial radiotherapy (CRT) as central nervous system (CNS) prophylaxis. The dexamethasone based DCOG ALL-6 protocol in the mid-eighties was the first protocol that did not use CRT as CNS prophylaxis. The ALL-7 and ALL-8 protocols were prednisolone-based protocols, similar to the BFM-protocols used in Germany. In this thesis most children were treated according to the DCOG ALL-9 protocol, based on the previous, dexamethasone based, ALL-6 protocol, which consisted of an induction, CNS prophylaxis, consolidation (high risk only) and maintenance phase, for a total of 109 weeks. Patients with peripheral white blood cell counts over 50 x 10^9/l, T-cell phenotype and/or mediastinal mass, extramedullary leukemia, patients with t(9;22), 11q23 with MLL gene rearrangements and non-responders to induction chemotherapy, were stratified to a high risk (HR) treatment schedule. Patients in the HR group received an intensive consolidation phase after induction. CNS prophylaxis consisted of recurrent intrathecal triple therapy with prednisolone, methotrexate (MTX) and cytarabine (Ara-C) combined with post remission MTX. Total cumulative doses of the chemotherapeutic agents used in the high risk and non-high risk (NHR) protocol are shown in table 1.

1.2 Osteogenic problems in ALL

1.2.1 Osteoporosis and fracture risk

One of the most important side effects of the current treatment protocols for pediatric ALL is osteoporosis. Due to the high cumulative dose of corticosteroids, bone mineral density (BMD) decreases during treatment. Osteoporosis is characterized by low bone mass and microarchitectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture. The definitions of osteopenia and osteoporosis in adults are based on T-scores, which compare the measured BMD with the BMD of young adults. Osteopenia is defined as a T-score between -1 and -2.5, and osteoporosis as a T-score below -2.5. In children, a T-score is not useful and age- and sex-adjusted Z-scores or standard deviation scores (SDS) are required. So far, there is still no consensus on the definitions of osteopenia and osteoporosis in children [7].

Table 1 Total cumulative doses of chemotherapeutic agents used in NHR and HR DCOG ALL-9 protocol

	NHR	HR
DEXA (mg/m^2) p.o.	1370	1244
MTX (mg/m^2) p.o. and i.v.	8100	13650
VCR (mg/m^2) i.v.	68	62
L-ASP (U/m^2) i.v.	24000	33000
6-MP (mg/m^2) p.o.	24500	24500
ARA-C (mg/m^2) i.v.	-	1920
DNR (mg/m^2) i.v.	-	175
CP (mg/m^2) i.v.	-	1920

DEXA = dexamethasone; MTX = methotrexate; VCR = vincristine; L-ASP = L-Asparaginase; Ara-C = Cytarabine; DNR = daunomycine; CP = cyclophosphaminde.

Prospective studies showed that during treatment for pediatric ALL BMD is already lower at diagnosis as compared to healthy children and continues to decrease during therapy [8, 9]. These studies also showed that the fracture risk

during and one year after cessation of therapy was more than six times higher in children treated for ALL as compared to healthy children [8].

In adults and children a reduction of one standard deviation in bone density is associated with at least a doubling of the fracture risk [10]. 14–39% of children with ALL sustain fractures, which can occur at presentation, during or after therapy [8, 11-13]. Pediatric ALL patients with fractures do not seem to differ in bone density from children without a fracture. The decrease in lumbar spine BMD seems to be a more important determinant of fractures than the absolute value of BMD [8, 11].

Several studies described long-term follow-up of ALL survivors. In general, normal to reduced BMD [12, 14-20] and elevated body fat [21, 22] were reported (table 2). Dexamethasone is associated with a higher incidence of side effects than prednisolone [23-25]. Until now it is unclear whether long-term side effects of dexamethasone differ from those of prednisolone in childhood ALL.

1.2.2 Avascular necrosis (AVN)

Avascular necrosis of the bone (AVN) or osteonecrosis is a potentially disabling complication of the treatment of childhood ALL. The reported incidence of symptomatic AVN in pediatric ALL is 4-12.5% with a higher incidence in children older than 10 years at diagnosis [25-28]. AVN mostly affects the weight bearing joints resulting in progressive joint damage, sometimes necessitating total joint replacement. Symptoms consist of pain, limited range of motion, limping, joint destruction following bone collapse and arthritis [29]. This complication mimics the familial occurrence of bone marrow edema syndrome (early phase of AVN) which has been associated with elevated levels of Lipoprotein(a) [Lp(a)] [30]. In children Lp(a) over-expression has also been reported to be associated with venous thrombosis [31, 32] and with Legg-Perthes disease [33]. So far the role of LP(a) in the occurrence of AVN as a complication of therapy for childhood ALL is unknown.

1.3 Determinants of osteogenic side effects in ALL

As bone mass is acquired during childhood and adolescence, disturbance of this process can result in a lower peak bone mass in later life resulting in osteoporosis. BMD is determined by several factors, like gender, race, physical activity, calcium intake, smoking and alcohol consumption [34]. In girls, pubertal stage is the most

Table 2. Studies on growth, bone mineral density and body composition in childhood ALL survivors

Author	*N* [a]	*CRT* [b]	*Age F-up (range) yrs* [c]	*F-up (range) yrs* [d]	*Growth*	*Long term effect on Bone mineral density*	*Body composition*
Brennan *et al.* [16]	31	31	23 (18.8 - 33.0)	17.8 (6.8 - 28.6)	↓	↓	-
Gilsanz *et al.* [17]	42	30	11.8 (0.5)*	3.5 (0.5-8.2)	↓	↓ after CRT	-
Jarfelt *et al.* [20]	35	19	(20-32)	> 10	↓ after CRT	n	↑ BMI in males after CRT
Arikoski *et al.* [14]	29	20	17 (12 - 30)	8 (2 - 20)	n	↓in males after CRT	-
Thomas *et al.* [19]	74	49	30 (7.2)*	24.4 (4.8)*	↓ in males	n	n
Warner *et al.* [22]	35	35	-	6.6 (3.3)*	n	-	↑ percentage fat in females
Van der Sluis *et al.* [18]	23	0	17.2 (12.2 - 25.4)	9.6 (7.9 - 11.4)	n	n	n
Marinovic *et al.* [12]	29	0	8.9	2.2 (0.1 - 3.1)	n	Higher increase in BMD compared with healthy controls	n
Brennan *et al.* [15]	53	0	11.2 (6.4 - 17.5)	4.6 (1.2 - 8.3)	normal	n	↑ BMI

*- = information not available; F-up = follow-up; ↓ decreased as compared to healthy controls; ↑ increased as compared to healthy controls ; yrs = years; n = normal. a) number of survivors; b) CRT = number of patients that received cranial adiotherapy; c) age at follow-up (median and range). * = mean (SD); d) median follow-up time (median and range). * = mean (SD)*

important determinant of BMD, whereas in boys weight is the most important determinant [35].

The fact that uniformly treated children show a large variety in reduction of BMD and subsequent problems like fractures [8], suggests a genetic variation in factors that influence these problems. It has been estimated that up to 75% of the variation in BMD is genetically determined [36, 37]. Over the last years several genes and polymorphisms have been associated with BMD.

Polymorphisms of the vitamin D receptor gene (*VDR*) are among the most frequently studied polymorphisms associated with BMD in adult populations, but results are conflicting [38-41]. A cluster of linked sites near exon 9 and the 3' UTR (untranslated region) [42] or polymorphisms in the binding sites of the Cdx-2 and GATA transcription factors in the 5'-promotor region of the VDR are reported to be associated with a lower BMD in elderly women [41, 43]. The effects of 3'-polymorphisms of *VDR* on BMD have been reported in only a few pediatric studies, with conflicting results [44-47], whereas no studies exist on the effects of the 5'-polymorphisms on BMD in children.

Another polymorphism frequently investigated in association with osteoporosis is a G to T substitution in the Sp1 binding site of the collagen type Iα1 gene (*COLIA1*). Most studies have been performed in elderly women [48-50]. Results from studies in children are conflicting. Some did found a relation between bone mass and *COLIA1* [51], where others did not [52].

Two polymorphisms in the first intron of the gene at the 5'-end of the estrogen receptor alpha gene (*ESR1*) influence BMD in postmenopausal women [53-55] and response to hormone replacement therapy [55-57], although other studies could not replicate these results [58-60]. Only one study has been performed in healthy children in which haplotype 1 of the polymorphism was associated with lower lumbar spine BMD [61].

In healthy adults polymorphisms in the glucocorticoid receptor gene (GR) gene are associated with a lower BMD, like the BclI polymorphism and the N363S [62]. Several polymorphisms in GR are known to modulate glucocorticoid sensitivity [63-65], and thus might be responsible for the variation in bone density in pediatric ALL patients treated with long term and high dose corticosteroids. There are currently no studies that report on GR polymorphisms and BMD in children treated for ALL.

Lp(a) is a complex of low-density lipoprotein (LDL) and a high molecular weight glycoprotein called apolipoprotein(a) [Apo(a)]. Plasma Lp(a) concentration

shows wide quantitative variation among individuals. This variation in concentration of Lp(a) is inheritable and inversely related to the number of kringle IV repeats in the gene for apo(a) (*LPA*) [66, 67]. This gene is located on chromosome 6 [68]. The size of the Apo(a) protein is determined by the number of repeats of kringle IV type 2 in the *LPA* gene and the variability in apo(a) size effects the plasma concentration of Lp(a) [66]. High Lp(a) levels are associated with familiar AVN. In cardiovascular diseases Lp(a) levels are determined by the number of kringle IV repeats in the *LPA* gene. The influence of lipid profiles or *LPA* kringle IV repeats on the occurrence of AVN in pediatric ALL has not been investigated.

Relling *et al.* investigated the role of other genetic polymorphisms associated with AVN in pediatric ALL. Among 16 single nucleotide polymorphisms (SNPs), she was able to show that only polymorphisms in the vitamin D receptor and thymidylate synthase are independent predictors for osteonecrosis [69]. Also, other polymorphisms may play a role in the development of AVN, like polymorphisms in the folate pathway (e.g. methylenentetrahydrofolate reductase; [70]) and polymorphisms in cytochrome P_{450} [71].

1.4 Hodgkin's Lymphoma (HL)

Hodgkin's lymphoma (HL) was first described in 1832 and has two incidence peaks in age distribution. The first peak is between the ages of 15 and 30 years and the second is between 45 and 55 years. HL is very rare in children under 15 years of age (incidence $0.6/10^6$) [72, 73].

The treatment of pediatric HL consists of radiotherapy, chemotherapy or a combination of both. Most pediatric oncology centers in the world use a treatment schedule consisting of both chemotherapy and radiotherapy. In Rotterdam (Erasmus MC – Sophia Children's Hospital) and Amsterdam (Academic Medical Center) since 1985, pediatric HL has been treated with a protocol in which only chemotherapy was used. Pediatric HL has a very good prognosis: an event free survival (EFS) up to 93% and an overall survival up to 96% has been reported [74-79]**.** Because of the improved survival rates, long-term side effects after treatment gain importance. Both chemotherapy and radiotherapy

have serious potential side effects, especially when used in children. In general, long-term side effects of chemotherapy are related to dose and to the kind of chemotherapy (e.g. alkylating agents, anthracyclines), whereas the toxicity of radiotherapy is related to dose, fractionating and extent of the irradiation field.

Potential long term effects of the treatment for HL are endocrine disorders [80, 81], secondary malignancies [82-88], heart failure [86, 89-91] and impairment of pulmonary function [86, 92]. Most studies on long-term effects of disease and treatment describe adult populations. Studies in childhood survivors are dominated by late effects due to radiotherapy. So far endocrine studies in children with HL treated with chemotherapy only are not available.

1.5 Endocrine late effects of treatment for childhood HL

This thesis focuses on chemotherapy induced endocrine late effects on growth, bones, body composition, thyroid and gonads.

1.5.1 Growth, bones and body composition

Reduced growth in children during treatment for HL is caused by the disease related morbidity, such as recurring infections, an increase in nutritional requirements, malnutrition during treatment and treatment itself (both chemotherapy and radiotherapy) [93-95].

Irradiation of parts of the spine contributes to poor growth by decreasing the growth of individual bones of the spine. Chemotherapy induced growth impairment can be caused by disturbance in growth hormone secretion [95] or by direct interference with bone growth [96]. This may result in impaired final height, but also in disproportional growth. Most of the loss in height after radiotherapy and chemotherapy is due to loss in sitting height [97]. This is not surprising, considering the fact that the spine contains a total of 48 growth plates [98]. Several treatment schedules for HL involve the use of high doses of corticosteroids, which cause osteopenia and osteoporosis [8, 18]. Corticosteroids interfere with both osteoblast and osteoclast function, resulting in increased in bone resorption.

Apart from these direct effects, there may be also indirect effects of chemotherapy on growth and BMD. Firstly, gonadal damage caused by therapy may result in lack of estrogens necessary for the pubertal growth spurt and increase of BMD during puberty in females, but also in males [99-104]. Secondly,

some chemotherapeutic agents might cause renal damage. This may result in dysregulation of the calcium and vitamin D metabolism resulting in lower BMD.

Only scarce data are available on body composition in survivors of childhood HL. Higher fat mass and body mass index (BMI) increases the risk of cardiovascular incidents and metabolic syndrome in later life [105, 106].

1.5.2 Thyroid

After radiotherapy of the cervical region a large proportion of the childhood HL survivors show disorders of the thyroid like hypothyroidism, thyroid nodules and thyroid cancer [89, 107-114]. For most of the protocols, the mean radiation dose to the thyroid was 35 Gy. Hypothyroidism is the most common thyroid problem after treatment for childhood HL. Up to 40% of the patients treated with radiotherapy during childhood had impaired thyroid function [108-110]. In patients under the age of 17, radiation dose was the most important risk factor for developing hypothyroidism [108]. Hyperthyroidism (mainly Graves' disease) may also occur after radiotherapy, although much less frequently than hypothyroidism [108, 110, 111]. Chemotherapy does not enhance the damage to the thyroid axis caused by radiotherapy, however studies in which children were not irradiated are scarce [115].

The risk of thyroid cancer after radiotherapy is up to 18 times higher than in the normal population [86, 108, 110]. In HL survivors treated during childhood with chemotherapy only, no cases of thyroid cancer have been reported, but cohort studies are still small and follow-up relatively short [74, 75].

1.5.3 Gonads

An important side effect of both radiotherapy and chemotherapy is gonadal dysfunction. This can result in reduced fertility and subsequent loss of bone mass. Azoospermia or oligospermia are potential long-term side effects in male childhood HL patients, especially when alkylating agents, e.g. mustine or procarbazine, are used [81, 116]. In female HL survivors both alkylating agents and abdominal radiotherapy can cause severe ovarian damage, eventually leading to premature ovarian failure (POF).

In male childhood HL survivors serum luteinizing hormone (LH) and follicle stimulating hormone (FSH) are generally higher after alkylating chemotherapy as compared to treatment without alkylating chemotherapy [76, 117, 118].

However, neither LH nor FSH are very sensitive predictors of the ovarian reserve [119].

Apart from LH and FSH, inhibin B and Anti-Müllerian hormone (AMH) have become available as markers for gonadal function. Inhibin B is produced by Sertoli cells in males and granulosa cells in females. It provides a negative feedback to FSH. Inhibin B is strongly correlated with sperm counts in both healthy and subfertile men [120-122]. This hormone is decreased in women with known fertility problems and undetectable in postmenopausal women [123-125]. Inhibin B is one of the first endocrine markers to change in perimenopausal women, even before changes in FSH levels can be detected [126]. AMH is produced by granulosa cells of early developing (pre-)antral follicles of the ovary, and levels decrease when the number of follicles decreases with age [127]. A recent study showed a strong correlation between age at menopause and AMH levels randomly measured during the reproductive lifespan in a group of healthy women [128]. In addition, recently AMH was shown to be a good predictor for the success of artificial reproductive technology [129]. So far, studies in which these markers were used for the assessment of gonadal damage in childhood HL patients treated with chemotherapy only are not available

1.6 Aims and outline of the thesis

This thesis describes endocrine effects of the treatment for pediatric ALL and HL. Growth, BMD and body composition have been evaluated in both groups of patients. Furthermore, avascular necrosis was studied in patients treated for pediatric ALL and gonadal and thyroid functions were studied in long-term survivors of pediatric HL.

The first group consist of patients treated for pediatric ALL. First aim of this study was to determine whether SNPs in genes associated with BMD and osteoporosis in adults, influence growth, BMD and body composition in pediatric ALL patients and possibly indentify the patients at highest risk for osteoporosis during treatment. Second aim was to determine whether polymorphisms in the gene for apo(a) could identify patients at risk for AVN during treatment of pediatric ALL. Third aim was to determine the long-term effects of treatment on growth, BMD and body composition in pediatric ALL.

The second group consists of pediatric HL survivors. Since most of the treatment protocols used in pediatric HL use radiotherapy in combination with chemotherapy, little data exists on the effects of chemotherapy alone. Aim of this study was to determine the long-term effects of chemotherapy only on growth, BMD, body composition, thyroid function and gonadal function in HL survivors.

The first part of this thesis describes studies in patients treated for pediatric ALL. **Chapter 2** reports on the effects of different genetic polymorphisms on growth, BMD and body composition during therapy and the first year after therapy in pediatric ALL patients. **Chapter 3** reports on the effects of apo(a) gene polymorphisms on the occurrence of AVN in these patients. **Chapter 4** shows the results of a long-term follow-up study of patients treated for pediatric ALL, in which prednisone-based therapies were compared to dexamethasone-based therapies.

The second part of this thesis describes the long-term follow-up studies in patients treated for pediatric HL with chemotherapy only. **Chapter 5** shows the results on BMD, body composition and growth in long-term survivors of childhood HL. In **chapter 6** the long-term effects on male gonadal function are reported, whereas in **chapter 7** long-term effects on female gonadal function are shown. **Chapter 8** reviews literature data on endocrine long-term effects of the treatment for pediatric HL patients. In **chapter 9** the results and clinical implications of this thesis and possible future research are discussed. Finally, **chapter 10** is a Dutch summary of this thesis.

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Part 1

Endocrine Effects of the Treatment for Childhood Acute Lymphoblastic Leukemia

Chapter 2

Pharmacogenetic Risk Factors for Altered Bone Mineral Density and Body Composition in Pediatric Acute Lymphoblastic Leukemia

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Abstract

Background. This study investigates pharmacogenetic risk factors for bone mineral (apparent) density (BM(A)D) and body composition in pediatric acute lymphoblastic leukemia (ALL).

Design and Methods. We determined the influence of SNPs in 4 genes (vitamin D receptor (*VDR: BsmI/ApaI/TaqI* and *Cdx-2/GATA*), collagen type I alpha 1 (*SpI*), estrogen receptor 1 (*ESR1: PvuII/XbaI*), glucocorticoid receptor (*BclI*)) on body composition, BM(A)D and fracture risk during dexamethasone-based pediatric ALL treatment. Body composition and BMD were measured repeatedly during and after treatment using dual energy x-ray absorptiometry.

Results. Non-carriers of *VDR* 5'-end (*Cdx-2/GATA*) haplotype 3 revealed a significant larger fat gain than carriers (Δ%fat: non -carriers: +1.76SDS, carriers: +0.77SDS, p<0.001). At diagnosis and during therapy, lumbar spine BMD was significant higher in non-carriers of *VDR* 5'-end (*Cdx-2/GATA*) haplotype 3 than in carriers. The other SNPs did not influence BMD or fracture risk during/after treatment. The year after treatment completion, lean body mass increased in non-carriers of *ESR1* (*PvuII/XbaI*) haplotype 3 and decreased in carriers (Δ lean body mass: non-carriers:+0.28SDS, carriers: -0.55SDS, p<0.01).

Conclusion. Only *VDR* 5'-end (*Cdx-2/GATA)* haplotype 3 was identified as protective factor against excessive fat gain and as a risk factor for lower lumbar spine BMD during treatment. Carrying *ESR1* (*PvuII/XbaI*) haplotype 3 negatively influenced recovery of lean body mass after pediatric ALL treatment.

Introduction

As the cure-rate of pediatric acute lymphoblastic leukemia (ALL) is high [1], research on treatment-related morbidity, like disturbance of body composition and bone mineral density (BMD), is required [2-4]. Leukemia and its treatment, especially involving corticosteroids [5] and methotrexate [6], may lead to reduced BMD. However, uniformly treated children show a large variation in disturbance of body composition, BMD reduction and fractures, suggesting a role for pharmacogenetics in the pathogenesis of these problems [4].

Several single nucleotide polymorphisms (SNPs) have been shown to influence BMD in adults, especially those of the vitamin D receptor gene (*VDR*) [7-12]. The extent of the influence of *VDR* SNPs on BMD may be dependent on age

and menopausal state [13]. In healthy children, only a few studies on the influence of *VDR* 3'-end SNPs (*BsmI*, *ApaI, TaqI*) on BMD have been reported, with conflicting results [14-17]. Effects of *VDR* 5'-promoter SNPs (*Cdx-2, GATA*) on BMD have not been investigated in healthy children. With regards to body composition (muscle strength and fat mass), it has been demonstrated that the *VDR BsmI* SNP determines body composition in premenopausal women [18].

Another polymorphism frequently reported to be associated with a low BMD for chronological age is the G→T substitution in the *Sp1* binding site of the collagen type I alpha 1 gene (*COLIA1*). This can result in increased expression of collagen type I alpha 1 in the bone matrix [7, 19-21]. Studies regarding the relationship between BMD and carrying *COLIA1* risk alleles in healthy children show conflicting results [22-25].

Haplotypes of the 5'-end of the estrogen receptor alpha gene (*ESR1)* in which the risk alleles of the *PvuI* and *XbaII* SNPs are combined, are associated with decreased BMD and fractures in postmenopausal women [26-28]. Only a few studies in healthy children have been carried out, showing conflicting results of the influence of *ESR1* risk alleles on BMD [25, 29]. On the other hand, the *PvuII* and *XbaI SNPs* are not related to body composition in healthy children [30].

In healthy adults, polymorphisms in the glucocorticoid receptor gene (*GR*), like *BclI* and *N363S*, have been suggested to modulate corticosteroid sensitivity [31, 32]. This in turn could result in reduced BMD [32, 33] and disturbed body composition [31, 34]. Since corticosteroids are considered to cause altered body composition and reduced BMD, we hypothesize that *GR* SNPs may influence variation in body composition and BMD in pediatric ALL.

To our knowledge this is the first study investigating the influence of genetic variation of the *VDR*, *COLIA1*, *ESR1* and *GR* on BMD, body composition and fracture risk in pediatric ALL patients during and after therapy. The aim of this study is to identify patients at risk for a low BMD for chronological age and a disturbed body composition, in order to develop early preventative interventions.

Design and Methods

Patients

In this prospective study, children with newly diagnosed ALL were treated according to the dexamethasone-based protocol of the Dutch Childhood Oncology Group (DCOG-ALL9) [35]. High-risk criteria were white blood cell count $\geq 50 \times 10^9$/L,

T-cell immunophenotype, mediastinal mass, central nervous system involvement, testes infiltration, t(9;22) and 11q23/*MLL* gene rearrangements. The treatment schedules included dexamethasone given in repetitive pulses (cumulative dose: 1244 mg/m^2 (high risk) and 1370 mg/m^2 (non-high risk). Total cumulative dose of methotrexate was 13650 mg/m^2 in the high-risk protocol and 8100 mg/m^2 in the non-high-risk protocol. No patient received central nervous system irradiation.

To determine a potential selection bias, we compared patient characteristics of participants of the current study with those of the total Rotterdam DCOG-ALL9-treated cohort. The Medical Ethical Committee approved the study. Written informed consent according to the Helsinki agreement was obtained from all parents and patients ≥12 years.

Polymorphisms

After reaching complete remission, germ-line genomic DNA was extracted from a minimum of 5.0x10^6 peripheral blood mononuclear cells using TRIzol reagent (Gibco BRL,Life Technologies) according to the manufacturer's protocol. The DNA was quantified using spectrophotometry. Figure 1 shows positions of the SNPs, which were detected by real-time PCR and hybridization probes (Taqman).

We determined three SNPs at the 3'-end of the *VDR* gene (*BsmI* (E8-G+284A, rs1544410), *ApaI* (E9-T-48G, rs739837), and *TaqI* (E9-T32C, rs731236) [12]. Haplotypes were named as previously described [9, 12]. In our patients haplotype 1 (baT), haplotype 2 (BAt), haplotype 3 (bAT) and haplotype 4 (BAT) occurred, which combined to eight genotypes encoded 1/1, 1/2, 1/3, 1/4, 2/2, 2/3, 2/4 and 3/3 (3/4 and 4/4 not observed).

Two other *VDR* 5'-promoter region SNPs were studied; the G→A substitution in the *Cdx-2* binding site (1e-G-1739A, rs11568820) and an A→G substitution in the *GATA* binding site (1a-A-1012G, rs4516035) [8, 9, 36]. Both 5'-promoter polymorphisms were combined to haplotype 1 (GA), haplotype 2 (GG) and haplotype 3 (AG), combining to six genotypes encoded as 1/1, 1/2, 1/3, 2/2, 2/3 and 3/3.

The *Sp1* polymorphism is a G→T substitution affecting a binding site of the Sp1 transcription factor in the first intron of *COLIA1 (int1-G+1245T,* rs1800012*)* [37]. The polymorphism results in three genotypes GG, GT and TT.

We genotyped two polymorphisms in the first intron of *ESR1*: *PvuII* (int1-T-397C, rs2234693) and *XbaI* (int1-A-351G, rs9340799) [28]. Three haplotype

alleles were encoded as haplotype 1 (px), haplotype 2 (PX), and haplotype 3 (pX), combining to six genotypes 1/1, 1/2, 1/3, 2/2, 2/3 and 3/3.

We determined two SNPs of the *GR*: the *BclI* (int2-C-646G, rs not available) which combined to the genotypes CC, CG and GG and the *N363S* (e2-A1218G, rs6195) combining to the genotypes AA, AG and GG [38].

End points

Anthropometry data were measured in all patients. Height was measured with a Harpenden stadiometer and weight with a standard clinical balance. The body mass index (BMI) was calculated as weight/height^2. Height and BMI of the patients were compared with reference values of healthy controls matched for age and sex and expressed as standard deviation scores (SDS) [39, 40].

In patients aged≥4 years, dual energy X -ray absorptiometry (DXA, Lunar DPX-L) provided estimates of lean body mass (LBM), percentage fat of the total body (%fat_{TB}), BMD of the total body (BMD_{TB}) and BMD of the lumbar spine (BMD_{LS}). To correct for bone size we calculated bone mineral apparent density (BMAD) of the lumbar spine with the model $BMAD_{LS}=BMD_{LS}x(4/(\pi x width))$. 'Width' is the mean width of the second to the fourth lumbar vertebrae. This model was validated by *in vivo* volumetric data obtained from magnetic resonance imaging [41]. All DXA results were expressed as age-matched and sex-matched SDS [42]. Special pediatric software was used for children with a weight <30 kg.

Symptomatic fractures, confirmed by radiography, were registered. Fracture incidence rates of the various allelic variants were calculated. In addition, incidence-rate ratios for non-carrier versus carriers were calculated.

Habitual physical activity measured in minutes/week included physical education classes, organized sports, recreational activities, habitual walking/ cycling [43]. Calcium intake was determined by a detailed food-frequency questionnaire of dairy products [44]. Serum calcium, 1,25-dihydroxy-vitamin D and PTH were assessed. Because, over time, PTH concentrations were measured on three different immunoanalyzers, concentrations of PTH were expressed as the number of standard deviations above the upper limit of the reference range of the immunoassay used [45].

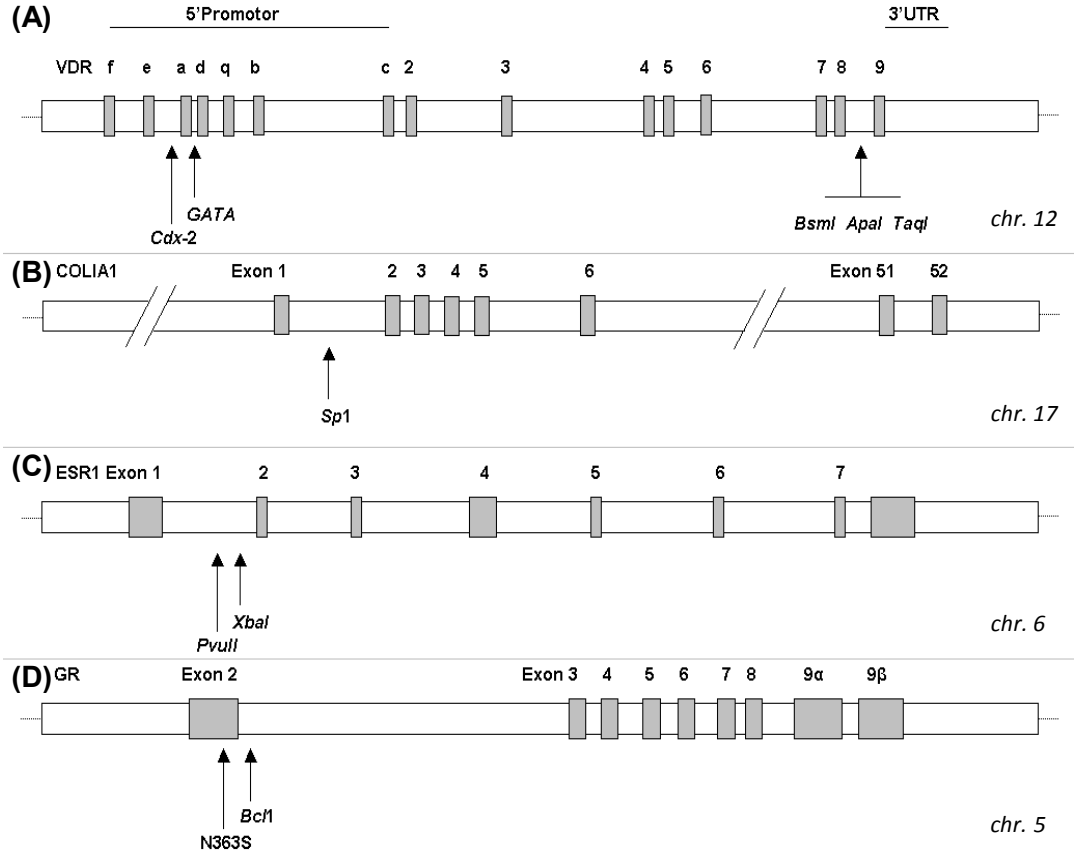

Figure 1. Genomic structure and positions of the single nucleotide polymorphisms investigated in current study. (A) The vitamin D receptor gene (VDR). (B) The collagen type I alpha 1 gene (COLIA1). (C) The estrogen receptor alpha gene (ESR1). (D) The glucocorticoid receptor gene (GR). *Abbreviations: UTR = untranslated region, chr. = chromosome.*

Measurements were performed at diagnosis, after 32 weeks, 1 year, 2 years (completion of therapy) and 3 years (1 year after completion of therapy). Differences between non-carriers and carriers in change of end points during the two-year treatment period (Δ_1) and during the first year after completion of chemotherapy (Δ_2) were investigated. To compare positions of the curves of the different carrier groups, areas under the curves were calculated.

Statistical analysis

SNPs were tested for deviation from the Hardy Weinberg Equilibrium (HWE) by comparing the observed and expected genotype frequencies using a chi-square test. We calculated areas under the curves using the trapezium rule. A Mann-Whitney U-test/chi-square-test was used to compare baseline patient characteristics and areas under the curves for the different carrier groups. Anthropometry, body composition and BMD at diagnosis were compared with normal reference values using a one-sample T-test. Fracture incidence-rate ratios were tested using Poisson statistics. These statistical analyses were performed with SPSS 15.0 (SPSS Inc. Chicago, IL, USA). Differences between the carrier groups in changes of end points (Δ_1 and Δ_2) were analyzed using repeated measurements analysis (SAS PROC MIXED, SAS Institute Inc., North Carolina, USA), with an unstructured repeated covariance type. We pooled heterozygous and homozygous carriers under a dominant inheritance model. In view of the multiple comparisons, P-values of $\leq$0.01 were considered to be significant. All analyses were carried out according to the intention-to-treat principle; for children who did not complete the study data prior to elimination were included.

Results

Patients

Sixty-nine patients (39 males) were included, with a mean age of 7.4 (range 1.6-16.8) years. Twenty patients were treated with the high-risk protocol and the remaining children received non-high-risk treatment. Age, gender and risk-group stratification of the included patients were similar to that of the total DCOG-ALL9-treated cohort, which indicated that the sample constituted a representative selection of the Rotterdam cohort.

Genotype distribution

The distribution of the genotypes of *VDR* 3'-end (*BsmI/ApaI/TaqI*) and 5'-end (*Cdx-2/GATA*), *COLIA1* (*Sp1*), *ESR1* (*PvuII/XbaI*), and *GR* (*BclI*) were in HWE (Table 1). No homozygous carriers and only three heterozygous carriers of the *GR* (*N363S*) were determined (data not shown).

Table 1. Genotype distribution in pediatric ALL patients

VDR 3'-end (BsmI/ApaI/TaqI)				**VDR 5'-end (Cdx-2/GATA)**			
Gen	Haplo	N	%	Gen	Haplo	N	%
baT-baT	*1/1*	*17*	*25*	GA-GA	*1/1*	*13*	*20*
baT-BAt	*1/2*	*16*	*23*	GA-GG	*1/2*	*19*	*30*
baT-bAT	*1/3*	*8*	*12*	GA-AG	*1/3*	*15*	*23*
baT-BAT	*1/4*	*2*	*3*	GG-GG	*2/2*	*5*	*8*
BAt-BAt	*2/2*	*14*	*21*	GG-AG	*2/3*	*9*	*14*
BAt-bAT	*2/3*	*5*	*7*	AG-AG	*3/3*	*3*	*5*
Bat-BAT	*2/4*	*4*	*6*				
bAT-bAT	*3/3*	*2*	*3*				
Total		*68*				*64*	
HWE p-value		*0.30*				*0.72*	

ESR1 (PvuII/XbaI)				**COLIA1 (Sp1)**			**GR (BclI)**		
Gen	Haplo	N	%	Gen	N	%	Gen	N	%
px-px	*1,1*	*20*	*29*	GG	*49*	*73*	CC	*20*	*41*
px-PX	*1,2*	*29*	*43*	GT	*17*	*25*	CG	*22*	*45*
px-Px	*1,3*	*4*	*6*	TT	*1*	*2*	GG	*7*	*14*
PX-PX	*2,2*	*10*	*15*						
PX-Px	*2,3*	*4*	*6*						
Px-Px	*3,3*	*1*	*1*						
Total		*68*			*67*			*49*	
HWE p-value		*0.84*			*0.73*			*0.36*	

VDR = vitamin D receptor gene, COLIA1 = collagen type I alpha 1 gene, ESR1 = estrogen receptor alpha gene, GR = glucocorticoid receptor gene, gen = genotype, haplo = haplotype, N = number, HWE = Hardy Weinberg equilibrium.

Baseline data

At diagnosis, $\%fat_{TB}$ of our sample was not different compared with healthy peers. The patients showed a lower BMI and LBM at diagnosis than healthy peers (BMI=-0.51SDS, $p<0.01$ and LBM=-0.67SDS, $p<0.001$). Baseline BMD_{TB} of our ALL patients was not different from healthy peers, whereas BMD_{LS} of the patients was lower than BMD_{LS} of healthy peers (BMD_{LS}=-0.53SDS, $p=0.01$). However, after correction for bone size, the calculated $BMAD_{LS}$ showed no differences between patients and healthy peers ($BMAD_{LS}$=-0.21SDS, $p=0.25$).

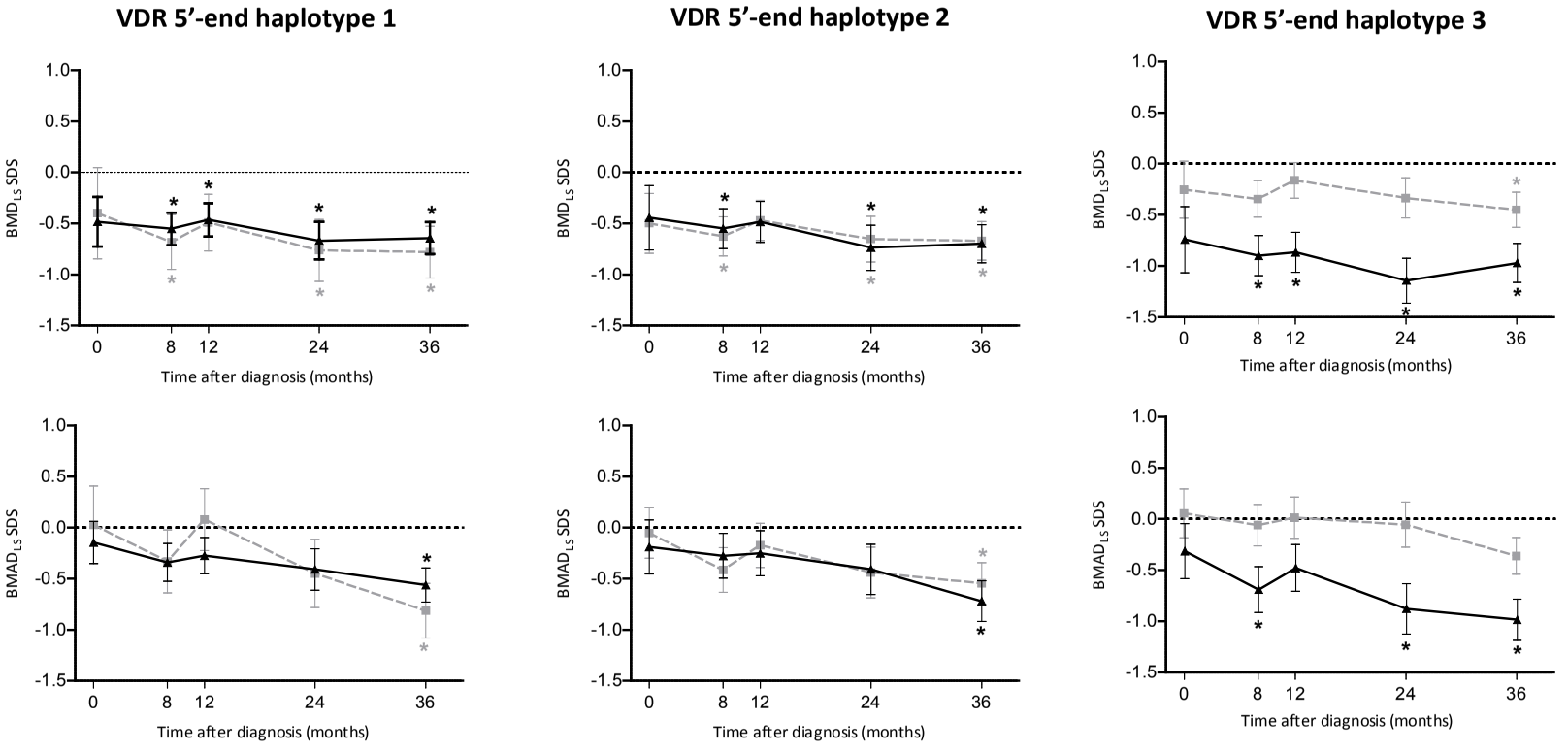

Figure 2. Bone mineral (apparent) density of the lumbar spine in non-carriers versus carriers of haplotypes of the VDR 5'-end (Cdx-2/ GATA) (mean ± SEM). Abbreviations: BM(A)D_{LS}=bone mineral (apparent) density of the lumbar spine, SDS=standard deviation score, non-carrier=—■—, carrier=--▲--, *=significant different from 0.

Table 2a. Change of anthropometry, bone mineral density and body composition for the investigated genetic variations of the *VDR*, *COLIA1*, *ESR1* and *GR* genes

		VDR 3'-end (BsmI/ApaI/TaqI)						***VDR 5'-end (Cdx-2/GATA)***					
		Haplotype 1		**Haplotype 2**		**Haplotype 3**		Haplotype 1		**Haplotype 2**		**Haplotype 3**	
		Non-carrier	Carrier	Non-carrier	Carrier	Non-carrier	Carrier	Non-carrier	Carrier	Non-carrier	Carrier	Non-carrier	Carrier
1	Height	-0.56	-0.65	-0.68	-0.57	-0.64	-0.54	-0.87	-0.51**	-0.54	-0.66	-0.56	-0.66
	BMI	1.30	1.51	1.55	1.36	1.49	1.24	0.99	1.48	1.39	1.33	1.58	1.05
	BMD_{LS}	0.17	-0.32	-0.33	-0.03	-0.08	-0.44	-0.36	-0.19	-0.15	-0.29	-0.08	-0.40
	$BMAD_{LS}$	-0.01	-0.40	-0.28	-0.25	-0.34	0.01	-0.48	-0.26	-0.39	-0.22	-0.11	-0.57
	BMD_{TB}	-0.68	-1.20	-1.33	-0.78	-0.97	-1.15	-1.06	-1.08	-0.99	-1.17	-0.96	-1.23
	$\%fat_{TB}$	1.09	1.50	1.51	1.25	1.31	1.50	0.59	1.47**	1.25	1.39	1.76	0.77 *
	LBM	0.09	-0.40	-0.46	-0.06	-0.20	-0.34	-0.34	-0.20	-0.07	-0.45	-0.25	0.24
2	Height	0.42	0.16	0.24	0.26	0.14	0.56**	0.61	0.10*	0.09	0.36	0.28	0.20
	BMI	-0.13	-0.45	-0.34	-0.33	-0.43	-0.02	-0.23	-0.38	-0.30	-0.38	-0.58	0.00 †
	BMD_{LS}	-0.01	0.02	-0.02	0.02	-0.06	0.24	-0.02	0.02	-0.02	0.04	-0.12	0.17
	$BMAD_{LS}$	-0.17	-0.23	-0.20	-0.21	-0.17	-0.31	-0.36	-0.15	-0.11	-0.31	-0.31	-0.11
	BMD_{TB}	0.44	0.22	0.21	0.37	0.25	0.45	0.54	0.23	0.32	0.30	0.18	0.46
	$\%fat_{TB}$	-0.32	-0.79	-0.63	-0.63	-0.73	-0.27	-0.66	-0.66	-0.49	-0.81	-0.85	-0.42
	LBM	0.23	0.22	0.26	0.18	0.12	0.57**	0.61	0.10**	0.03	0.42**	0.11	0.38

*Abbreviations: VDR = vitamin D receptor gene, COLIA1 = collagen type I alpha 1 gene, ESR1 = estrogen receptor alpha gene, GR = glucocorticoid receptor gene, 1 = change during treatment, 2 = change after treatment discontinuation, BMI = body mass index, $BM(A)D_{LS}$ = bone mineral (apparent) density of the lumbar spine, BMD_{TB} = bone mineral density of the total body, $\%fat_{TB}$ = percentage of fat of the total body, LBM = lean body mass. Values are expressed as mean ΔSDS. Difference between non-carriers and carriers: *p≤0.01 and **0.01<p<0.05 (ANOVA).*

Table 2b. Change of anthropometry, bone mineral density and body composition for the investigated genetic variations of the *VDR*, *COLIA1*, *ESR1* and *GR* genes

		COLIA1 (Sp1)		***ESR1 (PvuII/XbaI)***				***GR (BclI)***			
				Haplotype 1		**Haplotype 2**		**Haplotype 3**			
		Non-carrier	Carrier	Non-carrier	Carrier	Non-carrier	Carrier	Non-carrier	Carrier	Non-carrier	Carrier
1	Height	-0.60	-0.68	*-0.55*	*-0.64*	*-0.65*	*-0.60*	*-0.62*	*-0.64*	*-0.44*	*-0.62*
	BMI	1.50	1.36	*1.23*	*1.50*	*1.78*	*1.24*	*1.38*	*1.81*	*1.11*	*1.48*
	BMD_{LS}	-0.04	-0.44	*-0.57*	*-0.03*	*-0.14*	*-0.16*	*-0.02*	*-1.09*	*-0.18*	*0.06*
	$BMAD_{LS}$	-0.26	-0.28	*-0.50*	*-0.19*	*-0.29*	*-0.25*	*-0.20*	*-0.64*	*-0.24*	*0.03*
	BMD_{TB}	-0.96	-1.13	*-1.62*	*-0.82*	*-0.76*	*-1.13*	*-0.91*	*-1.72*	*-1.40*	*-0.80*
	$\%fat_{TB}$	1.44	1.13	*1.13*	*1.44*	*1.61*	*1.22*	*1.36*	*1.40*	*1.47*	*1.15*
	LBM	-0.19	-0.30	*-0.32*	*-0.20*	*-0.12*	*-0.28*	*-0.22*	*-0.36*	*-0.33*	*-0.10*
2	Height	0.14	0.30	*0.00*	*0.32*	*0.19*	*0.29*	*0.28*	*0.04*	*0.29*	*0.27*
	BMI	-0.35	-0.26	*-0.29*	*-0.35*	*-0.42*	*-0.28*	*-0.28*	*-0.76*	*-0.01*	*-0.19*
	BMD_{LS}	0.09	-0.17	*0.37*	*-0.09***	*-0.11*	*0.07*	*0.00*	*0.02*	*-0.09*	*0.11*
	$BMAD_{LS}$	-0.06	-0.55**	*-0.11*	*-0.24*	*-0.25*	*-0.20*	*-0.23*	*0.12*	*-0.24*	*-0.23*
	BMD_{TB}	0.36	0.15	*0.50*	*0.24*	*0.27*	*0.31*	*0.27*	*0.55*	*0.15*	*0.36*
	$\%fat_{TB}$	-0.54	-0.84	*-0.32*	*-0.73*	*-0.81*	*-0.55*	*-0.61*	*-0.88*	*-0.55*	*-0.46*
	LBM	0.16	0.37	*0.22*	*0.23*	*0.11*	*0.29*	*0.28*	*-0.55**	*0.38*	*0.28*

*Abbreviations: VDR = vitamin D receptor gene, COLIA1 = collagen type I alpha 1 gene, ESR1 = estrogen receptor alpha gene, GR = glucocorticoid receptor gene, 1 = change during treatment, 2 = change after treatment discontinuation, BMI = body mass index, $BM(A)D_{LS}$ = bone mineral (apparent) density of the lumbar spine, BMD_{TB} = bone mineral density of the total body, $\%fat_{TB}$ = percentage of fat of the total body, LBM = lean body mass. Values are expressed as mean ΔSDS. Difference between non-carriers and carriers: *p≤0.01 and **0.01<p<0.05 (ANOVA).*

Baseline anthropometry, body composition and BM(A)D were not significantly different for non-carriers compared with carriers of any of the SNPs or haplotypes. In addition, there were no significant differences regarding age, calcium intake and physical activity.

Body composition during/after therapy

During treatment, the whole group of patients showed an increase in BMI (Δ_1BMI=+1.43SDS, p<0.001). Consequently, BMI became higher than BMI of healthy peers (area under the curve during treatment: p<0.001). Non-carriers and carriers of the different allelic variants showed no difference in increase of BMI during treatment (Table 2). After completion of treatment, BMI of the patients decreased (Δ_2BMI=-0.31SDS, p<0.01), but remained higher than BMI of healthy peers one year after completion of treatment (BMI=+0.60SDS, p<0.001). Furthermore, there was no influence of the carrier status of any of the genotypes on change of BMI after treatment. Both the areas under the curves of BMI during treatment and during the year after treatment did not present differences between non-carriers and carriers of the investigated risk alleles.

During treatment, $\%fat_{TB}$ in the patient group was higher than in healthy peers (area under the curve during treatment: p<0.001) and increased significantly ($\Delta_1\%fat_{TB}$=+1.32SDS, p<0.001). After completion of treatment, $\%fat_{TB}$ in the whole study group decreased ($\Delta_2\%fat_{TB}$=-0.60SDS, p<0.001), but remained higher than in healthy peers ($\%fat_{TB}$=+0.64SDS, p<0.001). A significant difference in gain of $\%fat_{TB}$ during treatment was found between non-carriers and carriers of the *VDR* 5'-end (*Cdx-2/GATA*) haplotype 3 (non-carriers: $\Delta_1\%fat_{TB}$=+1.76SDS, carriers $\Delta_1\%fat_{TB}$=+0.77 SDS; p<0.001 (Table 2)). This difference in fat gain between both groups was not evident in the first eight months of treatment, but became obvious during the remaining part of the treatment. No differences in $\Delta_1\%fat_{TB}$ between non-carriers and carriers of any of the other investigated risk alleles were found. Furthermore, $\Delta_2\%fat_{TB}$ of the non-carriers of the investigated SNPs/ haplotypes was similar to that of the carriers. Area under the curve of polymorphism were determined (data not shown).

investigated risk alleles. During treatment and during the year after treatment areas under the curves of LBM were not different in the various carrier groups.

BMD during/after therapy

During treatment, BMD_{LS} of the patients remained lower than of healthy peers (p<0.01). As BMD_{LS} of the whole group did not change either during or after

treatment, a year after completion of treatment it was still lower in the patients than in healthy peers (BMD_{LS}=-0.63SDS, p<0.001). $BMAD_{LS}$ was only lower in patients than in healthy peers after completion of treatment (p<0.01).

Figure 2 shows the effect of different haplotypes of the *VDR* 5'-end (*Cdx-2/GATA*) on BM(A)D_{LS}. Carriers of the *VDR* 5'-end haplotype 3 had a lower BMD_{LS} and $BMAD_{LS}$ than non-carriers (area under the curve BMD_{LS}: p=0.01, area under the curve $BMAD_{LS}$: p=0.03). There was no difference in BM(A)D_{LS} between non-carriers and carriers of haplotype 1 (area under the curve BMD_{LS}: p=0.68, area under the curve $BMAD_{LS}$: p=0.98) or haplotype 2 (area under the curve BMD_{LS}: p=0.91, area under the curve $BMAD_{LS}$: p=0.92) of the *VDR* 5'-end. No differences in areas under the curves of BM(A)D_{LS} were found between non-carriers and carriers of the other investigated risk alleles. Moreover, no differences were shown for Δ_1BM(A)D_{LS} and Δ_2BM(A)D_{LS} between non-carriers and carriers of the SNPs/ haplotypes (Table 2).

During treatment, BMD_{TB} decreased in patients (Δ_1BMD_{TB}=-1.00SDS, p<0.001). One year after diagnosis, the total group of patients developed lower levels of BMD_{TB} than healthy peers (p<0.01). During the year after treatment, BMD_{TB} of the patients increased (Δ_2BMD_{TB}=+0.29SDS, p<0.001), but remained lower than healthy peers (BMD_{TB}=-0.52SDS, p<0.001). No significant differences in Δ_1BMD_{TB} or Δ_2BMD_{TB} between non-carriers and carriers of the *VDR*, *COLIA1, ESR1* or *GR* risk alleles were found (Table 2). In addition, areas under the curves of BMD_{TB} differed neither during treatment nor during the year after completion of treatment between the various carrier groups.

Fractures

Nine patients sustained a fracture during therapy (n=5) or within one year after completion of treatment (n=4). Fractures involved the forearm (n=4), the tibia (n=3), the clavicle (n=1) and a vertebra (n=1). Except for the vertebral compression fracture, all fractures were preceded by minor trauma. The investigated SNPs/ haplotypes were not associated with an increased fracture risk (Figure 3).

Biomarkers

No differences in serum calcium, PTH and 1,25-dihydroxy-vitamin D were found between non-carriers and carriers of the *VDR* 5'-end (*Cdx-2/GATA*) haplotype 3 (Table 3). Moreover, the change of calcium, PTH and 1,25-dihydroxy-vitamin D

during therapy and during the year after treatment was not different between non-carriers and carriers of the *VDR* 5'-end haplotype 3.

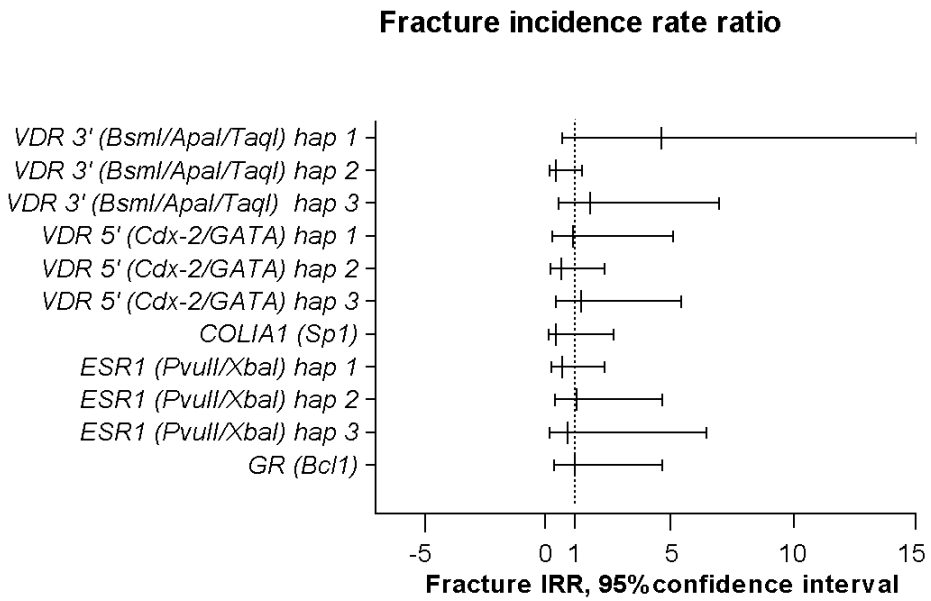

Figure 3. Fracture incidence-rate ratios for non-carriers versus carriers of the different allelic variants *Abbreviations: IRR=incidence-rate ratio, VDR=vitamin D receptor gene, COLIA1=collagen type I alpha 1, ESR1=estrogen receptor alpha gene, GR=glucocorticoid receptor gene, hap=haplotype.*

Discussion

Body composition and polymorphisms

None of the genetic variations in the investigated genes (*VDR*, *COLIA1*, *ESR1* and *GR*) influenced body composition during pediatric ALL treatment, except for haplotype 3 of the *VDR* 5'-promoter region (*Cdx-2/GATA*). Non-carriers of the *VDR* 5'-end haplotype 3 had a larger gain of body fat during treatment than carriers, suggesting a role for vitamin D in the regulation of body fat during pediatric ALL treatment. The *Cdx-2* A-allele increases *VDR* transcription in the small intestine and may consequently increase calcium absorption [36]. Therefore, we hypothesized that non-carriers of the *VDR* 5'-end haplotype 3 (without the *Cdx-2*

A-allele) may have relatively lower serum calcium resulting in a relative hyperparathyroidism. This could lead to increased intracellular calcium within adipocytes, inducing lipogenesis [46]. However, we found no significant differences in serum calcium or PTH between non-carriers and carriers of the *VDR* 5'-end haplotype 3. Therefore, it is questionable whether this mechanism indeed plays a role in regulation of body fat during ALL treatment in children.

Table 3. Biochemical markers in non-carriers and carriers of the *VDR* 5'-end haplotype 3

		***VDR* 5'-end (*Cdx-2/GATA*) Haplotype 3**			
		Non-carrier	Carrier	*p*	*p* of change
Calcium (mmol/L)	Diagnosis	*2.30*	*2.29*	*0.87*	
	Cessation of treatment	*2.36*	*2.36*	*0.93*	Δ1: *0.84* Δ2: *0.81*
	1 Year after cessation of treatment	*2.38*	*2.37*	*0.78*	
PTH (SD above upper limit)	Diagnosis	*-2.93*	*-2.05*	*0.17*	
	Cessation of treatment	*-1.90*	*-1.09*	*0.26*	Δ1: *0.92* Δ2: *0.27*
	1 Year after cessation of treatment	*-0.54*	*-0.52*	*0.98*	
1,25-dihydroxy-vitamin D (pmol/L)	Diagnosis	*107.2*	*104.8*	*0.88*	
	Cessation of treatment	*134.4*	*128.3*	*0.70*	Δ1: *0.86* Δ2: *0.30*
	1 Year after cessation of treatment	*134.5*	*145.9*	*0.25*	

Abbreviations: VDR = vitamin D receptor gene, Δ1 = change during treatment, Δ2 = change after treatment discontinuation, SD = standard deviation.

The current study suggests that carrying *ESR1* (*PvuII/XbaI*) haplotype 3 negatively influences recovery of LBM after completion of treatment, whereas none of the other genes (*VDR*, *COLIA1* and *GR*) influence body composition after ALL treatment. In adults, polymorphisms in *ESR1* have been described to be

associated with measures of adiposity [47], although studies on the influence of *ESR1* on LBM are not available. In healthy children the *PvuII* and *XbaI* SNPs were not related to body composition [30].

BMD and polymorphisms

We found a lower BMD_{LS} in patients carrying the *VDR* 5'-end (*Cdx-2/GATA*) haplotype 3 than in non-carriers, which was already present at diagnosis. Despite this lower BMD_{LS}, carriers of the *VDR* 5'-end haplotype 3 did not show a larger treatment-related loss of BMD_{LS}. Two previous reports showed that presence of the Cdx-2 A-allele protected against the loss of BMD and subsequent osteoporotic fractures in elderly individuals [8, 36]. This illustrates that aging may influence the effect of genetic variation on BMD [48]. Moreover, the present study reports on genotype-based differences in development of BMD following ALL treatment, while the majority of adult reports examined BMD differences between genotypes without chemotherapy.

We did not find any association between *VDR* 3'-end (*BsmI/ApaI/TaqI*) haplotypes and BMD. This is in line with the fact that *VDR BsmI* and *ApaI* SNPs did not influence corticosteroid-induced bone loss in adults receiving corticosteroids for rheumatoid arthritis [49]. No studies are available on the effects of the *VDR* 3'-end SNPs on BMD in ALL patients treated with corticosteroids. Studies on the relation between BMD and *VDR* 3'-end SNPs in healthy children show conflicting results that may be explained by gene-environment interactions, like dietary calcium intake [48, 50]. In the current study however, calcium intake was adequate and not different for non-carriers compared with carriers. Moreover physical activity could interact with the gene-effect, although it was not different for non-carriers compared with carriers. The fact that the individuals in whom the questionnaire was validated had a higher age than our studied ALL patients may have masked a possible gene-physical activity interaction.

We found no influence of polymorphisms of the *COLIA1* (*Sp1*), *ESR1* (*PvuII/XbaI*) and *GR* (*BclI*) on BM(A)D. Because the number of included patients was relatively low, validation of the results in larger cohorts is recommended to confirm our results and to exclude the risk of false-negative findings. So far, no studies on the influence of genetic variation of the *COLIA1*, *ESR1* and *GR* on BM(A)D have been performed in pediatric ALL patients. Several studies in the elderly reported an association between the *Sp1* polymorphism and a lower BMD and increased fracture risk [7, 21]. In pediatric populations this association is less

clear. A lower BMD in healthy children carrying the *Sp1* T-allele has been reported but this was mainly due to differences in bone size [19, 23]. The effect of the *ESR1* polymorphism on steroid-induced bone loss has not been described previously. Regarding the *GR* SNPs, the *N363S* was associated with BMD in healthy adults [32]. Our study included no homozygous and three heterozygous carriers of the *N363S* SNP, so no conclusion could be drawn on the effects of this SNP on BMD in pediatric ALL.

Fractures

The evaluated risk alleles did not influence fracture risk in our cohort of pediatric ALL patients. This lack of association might be explained by other factors contributing to fracture risk, like an increased tendency to fall due to vincristine neuropathy during ALL treatment.

Conclusion

This is the first study investigating the influence of genetic variation of the *VDR*, *COLIA1*, *ESR1* and *GR* on body composition, BMD and fracture risk in pediatric ALL. We found the *VDR* 5'-end (*Cdx-2/GATA)* haplotype 3 as a protective factor for excessive fat gain during therapy. Moreover, this haplotype 3 of the *VDR* 5'-promoter was determined as a risk factor for a lower BM(A)D_{LS} at diagnosis, which remained a risk factor for a lower BM(A)D_{LS} over the course of ALL treatment. Carriage of *ESR1* (*PvuII/XbaI*) haplotype 3 negatively influenced recovery of LBM after completion of treatment.

Authorship and Disclosures

M.L.W. collected data, performed statistical analysis, interpreted data and wrote the manuscript. R.D.B. collected and interpreted data and wrote the manuscript. S.M.P.F.M. designed research, interpreted data and supervised the manuscript. A.G.U. performed genetic analyses and supervised the manuscript. W.C.J.H. supervised the statistical analysis and interpreted data. R.P. and M.M.H. designed research, interpreted data and supervised the manuscript. The authors reported no potential conflicts of interest.

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Chapter 3

Repeats in the Kringle IV Encoding Domains in the Apo(a) Gene and Serum Lipoprotein(a) Level Do not Contribute to the Risk for Avascular Necrosis of the Bone (AVN) in Pediatric Acute Lymphoblastic Leukemia

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Abstract

Avascular necrosis of the bone (AVN) or osteonecrosis is a potentially disabling complication of the treatment of childhood ALL. Familial occurrence of AVN is associated with elevated levels of lipoprotein(a) [Lp(a)]. In cardiovascular diseases, the concentration of Lp(a) has been shown to be inversely related to the number of kringle IV repeats in the apo(a) gene. Lp(a) serum levels and kringle IV repeats of the *LPA* gene were studied in 10 children treated for ALL with and 14 without symptomatic AVN compared with a group of 11 healthy adult controls. All children were treated according to the national treatment protocol (DCOG-ALL9), a 2-year dexamethasone based protocol without cranial irradiation. No significant differences in Lp(a) serum levels or kringle IV repeats between ALL patients with AVN and patients without AVN or healthy controls were found. The sum of the repeats of allele 1 and allele 2 was negatively correlated with Lp(a) plasma level when all groups were pooled together. No significant differences in cholesterol, HDL, triglycerides, apo-A1 and apo-B were found between patients with or without AVN. These findings suggest that Lp(a) does not contribute to the development of symptomatic AVN in children with ALL.

Introduction

Avascular necrosis of the bone (AVN) or osteonecrosis is a potentially disabling complication of the treatment of childhood ALL. The reported incidence of symptomatic AVN in pediatric ALL is 4-12.5% with a higher incidence in older children [1-4]. AVN mostly affects the weight bearing joints resulting in progressive joint damage, sometimes necessitating total joint replacement. Symptoms consist of pain, limited range of motion, limping, joint destruction following bone collapse and arthritis [5].

The pathogenesis of AVN is multi-factorial. It is commonly accepted that the final common pathway for the development of AVN involves a compromise in blood flow to the bone [6]. Suggested causes for AVN include vascular occlusion or ischemia [7], altered fat metabolism and fat emboli [8], intravascular coagulation [9, 10] and inhibition of angiogenesis [11]. In general the use of corticosteroids can cause AVN [6, 12-14]. In ALL, methotrexate can also cause AVN [1-4]. The fact that only a subset of ALL patients develops AVN suggests a role for genetic variation.

In the past, familial occurrence of bone marrow edema syndrome (early phase of AVN) has been described to be associated with elevated levels of Lipoprotein(a) [Lp(a)] [15]. In children Lp(a) has also been reported to be associated with venous thrombosis [16, 17] and with Legg-Perthes disease [18].

Lp(a) is a complex of low-density lipoprotein (LDL) and a high molecular weight glycoprotein called apolipoprotein(a) [Apo(a)]. Plasma Lp(a) concentration shows wide quantitative variation among individuals. This variation in concentration of Lp(a) is inheritable and inversely related to the number of kringle IV repeats in the gene for apo(a) (*LPA*) [19, 20]. This gene is located on chromosome 6 [21]. The size of the Apo(a) protein is determined by the number of repeats of kringle IV type 2 in the *LPA* gene and the variability in apo(a) size effects the plasma concentration of Lp(a) [19].

High Lp(a) levels are associated with familiar AVN. In cardiovascular diseases Lp(a) levels are determined by the number of kringle IV repeates in the *LPA* gene . However, the influence of lipid profiles or *LPA* kringle IV repeats on the occurrence of AVN in pediatric ALL has never been investigated. We hypothesized that children developing symptomatic AVN during ALL therapy might have a higher number of kringle IV repeats in the *LPA* gene, and consequently have higher Lp(a) levels, as compared to those who do not develop AVN and that children who develop AVN during ALL treatment. We studied lipid status, with a special focus on Lp(a) in children with ALL with and without symptomatic AVN.

Materials and Methods

Subjects

The study group consisted of 10 children who developed symptomatic AVN during ALL treatment (5 male/ 5 female) with a median age of 15.0 yr. and 14 children who did not develop AVN (6 male/ 8 female) with a median age of 6.6 yr. Furthermore, kringle IV repeats were determined in a control group of 11 healthy adult Caucasian subjects (3 male/ 8 female; median age 33.7 yr.). In the AVN group 2 patients were diagnosed with a T-ALL and 9 patients with a precursor-B ALL, 4 patients were treated according to the high-risk protocol (HR) and 7 according to the non-high risk protocol (NHR). In the non-AVN group 5 patients were diagnosed with a T-ALL and 9 patients with a precursor-B ALL, 6 patients were treated according to the high-risk protocol (HR) and 8 according to the non-high risk protocol (NHR). All children were treated according to the Dutch national

treatment protocol (DCOG-ALL9) which is based on dexamethasone given in 2-week repetitive courses during 2 years (cumulative dose dexamethasone NHR 1370 mg/m^2, HR 1244 mg/m^2; methotrexate NHR 8100 mg/m^2, HR 13650 mg/m^2). Symptomatic AVN is defined as pain of the extremities, not related to treatment with vincristine and confirmed by the typical findings on MRI.

Determination of Kringle VI repeat number in the Apo (a) gene.

Peripheral blood mononuclear cells (PBMC's) were isolated from EDTA-treated blood samples by a lymphoprep (Axis-Shield PoC AS, Oslo, Norway, 1077 mg/ml) centrifugation step at 1500 rpm for 15 min. Cells were washed once in PBS and resuspended in PBS to a final concentration of 20x10^6 cells/ml. The cell suspension was mixed with an equal volume of 1% incert agarose (FMC Bioproducts, Rockland, USA) in PBS kept at 50^{o}C, and poured into 100 µl plug-forming moulds. Cells were subsequently lysed by incubating the agarose blocks in 5 volumes of 0.5M EDTA, 1% Sodium-N-lauroyl sarcosine (pH=9.5), 0.5 mg/ml proteinase K (Roche Diagnostics, Almere, the Netherlands) at 50^{o}C overnight. Blocks were extensively washed in TE-buffer, and either stored for long-term storage in 0.5M EDTA at 4^{o}C or used immediately. TE-buffer washed blocks were incubated for 2 hr at room temparature or alternatively 4^{o}C overnight in the appropriate restiction enzyme buffer that was supplemented with 1.0 mM DTT and 2.0 mM spermidine. DNA was digested by incubating the blocks for 6 hrs at 37^{o}C in the restriction enzyme buffer supplemented with 1.0 mM DTT, 2.0 mM spermidine, 0.3 mg/ml BSA and 40 units restriction enzyme. Following depletion of the restriction enzyme buffer, blocks were melted at 65^{o}C and carefully loaded in the slots of a 1% Pulse Field Certified Agarose gel (Bio-Rad Laboratories, Hercules, CA) in 1x TBE buffer at 14^{o}C. The 5 Kb ladder (Bio-Rad Laboratories) and the Lambda ladder PFGE marker (New-England Biolabs, Beverly, MA) were used as DNA size standards. DNA fragments were separated by pulsed field gel electrophoresis (PFGE) using the CHEF-DR^{TM} III electrophoresis system (Bio-Rad Laboratories) at 6 volts/cm for 18 hrs using a variable switch-time from 1 to 15 seconds. After electrophoresis, DNA fragments were stained by incubating the gel for 30' in 1 µg/ml ethidium bromide in 1x TBE solution, and photographed. DNA fragments were transferred to a Hybond N$^+$ membrane (Amersham Pharmacia Biotech, Buckinghamshire, England) using the Southern-blotting technique [22]. The 1.2 Kb *Kpn1-BamH1* intron fragment from the Apo (a) gene cloned in pBluescript (Stratagene, La Jolla, CA, USA) was kindly provided by Prof. Dr. G.

Utermann, and labeled with (α-^{32}P)dCTP (ICN, 111TBq/mmol) using a Random Primed DNA Labeling kit (Roche Diagnostics). Following prehybridization for 6 hrs at 60^{o}C in the presence of excess hering sperm DNA, the blots were incubated overnight at 60^{o}C with 1x10^{6} cpm/ml of probe. After extensive washing, blots were exposed to X-ray film (Kodak). Kringle IV repeat numbers were calculated based upon the DNA fragment size relative to the DNA size standards.

The effect of apo(a) alleles on Lp(a) levels appears to be additive. Therefore the sum rather than the mean of the number of kringle IV repeats was used to calculate the correlation of apo(a) alleles with Lp(a), as has been described before [21].

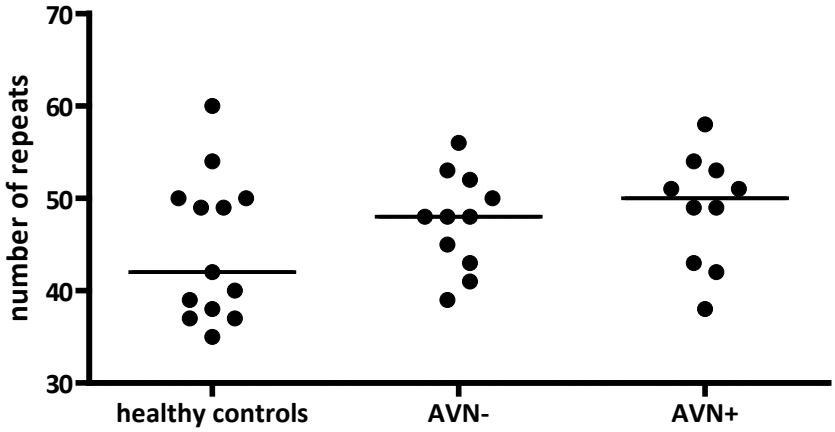

Figure 1. Lp(a) (g/l) serum levels on pediatric ALL patients with and without AVN and healthy controls. *Controls = healthy control group, AVN- = ALL patients without AVN, AVN+ = ALL patients with AVN. The lines indicate the median values. No significant differences (Kruskal-Wallis p=0.14)*

Laboratory measurements

Lp(a), apo-A1 and Apo-B levels were determined using the immuno-nephelometric detection method (Beckman-Coulter, Mijdercht, the Netherlands). Variation coefficients are 3.4%, 2.9% and 1.8% respectively. The lower limit of detection for Lp(a) is 0.02 g/l. Cholestorol, LDL, HDL and triglycerides were determined using an enzymatic color reaction (Roche Diagnostics, Almere, the Netherlands). Variation coefficients are 2.3%, 2.9%, 6.8% and 3.7% respectively.

Statistics

Differences in Lp(a) levels and kringle IV repeats of the *LPA* gene between controls, patients with AVN and patients without AVN were tested using the Kruskal-Wallis tests. Correlations were calculated using the pearson correlation. Differences in lipid profiles between patients with and patients without AVN were tested using the Mann-Whitney U test. All analyses were performed using SPSS 12.0.1. P-values <0.05 were considered significant.

Results and Discussion

The difference in the occurrence and severity of symptomatic AVN between pediatric ALL patients treated according to one and the same treatment protocol suggests differences in susceptibility based on genetic variation. In this study we investigated the relation between Lp(a) levels and the number of kringle IV repeats in the *LPA* gene and their effects on symptomatic AVN in children with ALL. Lp(a) levels are shown in figure 1. ALL-patients with symptomatic AVN had a median Lp(a) serum level of 0.086 g/l (range <0.02 g/l-0.848 g/l), whereas patients without AVN had a median Lp(a) level of 0.066 g/l (range < 0.020 g/l-0.362 g/l; p=0.79). In the healthy adult controls median Lp(a) level was 0.105 g/l (range <0.020 g/l-0.680 g/l). All levels were within normal ranges and no significant differences were found between the 3 groups.

In figure 2 the sum of kringle IV repeats in both alleles of the *LPA* gene is shown for children with AVN, children without AVN and healthy controls. Again, no significant differences were found between the 3 groups. We did find a significant negative correlation between Lp(a) serum levels and the sum of the repeats of allele 1 and allele 2 of the *LPA* gene as shown in figure 3 (r=-0.36; p=0.05) as was described previously by others in adults [20, 23]. In previous studies, although high Lp(a) serum levels have been associated with familiar AVN [15, 24], a large variation in Lp(a) protein levels was observed in individuals with the same number of kringle IV repeats [25]. We did not find higher Lp(a) levels in pediatric ALL patients with symptomatic AVN, which is consistent with our finding that AVN was not related with the number of kringle IV repeats.

So far most studies that reported an association between Lp(a) levels and AVN were performed in adults [26-28]. These studies showed a clear association between high levels of Lp(a) and increased risk of idiopathic osteonecrosis. To our knowledge, no studies of Lp(a) and AVN have been performed in childhood ALL

patients. In children there are reports on the association between Lp(a) and childhood thrombosis [16, 17]. There are two studies in non-ALL children with idiopathic osteonecrosis, but they reported contradictory results [18, 28]. Glueck *et al.* reported a correlation between high Lp(a) levels and the occurrence of Legg-Perthes disease [18], whereas Posan *et al.* did not find a significant difference in Lp(a) levels between children with Legg-Perthes disease and healthy controls [28]. In adults, elevated plasma levels of Lp(a) are associated with increased risk of atherosclerosis, coronary heart disease and vascular dementia [29-32]. Lp(a) is structurally very similar to plasminogen, but does not have the same fibronolytic activity. Instead, Lp(a) inhibits the activation of plasminogen and the conversion of plasminogen to plasmin. It is thought that this reduction in fibrinolytic activity is one of the pathophysiologic mechanisms to cause thrombo-embolic diseases in adult patients with increased serum levels of Lp(a) [21].

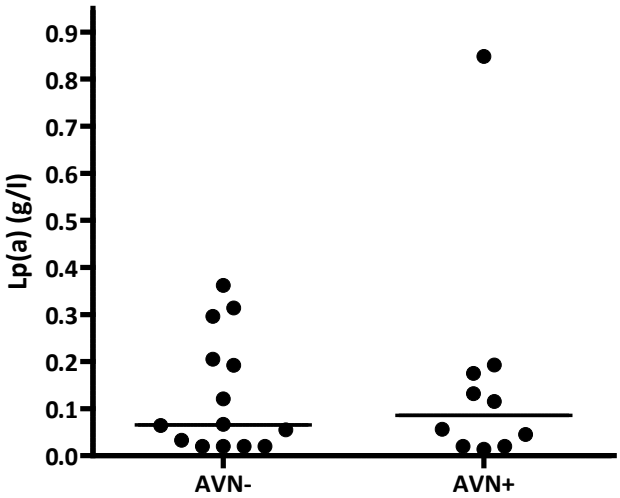

Figure 2. Sum of kringle IV repeats (allele 1 + allele 2) of the LPA-gene in pediatric ALL patients with and without AVN comapred with healthy controls. *AVN- = patients without AVN, AVN+ = patients with AVN. The lines indicate median values.*

Apart from the distinct role of Lp(a) in the development of AVN, hyperlipidemia has been suggested as an etiological factor for osteonecrosis [8, 33]. Lipid profiles of the ALL showed no significant differences in cholesterol, HDL, LDL, triglycerides and apo-B between pediatric ALL patients with or without symptomatic AVN (table 1). There were no differences in LDL/HDL ratios or apo-B/apo-A1 ratios between patients with or without AVN were found. Almost all

reported data on the role of lipid profiles in corticosteroid induced AVN is derived from studies in rabbits [8, 34] and pigs [35, 36]. Motomura *et al.* showed that treatment with lipid lowering medication (probucol) prevented steroid induced AVN in rabbits [33]. Other studies reported a correlation between increased apo-B/apo-A1 ratios in adults with idiopathic osteonecrosis [37] or higher LDL/HDL ratios in rabbits treated with corticosteroids [38] and AVN. In contrast to the studies in rabbits, in the present study, lipids were not assessed during corticosteroid therapy but 5 weeks later just before the next block of dexamethasone therapy. This might explain the rather normal lipid levels in our cohort of patients. It might be that like in the animal studies, increased lipid levels during corticosteroid administration may play a more important etiological role in the development of therapy related AVN. However, this was not investigated in our study.

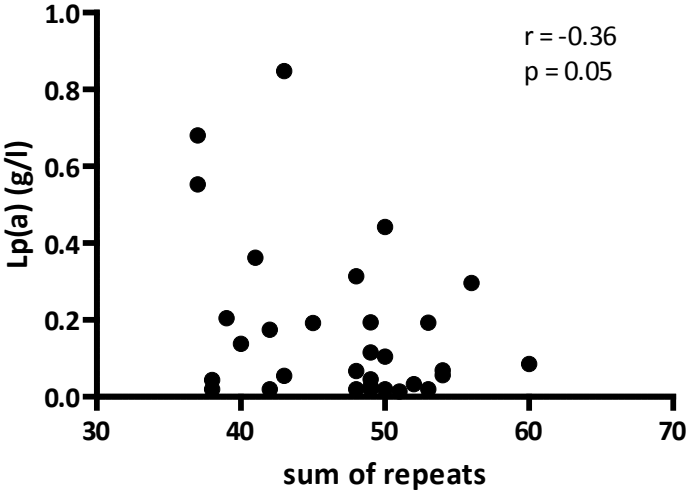

Figure 3. Correlation between Lp(a) levesl and sum of the kringle IV repeats in both alleles (patients and controls)

Still, individual differences in vulnerability with respect to developing AVN in ALL suggests a role for genetic variation. Other genetic polymorphisms associated with AVN in pediatric ALL, Relling *et al.* showed in a group of pediatric ALL that polymorphisms in the vitamin D receptor and thymidylate synthase are independent predictors for osteonecrosis [39]. Also, other polymorphisms have been suggested to play a role in the development of AVN, like polymorphisms in

the folate pathway (e.g. methylenentetrahydrofolate reductase; [40]) and polymorphisms in cytochrome P_{450} [41]. *LPA* polymorphisms have not been included in these studies.

This is the first study which investigated the role of lipid metabolism in developing symptomatic AVN during treatment for pediatric. We conclude that Lp(a) and the number of kringle IV repeats of the *LPA* gene do not contribute to an increased risk of symptomatic AVN in children treated for ALL.

Table 1. Lipid profile of ALL patients with without symptomatic AVN.

	AVN-	AVN+	p-value
Lp(a) (g/l)	0.066	0.086	0.80
	(<0.02-0.362)	(<0.02-0.848)	
Apo A1 (g/l)	1.04	1.25	0.058
	(0.93-1.30)	(1.04-1.30)	
Apo B (g/l)	0.76	0.72	0.90
	(0.44-0.90)	(0.52-1.05)	
HDL (mmol/l)	2.06	2.38	0.16
	(1.16-3.10)	(1.58-3.43)	
LDL (mmol/l)	1.22	1.29	0.43
	(0.96-2.65)	(0.95-3.10)	
Cholesterol (mmol/l)	4.1	4.3	0.23
	(3.1-4.8)	(3.6-5.4)	
TG (mmol/l)	0.82	0.75	0.87
	(0.52-1.38)	(0.35-1.51)	

Median values (range). AVN- = patients without AVN, AVN+ = patients with AVN, TG = triglycerides. P-value: Mann-whitney U test AVN- vs. AVN+.

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Chapter 4

No Difference between Prednisolone and Dexamethasone Treatment in Bone Mineral Density and Growth in Long Term Survivors of Childhood Acute Lymphoblastic Leukemia

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Abstract

Background. Dexamethasone is known to have both more potent leukemic activity and is associated with a higher incidence of side effects than prednisolone. In this study we compared the long-term effects of dexamethasone and prednisolone on bone mineral density (BMD), body composition and growth in long-term survivors of ALL in first complete remission. *Procedure.* Ninety patients (51 male, 39 female; 8,6-38,5 yr), treated with either a prednisolone containing protocol (n=47; n=19 also with CNS-irradiation) or a dexamethasone containing protocol (n=43; no cranial irradiation) participated in this cross-sectional single center study. Mean follow-up was 12.7 yr (2.0-29.7 yr). BMD of lumbar spine and total body, and body composition were expressed as standard deviation scores (SDS) using dual energy X-ray absorptiometry. Bone mineral apparent density of the lumbar spine (BMAD) was calculated to correct for bone size. *Results.* There was no difference in height, height corrected for target height, BMD or lean body mass between prednisolone and dexamethasone treated patients. Prednisolone treated patients had an increased percentage body fat (SDS 0.46; $p<0.05$) and increased body mass index (SDS 0.88; $p<0.01$) compared to normal. Dexamethasone treated patients had only an increased body mass index (SDS 0.52; $p<0.05$). Height, total body BMD and lean body mass were lower in patients treated with cranial irradiation as compared to non-irradiated patients, but differences in the latter two disappeared when corrected for height. BMAD was normal after CNS-irradiation. *Conclusions.* Long term survivors of ALL treated with prednisolone or dexamethasone containing regimens do not differ in height, BMD, or body composition.

Introduction

The cure rate of pediatric acute lymphoblastic leukemia (ALL) increased markedly over the last decades [1, 2]. Therefore, the long-term effects of the treatment become increasingly important. In healthy children bone mineral density increases until peak bone mass is reached [3]. A serious disease, such as ALL, may interfere with the important process of growth and bone accumulation and predispose these children to osteoporosis and growth retardation. Factors influencing growth and bone mass during ALL are the disease itself [4, 5], paracrine secretion of lymphokines [6, 7], and decreased physical activity [8, 9].

Furthermore, the treatment may play a major role, especially the use of high doses of corticosteroids [10, 11] and methotrexate [12-14] and the use of cranial irradiation [15, 16].

Concern exists about the long-term effects of high-dose corticosteroids in children treated for ALL. Dexamethasone is known to have both more potent leukemic activity and is associated with a higher incidence of side effects than prednisolone [17-19]. Whether or not the use of dexamethasone results in more long-term side effects on growth and bone mineral density (BMD) than prednisolone is unknown. Most reports on long-term effects on BMD or body composition describe a heterogeneous group of patients with regard of malignancy. A few studies described follow-up of long-term ALL survivors. These studies reported normal to reduced BMD [20-24] and elevated fat mass [25, 26]. None of these studies compared long-term effects of dexamethasone vs. prednisolone in long term survivors of childhood ALL.

We studied in a single center cohort of long-term survivors of childhood ALL, all treated according to Dutch national protocols. Aim of this study was to compare bone mineral density, body composition and growth between children treated with prednisolone and children treated with dexamethasone as part of the combination chemotherapy.

Subjects and methods

Subjects

Ninety long-term survivors of ALL (51 male, 39 female) participated in this cross-sectional single center study. All patients were treated with national Dutch treatment protocols in the Erasmus MC-Sophia children's hospital Rotterdam [2, 27-31]. All patients were in continuous first complete remission and were > 2 years after completion of therapy. None of the patients received bone marrow transplantation. Forty-seven patients were treated with prednisolone (cumulative dose 1225-22225 mg/m^2) and 43 children were treated with dexamethasone (cumulative dose 1244-1444 mg/m^2). Nineteen patients received cranial irradiation as part of their treatment (all in combination with prednisolone). These patients were excluded from the analysis when comparing the effects of prednisolone vs. dexamethasone. Twenty-six patients treated in the prednisolone group also received dexamethasone as part of their induction treatment (236 mg/m^2), no patients in the dexamethasone group received prednisolone. In the

prednisolone group 21 patients were treated with HD-MTX (mean total dose MTX: 13014 mg/m^2), whereas 3 patients in the dexamethasone group were treated with HD-MTX (5 g/m^2) and 40 children with MD-MTX (2 g/m^2) (mean total dose MTX: 8612 mg/m^2). The mean age (range) of the ALL patients at diagnosis was 7.0 yr (0.9-15.9 yr) and mean follow-up after diagnosis was 12.7 yr (2.0-29.7 yr). Mean follow-up in the prednisolone treated group was 15.4 yr (4.7-29.7 yr.), while the mean follow-up in the dexamethasone treated group was 9.5 yr (2.0-17.4 yr). Mean age (range) at follow-up was 21.2 (8.6-38.5) yr. Twenty-three patients treated according to the DCOG ALL-6 protocol were described earlier [24].

Anthropometry

Height was measured using a Harpenden stadiometer. Target height (TH) was calculated using the formulas: TH (cm) = [(height$_{father}$ + (height$_{mother}$ + 13)]/2 +4,5 for males and [(height$_{father}$ - 13)+ height$_{mother}$]/2 + 4.5 for females. Target height standard deviation score was calculated according to Dutch references [32]. Weight was measured on a standard clinical balance. The body mass index (BMI) was calculated as weight/(height)^2. Height and BMI were compared to Dutch normative values [32].

Bone mineral density (BMD) and body composition

BMD of lumbar spine and total body and body composition were measured using dual energy X-ray absorptiometry (DEXA; Lunar DPX-L and Lunar Prodigy, Madison, WI, USA). To correct for bone size bone mineral apparent density of the lumbar spine ($BMAD_{LS}$) was calculated using the model $BMAD_{LS}=BMD_{LS}$ x [4/(π x width)]. This model was validated by *in vivo* volumetric data obtained from magnetic resonance imaging of lumbar vertebrae [33]. DEXA of total body also provides estimates of body composition (lean body mass, percentage body fat and bone mineral content). Results were compared to a control group consisting of healthy schoolchildren and young adults (4-23 yr) measured on the DPX-L device [34] and healthy young adults (18-28 yr) measured on the Prodigy device. All values are expressed as standard deviation scores (SDS).

Questionnaires

During an interview at follow-up, current dietary calcium intake, medical history, previous fractures and use of other medication was determined. Information on type of corticosteroid used during therapy (prednisolone or dexamethasone) and

radiotherapy was collected from patient records. Dietary calcium intake was assessed by a detailed food frequency questionnaire of dairy products [35] and compared to the recommended daily intake of calcium in the Netherlands (800-1200 mg/day) [36].

Statistics

One-sample t-tests were performed to compare mean SDS values with zero (normal) and independent t-tests were used to compare irradiated and non-irradiated patients and to compare patients treated with prednisolone and dexamethasone. Regression analysis was used to correct for possible confounding factors in case of a significant difference. P values of <0.05 were considered statistically significant.

Results

Height

Overall the ALL survivors had a significantly reduced height at follow-up (SDS –0.44; p<0.001) as compared to healthy schoolchildren and young adults. Target height was normal (SDS –0.03). Patients treated with chemotherapy only had normal height. Corrected height (height SDS – target height SDS) was -0.51 SDS (p=0.06) in the prednisolone treated group and -0.21 SDS (p=0.26) in dexamethasone treated group. However, corrected height was not significantly different between the prednisolone and dexamethasone treated patients (figure 1).

A decreased height as compared to normal controls was found only in the CNS-irradiated group. Height corrected for target height was also decreased in the irradiated group. Corrected height was significantly lower in the CNS-irradiated group as compared to the non-irradiated group (p<0.05; figure 2).

Bone mineral density (BMD) and fractures

There were no significant differences in total body BMD, lumbar spine BMD or lumbar spine BMAD between prednisolone and dexamethasone treated patients (figure 1). CNS-irradiated patients had a significantly reduced total body BMD as compared to non-irradiated patients (p<0.05; figure 2). After correction for height, the difference in total body BMD in CNS-irradiated patients was no longer significant.

Three subjects reported two fractures, and 11 subjects reported one fracture during or shortly after therapy and all fractures were traumatic. No vertebral compression fractures were reported. Previous fractures were not associated with lower BMD, lumbar spine BMAD or calcium intake at time of evaluation. There was no difference in the percentage of fractures between prednisolone and dexamethasone treated patients, nor between irradiated and non-irradiated patients.

Sixteen patients had a calcium intake below the recommended intake (800-1200 mg/day). The mean calcium intake was 1200 mg/day (range 245-3211 mg/day; SD 645). There was no correlation between calcium intake and BMD or fracture prevalence.

Body composition and body mass index (BMI)

In the prednisolone treated group the percentage total body fat (%fat) was significantly increased (SDS 0.46; $p<0.05$), whereas %fat was normal in the dexamethasone treated group (figure 1). The difference in %fat between prednisolone and dexamethasone treated patients was not significant. Lean body mass and bone mineral content were normal in both prednisolone as well as dexamethasone treated patients.

Both lean body mass and bone mineral content were significantly lower in irradiated patients as compared to non-irradiated patients (figure 2). However, these differences disappeared completely after correction for height. Moreover, a significantly higher %fat was found in irradiated patients compared to non-irradiated patients. This difference remained significant after correction for height.

Mean BMI was significantly increased in all patients (figures 1 and 2). A total of 30 patients (33.3%) had a BMI $\geq$ 25 kg/m^2, which is the upper normal limit. Twenty-three patients had a BMI between 25 and 30 kg/m^2 (overweight), and 7 patients had a BMI $\geq$ 30 kg/m^2 (obesity).

Discussion

The improved survival rates after the use of multi-agent chemotherapy and radiotherapy in the treatment of ALL during childhood has raised the issue of long-term sequelae with respect to growth and bone formation. Several studies reported the adverse effects of chemotherapy on growth, BMD and body composition during ALL treatment [4, 5, 9, 10]. Corticosteroids are considered to

be the main cause of BMD reduction, by reducing bone formation and increasing bone resorption [10, 12]. Although several studies reported bone mineral density in long-term survivors of pediatric ALL, to our knowledge this is the first report in which prednisolone-based and dexamethasone-based protocols are compared with respect to the long-term effects on growth, BMD and body composition.

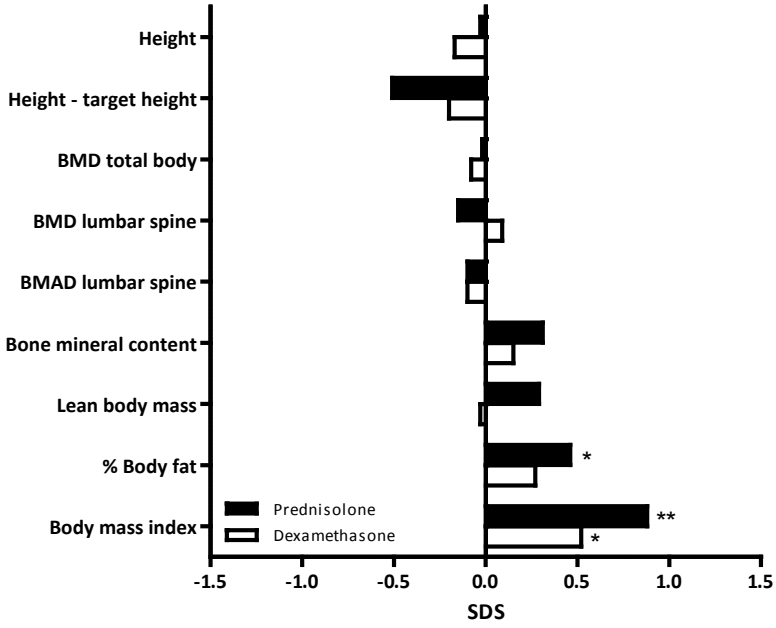

Figure 1. Long-term effects of prednisolone versus dexamethasone on height, bone mineral density and body composition. *Mean height, bone mineral density and body composition SDS according to corticosteroid group (non-irradiated patients only). * p<0.05 compared to healthy controls, ** p<0.01 compared to healthy controls. No significant difference in any of the parameters between prednisolone and dexamethasone treated patients.*

Our study showed no difference in height between patients treated with prednisolone or dexamethasone. Growth retardation is frequently reported in survivors of pediatric ALL, but this is especially due to the use of cranial irradiation [37-39]. Although chemotherapy alone can cause growth retardation during therapy, catch-up growth occurs after cessation of therapy [5]. The normal height in our patients treated with either prednisolone or dexamethasone containing chemotherapy confirmed these results.

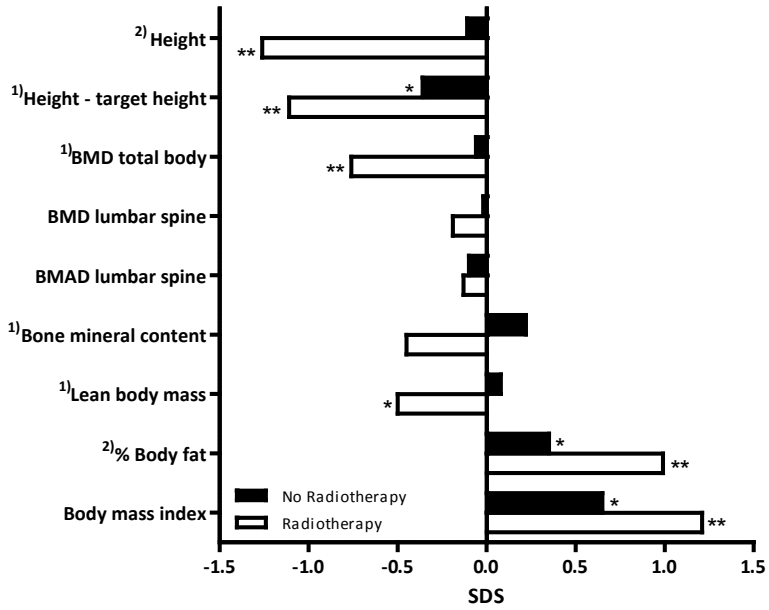

Figure 2. Long-term effects of cranial irradiation versus no cranial irradiation on height, bone mineral density and body composition. *Mean height, bone mineral density and body composition SDS according to radiotherapy group. * p<0.05 compared to healthy controls, ** p<0.01 compared to healthy controls, [1] p<0.05 radiotherapy vs. no radiotherapy, [2] p<0.01 radiotherapy vs. no radiotherapy.*

Our results show that bone mineral density was normal in long-term ALL survivors treated with prednisolone as well as those treated with dexamethasone. Several studies reported BMD in survivors of childhood leukemia [14-16, 22]. Most of these showed a reduced BMD after ALL treatment, with a history of treatment with cranial irradiation as very important risk factor for decreased BMD [15, 16, 22]. In patients treated with chemotherapy only, in general BMD recovered to normal values after cessation of therapy [14].

The present study confirmed that the use of radiotherapy has deleterious effects on BMD and body composition. Important is however that we show here that the presumed effects of CNS irradiation on BMD and lean body mass were both due to the reduced height of irradiated patients. Due to this underestimation of BMD, patients treated with cranial irradiation are often misclassified as having osteoporosis. In patients with a reduced height, the lumbar spine apparent bone mineral density (BMAD; g/cm^3) should be calculated to correct for bone size [33]. Although several studies reported lower bone mineral density after radiotherapy

[14-16], none of these studies reported results on BMAD, with the exception of one smaller study by Arikoski *et al.* who did report normal BMAD after cranial irradiation in 29 ALL survivors [20]. Our study confirmed in a larger cohort, using BMAD that there is no difference in osteoporosis. Although the clinical relevance of BMAD is limited in a healthy population with normal height [40], it is important in a population, which is known to have a reduced height [5, 41]. In conclusion, patients treated with CNS irradiation have a reduced height, and after correction for height do not have an increased risk for osteoporosis as compared to non-irradiated patients.

In our group 20% of the patients reported one or more fractures. This fracture prevalence is comparable to that found in our healthy control group (unpublished data) and data from English children [42]. Other studies did show an increased fracture rate during [5] and after therapy [43]. There was no difference between prednisolone or dexamethasone treated patients in fracture prevalence.

Increased BMI after treatment for ALL has been reported previously [25, 26, 44, 45]. These studies described mostly patients treated with CNS-irradiation, and the obesity found in these studies might be related to the growth hormone deficiency that is often present after cranial radiotherapy [16, 37]. BMI after chemotherapy has been reported to be normal [25, 45], whereas results on percentage body fat varied from normal in dexamethasone treated patients [46] to increased in prednisolone treated patients [25]. In this study, treatment with prednisolone resulted also in higher total body percentage fat and BMI as compared to healthy controls, whereas treatment with dexamethasone only results in a mildly elevated BMI. Percentage fat remains higher than normal in the patients treated with prednisolone, however the difference between prednisolone and dexamethasone treated patients is not significant. Although in the ALL survivors mean BMI was significantly increased after chemotherapy, the number of survivors with obesity (BMI $\geq$ 30 kg/m^2) is comparable to that found in a healthy Dutch population [47].

In conclusion, this study showed that long-term survivors of ALL treated with prednisolone or dexamethasone containing regimens do not differ in height, BMD or body composition. Patients treated with chemotherapy do have an increased BMI, but the prevalence of obesity is not increased.

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Part 2

Endocrine Effects of the Treatment for Childhood Hodgkin's Lymphoma

Chapter 5

Bone Mineral Density, Growth and Thyroid Function in Long-Term Survivors of Pediatric Hodgkin's Lymphoma Treated with Chemotherapy Only

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Abstract

Background. The aim of this study was to investigate the long-term side effects of treatment for childhood Hodgkin's lymphoma with chemotherapy only on growth, bone mineral density (BMD), body composition and thyroid function. *Procedure.* Eighty-eight patients (56 male, 32 female; 17.6-42.6 yr), treated for childhood Hodgkin's lymphoma from 1974-1998 with combination chemotherapy ABVD or EBVD with or without MOPP with the intention to avoid radiotherapy participated in this study. Median follow-up was 15.5 years (range 5.6-30.2 yr.). BMD of lumbar spine (BMD-LS) and total body (BMD-TB), and body composition were measured using dual energy X-ray absorptiometry. Bone mineral apparent density of the lumbar spine (BMAD-LS) was calculated to correct for bone size. Free thyroxin and thyroid stimulating hormone were measured. *Results.* Men treated with MOPP had a significantly reduced height with normal body proportions. Women treated with MOPP had decreased BMD-TB and BMAD-LS as compared to healthy controls. Percentage body fat was significantly increased in female patients treated without MOPP. BMI was significantly increased in MOPP treated male patients while lean body mass was normal in all patients. All patients, except one, treated with chemotherapy only had normal thyroid function. However, five patients who received additional radiation to the thyroid either had abnormal levels of TSH or fT4 or used thyroid hormones. *Conclusions.* Lean body mass was normal in all patients, thyroid function was normal in all but one patient. The use of MOPP leads to decreased height and increased BMI in men and decreased BMD-TB in women.

Introduction

Hogdkin's lymphoma in children can now successfully be treated with overall survival exceeding 90% [1-5]. Treatment for pediatric Hodgkin's lymphoma (HL) consists of radiotherapy, chemotherapy or a combination of both. With the excellent survival rates of pediatric cancer in general, the focus has shifted to the examination of long-term side effects However, most studies to date report on late effects in adult HL patients [6-10], whereas little is known about the long-term (endocrine) effects in survivors of childhood HL.

HL has its first peak-incidence during adolescence, a crucial period for growth and bone mass acquisition. During this period the human bone is most

vulnerable to malignant influences, such as cancer and its treatment [1]. Compromised growth in children during treatment for malignancies can be caused by the disease itself, by related morbidity, such as recurrent infections, an increase in nutritional requirements, malnutrition during treatment and/or by the treatment itself (both chemotherapy and radiotherapy) [11-13].

Several treatment protocols for HL involve the use of high doses of corticosteroids, which are known to cause osteoporosis [14-16]. Corticosteroids interfere with both osteoblast and osteoclast function, which subsequently results in increased bone resorption. So far, little is known about the long-term side effects of corticosteroids and other cytostatic drugs used to treat childhood HL on growth and bone mass development.

Thyroid dysfunction is a well-known side effect after radiotherapy of involved field or mantlefield when treating HL. In particular, radiotherapy to the cervical region causes hypothyroidism, thyroid nodules or thyroid cancer in a large proportion of the patients [17-24]. In contrast, the role of chemotherapy as a risk-factor for thyroid damage is unclear [25].

The aim of this cross-sectional follow-up study was to investigate the long-term effect of treatment for childhood HL on growth, bone mineral density and thyroid function with a special focus on patients treated with chemotherapy without adjuvant radiotherapy.

Patients and methods

Patients and treatment protocols

A total of 148 long-term survivors treated from 1974 to 1998 for pediatric Hodgkin's lymphoma (HL) in Erasmus MC-Sophia children's hospital and AMC-Emma children's hospital were identified. Twenty seven patients refused to participe (18.2%) and 33 patients were lost to follow-up (22.2%; fig. 1). Disease characteristics of the included 88 survivors and those not included are shown in table 1.

At the time of the cross-sectional follow-up study patients were in complete continuous remission for a median period of 15.5 years (range 5.6-30.2 yr.). The median age at follow-up was 27.0 yr (range 17.6-42.6 yr.). Patients were treated with combination chemotherapy ABVD or EBVD with or without MOPP as previously described [2, 5]. ABVD included adriamycine 25 mg/m^2, (epirubicin 30 mg/m^2 in EBVD), bleomycin 10 mg/m^2, vinblastine 6 mg/m^2 and dacarbazine

(DTIC) 250 mg/m^2 on day 1 and 15. MOPP included mechlorethamine 6 mg/m^2 and vincristine 2 mg/m^2 (max. 2.5 mg/dose) on day 1 and 8, prednisolone 40 mg/m^2/day and procarbazine 100 mg/m^2/day on days 1-14. Eighteen patients (3 treated without MOPP, 15 treated with MOPP) needed additional radiotherapy to the neck (n=6; 25-40 Gy), mantlefield (n=7; all 40 Gy), or mediastinum (n=5; 25-40 Gy) irradiation because of resistant disease or relapse after completion of chemotherapy. Patients who were irradiated were analyzed separately. The remaining patients were categorized into 3 treatment groups according to the number of MOPP cycles: no MOPP (n=22), 3-4 MOPP (n=22) and ≥6 MOPP cycles (n=26; median 6 cycles, range 6-12 cycles). None of the patients were treated with 1,2 or 5 MOPP cycles (fig. 1).

The medical ethic committee of the Erasmus MC approved this study and informed consent was obtained from all patients according to the Helsinki agreement.

Table 1. Comparison between included and not included patients.

		Included	Not included
Number (male/*female*)		56 / *32*	41 / *19*
Histology	NS	77.9%	58.5%
	MC	15.1%	15.4%
	LP	3.5%	6.1%
	LD	2.3%	0%
	NOS/Unknown	3.5%	16.9%
Stage	I	31.4%	35.4%
	II	43.0%	26.5%
	III	24.4%	12.3%
	IV	2.3%	6.2%
	Unknown	1.2%	15.4%
% of patients receiving Radiotherapy		19.8%	15.4%
Relapse rate		5.8%	4.6%
Median age at diagnosis (M/F)		11.4/14.0 y	10.7/14.0 y
Age range total group at diagnosis		3.7-17.2 y	3.2-19.8 y

NS = nodular sclerosing, MC = mixed-cellular, LP = lymphocyte predominant, LD = lymphocyte depleted, NOS = not otherwise specified. No significant differences between included and not included patients.

Anthropometry

Height was measured using a Harpenden stadiometer. Sitting-height was measured and sitting height/height ratios were calculated. Weight was measured on a standard clinical balance. The body mass index (BMI) was calculated as weight(kg)/(height(m))^2. Height, sitting height/height ratio and BMI were compared to Dutch normative values [26]. Values are expressed as standard deviation scores (SDS). No data on target height were available.

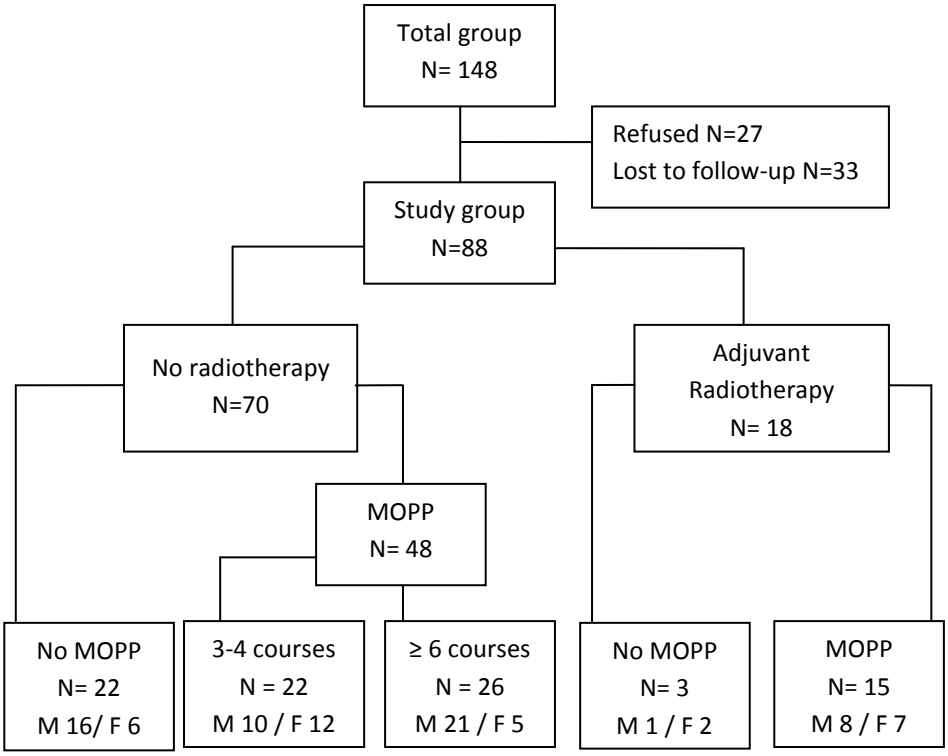

Figure 1. Trial profile. *no MOPP = treated without MOPP, MOPP = treated with MOPP. M= male, F = female*

Bone mineral density (BMD)

BMD of lumbar spine and total body and body composition were measured using dual energy X-ray absorptiometry (DEXA; Lunar DPX-L and Lunar Prodigy, Madison, WI, USA). To correct for bone size, bone mineral apparent density of the lumbar spine ($BMAD_{LS}$) was calculated using the model $BMAD_{LS}=BMD_{LS}$ x [4/(π x

vertebral width)]. This model has been validated by *in vivo* volumetric data obtained from magnetic resonance imaging of lumbar vertebrae [27]. A DEXA scan of the total body provides estimates of body composition, specifically lean body mass, percentage body fat and bone mineral content. Results were compared to a control group consisting of 2 groups of healthy adolescents and young adults (19 male and 46 female; median age 19.8 yr (range 18-25.3 yr)) who were measured using the Lunar DPX-L device [28] and healthy young Caucasian adults (62 male and 85 female; median age 21.9 yr (range 18-37.1 yr)) who were measured using the Lunar Prodigy device. Measurements on both devices are comparable [29].

Laboratory measurements

All blood samples were taken during regular consultation at the long-term effects outpatient clinic. Blood samples, obtained by venapuncture, were processed within 2 hours after withdrawal and serum was stored at -20˚C until assay. Free thyroxine (fT4) and thyroid stimulating hormone (TSH) were measured by chemoluminescence assays (Vitros ECI immunodiagnostic system, Ortho Diagnostics, Rochester, NY). Normal values, determined in our laboratory were 11-25 pmol/l (interassay VC% 4.7%-5.4%) for fT4 and 0.4-4.3 mU/l (interassay VC% 2.5%-4.1%) for TSH.

Questionnaires

During an interview at the cross-sectional follow-up study, patients were asked questions to determine current dietary calcium intake, medical history, previous fractures and use of other medication. Dietary calcium intake was assessed by a detailed standardised food frequency questionnaire of dairy products [30]. The estimate of dietary calcium intake derived from the questionnaire was compared to the recommended daily intake of calcium in the Netherlands (800-1200 mg/day) [31].

Statistics

Statistical analyses were performed using SPSS 15.0. Normal distribution of these variables was tested using the Kolmogorov-Smirnov test. All variables were found to be approximately normally distributed. One-sample t-tests were used to compare the mean SDS values of the anthropometry to healthy controls (SDS=0), a two-sample t-test was used to compare patients treated with MOPP and those treated without MOPP and to compare patients treated with chemotherapy only

and chemotherapy in combination with radiotherapy. In the chemotherapy only group, a one-way ANOVA was used to compare the BMD and body composition results in patients treated with and without MOPP and healthy controls. Pearson correlation was used to test for correlation between variables. A one-way ANOVA test was also used to test for differences in thyroid hormones between the different MOPP-groups (no MOPP, 3-4 MOPP and ≥ 6 MOPP cycles). In view of the multiple tests performed a p-value <0.01 was considered significant.

Results

Anthropometry

All patients had reached final height at the time of study. In patients treated with chemotherapy only, mean height SDS was -0.45 (95% CI -0.92 to +0.03; p=0.07) for male patients and -0.20 (95% CI -0.58 to +0.17; p=0.28) for female patients. In male patients treated with MOPP (MOPP+) height SDS was -0.68 (95% CI –1.15 to –0.21; p=0.007), whereas in female MOPP+ patients height SDS was -0.40 (95% CI –0.84 to 0.04; p=0.08). Both in male and female patients treated without MOPP (MOPP-), height SDS was not different from normal (SDS -0.42 (95% CI –0.92 to 0.61; p=0.63) and SDS -0.15 (95% CI –1.04 to 0.61; p=0.57), respectively). The difference in height SDS between male MOPP+ and MOPP- patients was not significant. Sitting height/height ratio was normal for all patients (fig. 2). There correlations between age at diagnosis and height at follow-up was not statistically significant (male: r=0.34, p=0.04; female r=0.01, p=0.96).

For the 18 patients treated with radiotherapy the mean height SDS was -0.82 (95% CI -1.39 to -0.25; p=0.047). The difference between patients treated with additional radiotherapy and patients treated with chemotherapy only was not significant. There was no significant difference in sitting height/height ratio between patients treated with radiotherapy and patients treated with chemotherapy only (SDS 0.09 vs. SDS -0.39; p=0.29; 95% CI for difference -0.36 to 1.19).

In patients treated with chemotherapy only BMI was significantly increased in male (0.94 SDS, 95% CI 0.40 to 1.48; p=0.002) but not female (0.70 SDS, 95% CI 0.018 to 1.38; p=0.045) MOPP+ patients. BMI was normal, as compared to healthy controls, in both male and female MOPP- patients (0.10 SDS (95% CI –0.67 to 0.87); p=0.78 and 0.50 SDS (95% CI –0.29 to 1.29); p=0.15,

respectively). There was no significant difference in BMI between MOPP+ and MOPP- patients, nor were there any significant differences between men and women (fig. 2). There was no significant difference in BMI between patients treated with chemotherapy only and patients treated with adjuvant radiotherapy (0.66 SDS vs. 0.44 SDS; p=0.55; 95% CI for difference -0.50 to 0.93).

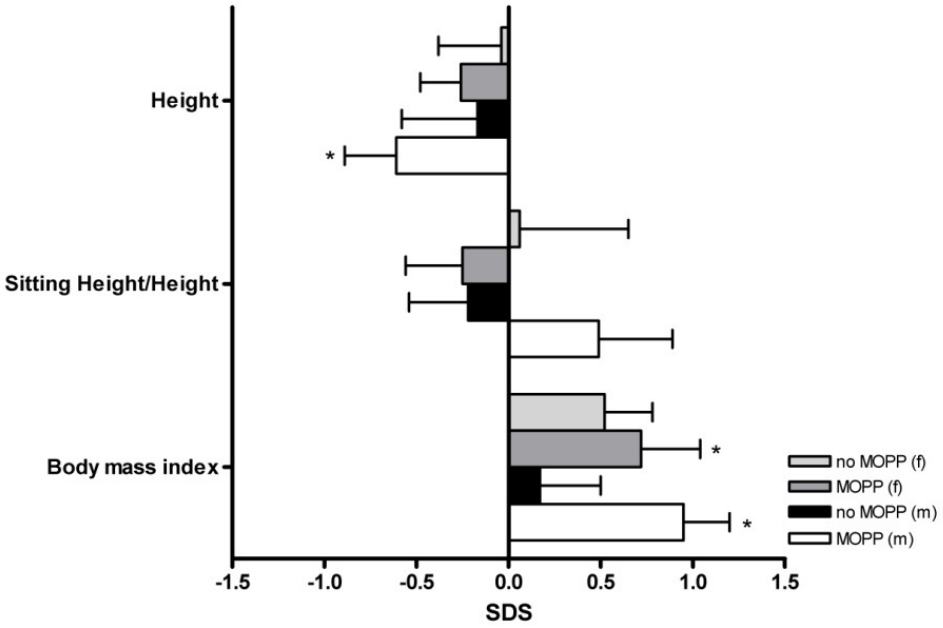

Figure 2. Height, sitting height and BMI SDS for long-term survivors of pediatric Hodgkin's lymphoma treated with chemotherapy only. *no MOPP = treated without MOPP, MOPP = treated with MOPP; f= female, m= male. * = p<0.01 as compared to healthy controls (SDS=0). Bars represent mean values, lines represent 1 standard error of the mean.*

Bone mineral density (BMD) and body composition

Results on BMD and body composition were analyzed separately for males and females. BMD of the total body and BMD and BMAD of the lumbar spine were comparable to healthy controls in all males treated with chemotherapy only (fig. 3). Female MOPP+ patients had a significantly lower total body BMD (mean difference -0.052 g/cm^2; p=0.006; 95% CI for difference -0.09 to -0.02). Lumbar spine BMAD (mean difference -0.0249 g/cm^3; p=0.011; 95% CI for difference -0.04 to -0.006) was lower compared to healthy controls, however this difference was

not significant. There were no significant differences in BMD or in BMAD between patients treated with chemotherapy only and patients treated with chemotherapy and radiotherapy. There were no significant correlations between cumulative prednisolone dose or number of MOPP courses and BMD of the total body or BMD of the lumbar spine or BMAD of the lumbar spine. However, there was a significant correlation between calcium intake and bone mineral density of the total body ($r=0.39$, $p<0.001$), but not lumbar spine ($r=0.33$, $p=0.019$).

Body composition measurements (percentage body fat and lean body mass) are shown in figure 4. Compared to the healthy controls, percentage body fat was significantly increased in female MOPP- patients (mean difference 5.4%; $p=0.001$; 95% CI for difference 2.12 to 8.65). Percentage body fat was also increased in female MOPP+ patients (mean difference 4.26%; $p=0.035$; 95% CI for difference 0.31 to 8.21), although this difference was not statistically significant. In male MOPP+ patients (mean difference 3.6%; $p=0.089$; 95% CI for difference -0.56 to 7.77) percentage body fat was increased, without being significant. Percentage body fat of male MOPP- patients was not significantly different compared to healthy controls (mean difference -0.34%; $p=0.91$; 95% CI for difference -6.10 to 5.37). The differences between MOPP+ and MOPP- patients were not significant in the group of male or in the group of female patients. Lean body mass was comparable to that in healthy controls in all patients. There were no significant correlations between cumulative dose of prednisolone or number of MOPP courses and the body composition measurements, nor were there any differences between patients treated with and without radiotherapy.

Thyroid

Thyroid tests (fT4 and TSH) were performed in a total of 62 patients (46 chemotherapy only and 16 treated with adjuvant radiotherapy). Four patients had abnormal TSH and/or fT4 levels: 2 male patients (TSH 4.8 mU/l, fT4 unknown and TSH 6.4 mU/l, fT4 8.8 pmol/l respectively) and 2 female patients (TSH 4.9 mU/l, fT4 14.0 pmol/l and TSH 3.3 mU/l, fT4 10.6 pmol/l) (fig. 5). Two additional patients were using thyroid hormones, but had normal TSH and fT4 at time of study. Another two patients reported a previous (partial) strumectomy after radiotherapy and no malignancies were found in these patients after pathological examination. Neither of these two patients used any medication. Among the 6 patients that had increased levels of TSH, decreased levels of fT4 or used thyroid hormones, five had received radiation to the thyroid. Only one patient was

treated with ABVD only. There were no significant differences in median levels of fT4 and TSH between patients treated with MOPP and patients treated without MOPP, nor were there any differences between men and women. There was no difference in fT4 or TSH levels between the different treatment groups (no MOPP, 3-4 MOPP courses, 6 or more MOPP courses; One-way ANOVA, p=0.13 and p=0.65 respectively).

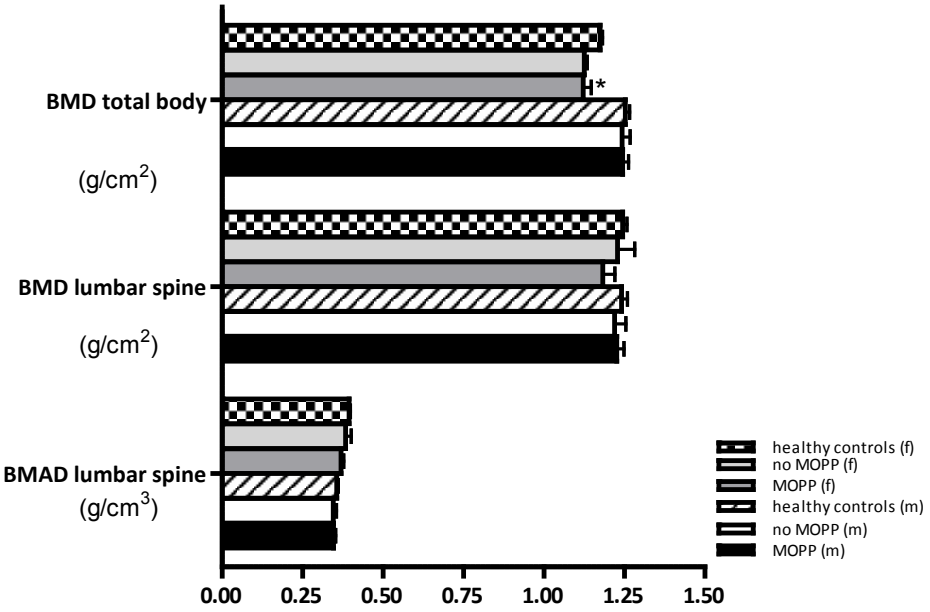

Figure 3. Bone mineral density of long-term survivors of pediatric Hodgkin's lymphoma treated with chemotherapy only and healthy controls. *no MOPP = treated without MOPP, MOPP = treated with MOPP; f = female, m = male. BMD = bone mineral density, BMAD = bone mineral apparent density. Bars represent mean values, lines represent 1 standard error of the mean. * = p<0.01 as compared to healthy controls.*

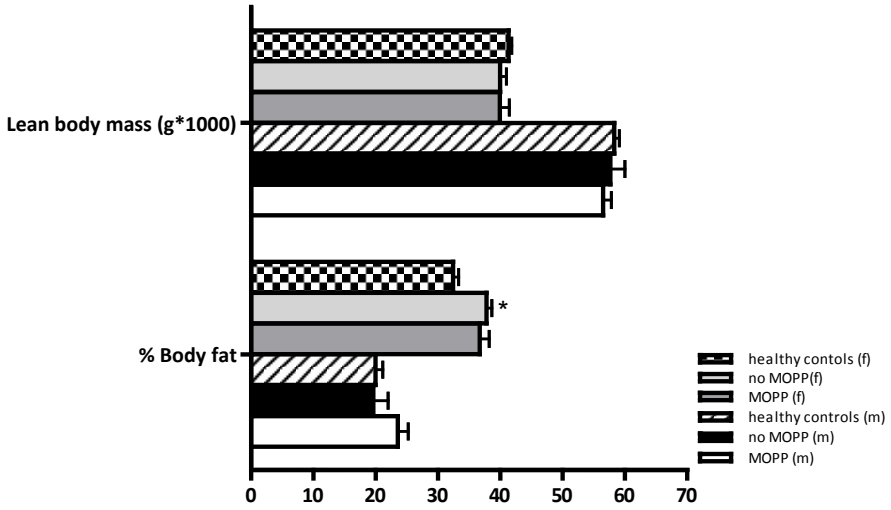

Figure 4. Body composition of long term survivors of pediatric Hodgkin's lymphoma treated with chemotherapy only and healthy controls. *no MOPP = treated without MOPP, MOPP = treated with MOPP; f= female, m= male. Bars represent mean values, lines represent 1 standard error of the mean. * = p<0.01 as compared to healthy controls.*

Discussion

Few data exist on the long term effects of the treatment for HL on bone mineral density (BMD) and growth and the studies performed thus far have focused solely on adult patients (6-10). This is the first study to describe height and BMD in a group of paediatric HL patients treated with chemotherapy only.

Chemotherapy can diminish growth during treatment [11-13]. However, children generally show some catch-up growth after treatment has finished, resulting in normal final height [15, 32]. In the current study however, male patients treated with MOPP were relatively shorter than the healthy controls, whereas female patients had normal height. The finding that only men had a reduced height might be explained by the fact that men were younger at the time of diagnosis and more often treated at or before the time of peak height velocity [33], whereas women were more often treated after their peak height velocity [33]. An interruption of growth during peak height velocity, as occurred in case of the male patients, might have had a greater impact on growth. The correlation

between age at diagnosis and height at follow-up in male patients, although only a trend, supports this assumption.

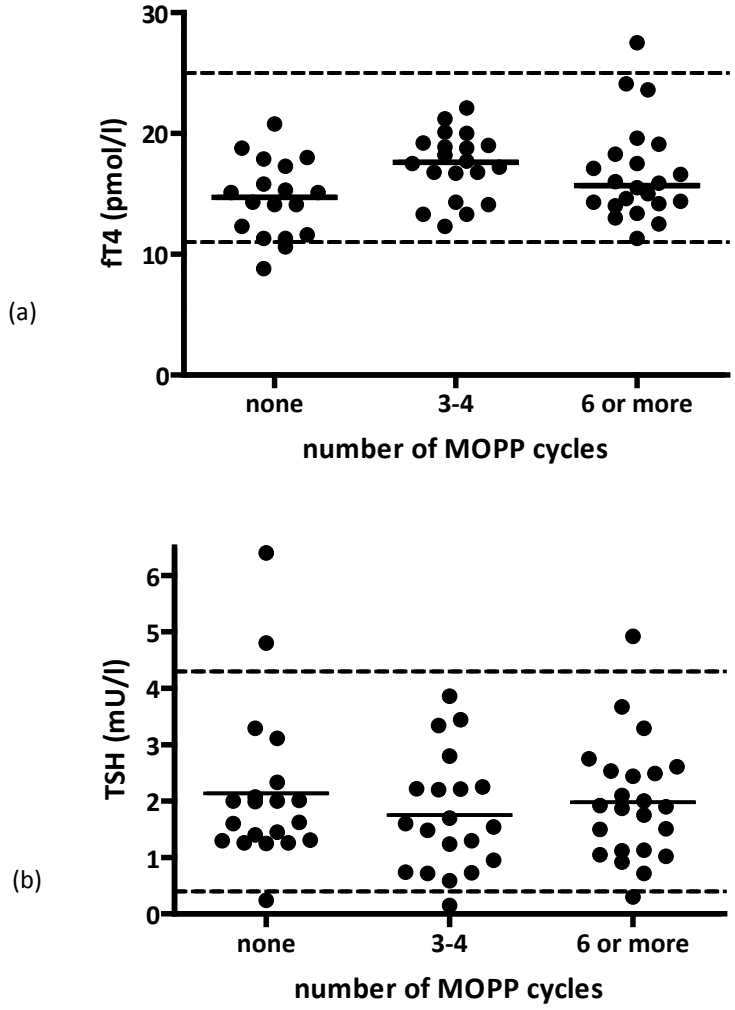

Figure 5. Free thyroxine (a) and thyroid stimulating hormone (b) levels in all long-term survivors of pediatric Hodgkin's lymphoma according to number of MOPP cycles received. *Straight lines indicate median values, dotted lines indicate normal values.*

Radiotherapy is known to cause loss of height [34] and a reduced final height [35]. In our study patients who were treated with radiotherapy because of relapse or refractory disease, were similar in final height when compared to those treated

with chemotherapy only. Unfortunately, no data on target height were available to calculate loss of height potential.

The present study is the first to describe BMD in male HL patients treated during childhood with chemotherapy only. BMD appeared to be normal for male patients. Holmes *et al.* did show a reduced BMD in adult men after MVPP chemotherapy, combined with radiotherapy in a small number of patients who were examined after a relatively short follow-up of only 3 years [36].

Only female MOPP+ patients had a significantly decreased total body BMD. After correction for bone size, there was a trend for BMAD to be decreased in female MOPP+ patients (p=0.011). In other patient groups treated with chemotherapy containing corticosteroids, such as children with acute lymphoblastic leukemia (ALL), BMD decreases during therapy and recovers after cessation of therapy, resulting in normal BMD [15]. Previous studies in female patients showed that BMD is decreased after treatment for HL at adult age, however this was mainly due to premature ovarian failure and not due to a direct effect of the therapy on the bone itself [37, 38]. This could also play a part in our female MOPP treated patients since we previously reported low AMH levels in these women indicating an impaired ovarian function [39].

Only one study reported on body composition in 23 survivors of childhood HL (treated with radiotherapy also) and showed an increased percentage body fat [40]. This finding was confirmed by our larger study of 88 patients. Treatment with prednisolone is reported to be a risk factor for increased percentage body fat in other groups of cancer survivors, mainly ALL [32, 40]. In male patients treated with MOPP that contained prednisolone percentage body fat tended to be higher. However we did not find a significant difference in percentage body fat between patients treated with and without MOPP. This indicates that prednisolone is not the only determinant of increased percentage body fat in patients treated for HL. A possible explanation for these differences found according to type of cancer (ALL versus HL) is that the total cumulative dose of prednisolone used to treat HL is much lower and the duration of the therapy is markedly shorter than in ALL [15]. Nysom *et al.* found normal levels of BMI in spite of an increased percentage fat and suggested it might be related to a decreased lean body mass, although this was not directly measured [40]. In our study lean body mass was normal in all patients, while BMI was significantly increased in male patients treated with MOPP. This might be partly explained by the impaired gonadal function in these MOPP treated male patients [41], although

a higher caloric intake cannot be excluded as a cause of the higher BMI since caloric intake was not assessed in these patients.

Our data showed that chemotherapy alone has no effect on thyroid function in patients treated for HL. Conversely, it is possible that radiotherapy has a negative effect on the thyroid. Of the six patients that either had abnormal levels of TSH or fT4 or used thyroid hormones, five also received radiation to the thyroid. Surprisingly, one man treated with ABVD only, without radiotherapy, used thyroid hormone. This could be a coincidence, however there is some evidence for an association between auto-immune mediated thyroid disease and HL (20), which may explain this phenomenon. No thyroid malignancies were found by palpation in any of our patients during the regular consultations at the long-term effects outpatient clinic, but imaging studies were not done. However, since thyroid malignancies tend to develop long after treatment (usually >20 years after treatment; [42]), even longer follow-up is needed to assess whether or not there is an increased risk for these malignancies after chemotherapy only.

In conclusion, after a median follow-up of 15 years, patients treated for HL at childhood age with chemotherapy alone, only male patients treated with MOPP have a decreased height with increased BMI. Lean body mass was normal in all patients. BMD was normal in male patients whereas in females treatment with MOPP was associated with a decreased total body BMD and lumbar spine BMAD. In patients treated with chemotherapy only the thyroid function was normal.

Acknowledgement

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Chapter 6

Inhibin B is a Valuable Serum Marker for Gonadal Dysfunction in Men Treated for Hodgkin's Lymphoma with Chemotherapy During Childhood

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Abstract

A long-term side-effect of both radiotherapy and chemotherapy is gonadal dysfunction. Aim of this study is to evaluate the long-term gonadal side-effects in 56 male patients treated from 1974-1998 for childhood Hodgkin's lymphoma with combination chemotherapy ABVD or EBVD (adriamycine or epirubicin, bleomycin, vinblastine and daunorubicin) with or without MOPP (mechlorethamine, vincristine, procarbazine and prednisolone) without radiotherapy using the best available markers. Patients were studied 15.5 yr (range 5.6-30.2 yr.) after cessation of therapy. Serum follicle stimulating hormone (FSH), luteinizing hormone (LH), inhibin B, testosterone and sex hormone binding globulin (SHBG) were determined. In men treated with MOPP, median FSH (16.6 U/l) and LH (5.7 U/l) were significantly ($p<0.001$) increased as compared to patients treated without MOPP (2.4 U/l and 2.5 U/l, respectively). Inhibin B (17.5 ng/l vs. 143 ng/l; $p<0.001$) and sperm concentration ($1.05*10^6$/ml vs. $49.5*10^6$/ml; $p<0.05$) were significantly decreased in patients with vs. patients treated without MOPP. Number of MOPP courses was correlated with FSH and inhibin B levels. Inhibin B showed a stronger correlation with sperm concentration (r_s=-0.83; $p<0.001$) than FSH. In conclusion, the use of MOPP chemotherapy causes permanent gonadal damage in men and inhibin B is the most valuable serum marker for gonadal function.

Introduction

The treatment of pediatric Hodgkin's lymphoma consists of radiotherapy, chemotherapy or a combination of both. With the current treatment strategies, childhood Hodgkin's lymphoma currently has an event free survival rate and an overall survival rate of over 90% [1-4]. Consequently, the improved survival rates for Hodgkin's lymphoma has led to a growing attention for long-term side effects [5].

An important long-term effect of both radiotherapy and chemotherapy in males is testicular dysfunction, which may subsequently result in infertility or subfertility [6]. Especially chemotherapy protocols containing alkylating agents (e.g. mechlorethamine or procarbazine) are known to cause severe gonadal damage in both adult and prepubertal patients [6, 7]. Even though spermatogenesis is not functional before puberty, the germinal epithelium can be

damaged irreversibly when alkylating agents are administered during early childhood due to the ablation of the non-proliferating spermatogonia [8-11]. Up to now, studies on gonadal function after treatment for pediatric Hodgkin's lymphoma mainly focus on patients treated with combined modality treatment and not with chemotherapy alone [1, 12-18].

Most follow-up studies of long term survivors of childhood cancer have assessed gonadal function and fertility using combinations of parameters like testicular volume, semen analysis and/or serum levels of luteinizing hormone (LH), follicle stimulating hormone (FSH) and testosterone. An increase in serum FSH is considered the first indirect indicator of testicular dysfunction [19]. In the last decade inhibin B has been identified as a good direct marker for assessment of gonadal function in andrology and fertility clinics. Inhibin B is produced by Sertoli cells, and is strongly correlated with spermatogenesis [19-21]. To date no studies are available in pediatric Hodgkin's Lymphoma using inhibin B.

Andrological evaluation of the infertile male routinely consists of classic sperm analysis according to World Health Organization (WHO) guidelines [22]. In recent years sperm DNA integrity has received growing attention as an additional diagnostic tool for the fertilizing potential of spermatozoa. Several studies have documented increased levels of sperm DNA fragmentation in male infertility [23-25]. Although chemotherapy leads to mutagenic effects in animal studies, little is known about sperm DNA damage that may prevail after childhood Hodgkin's lymphoma and consequent chemotherapy treatment [26]. It might be that persistent sperm DNA damage may either lead to impaired fertility or be transmitted to the offspring causing developmental abnormalities and cancer in the offspring [27], although after treatment with radiotherapy no such effect has been observed [28]. Scrotal ultrasound can also be valuable in identifying causes for male infertility [29, 30]

Aim of this study is to evaluate the long-term gonadal sequelae after treatment for childhood Hodgkin's lymphoma with combination chemotherapy only, using up to date fertility parameters and andrological evaluation, including for the first time inhibin B.

Patients and methods

Patients

A total of 100 male long-term survivors treated from 1974-1998 for pediatric Hodgkin's lymphoma in the Erasmus MC-Sophia Children's Hospital and AMC-Emma Children's Hospital were identified. From this group 13 patients refused participation (13%) and 31 patients were lost to follow-up (31%). There were no differences in age, disease characteristics and treatment between the included 56 male survivors and the 44 not included (table 1). Written informed consent was obtained from all participants, according to protocols approved by the ethical review board of the Erasmus MC. At the time of the study all patients were in complete remission > 5 years after therapy (median follow-up time 15.5 yrs.; range 5.6-30.2 yrs.) The median age at diagnosis was 11.4 yrs. (range 3.7- 15.9 yrs.). The median age at follow-up was 27.0 yrs. (range 17.7-42.6 yrs.).

Therapy

All patients were treated with chemotherapy as previously described (1,2) with ABVD or EBVD (adriamycine 25 mg/m^2, (epirubicin 30 mg/m^2 in EBVD), bleomycin 10 mg/m^2, vinblastine 6 mg/m^2 and dacarbazine (DTIC) 250 mg/m^2 on day 1 and 8 and with or without MOPP (mechlorethamine 6 mg/m^2 and vincristine 2 mg/m^2 (max. 2.5 mg/dose) on day 1 and 8, prednisolone 40 mg/m^2/day and procarbazine 100 mg/m^2/day on days 1-14 (1,2). Seven patients received additional involved field radiotherapy because of treatment failure with chemotherapy only, disease progression or relapse. None of the patients was treated with irradiation to the abdominal or pelvic region. Patients were categorized into 3 treatment groups according to the number of MOPP cycles: no MOPP (n=16), 3-4 MOPP (n=14) and ≥ 6 MOPP cycles (n=26). None of the patients were treated with 1,2 or 5 MOPP cycles.

History and measurements

All participants completed a detailed questionnaire regarding fertility-related disease or surgery, paternity and medication. Medical records were reviewed to establish the pubertal stage at diagnosis, the disease stage at diagnosis, the presence of B-symptoms (fever, night sweats and/or loss of >10% of body weight), age at diagnosis and duration of follow-up. Puberty was defined as Tanner stage G2 and higher or age above 14 years.

Table 1. Comparison between included and not included Hodgkin's lymphoma patients

		Included	Not included
Number		56	44
Histology	NS	71.4%	56.8%
	MC	19.6%	20.2%
	LP	1.8%	6.8%
	LD	3.6%	-
	Unknown/NOS	3.6%	15.9%
Stage	I	39.3%	45.5%
	II	30.4%	22.7%
	III	26.8%	9.1%
	IV	1.8%	6.8%
	Unknown	1.8%	15.9%
Radiotherapy (y)		19.8%	14.8%
Relapse (y)		5.8%	6.6%
Age at diagnosis		11.4 y	10.7 y
		(3.7-15.9)	(3.2-16.6)

NS = nodular sclerosing, MC = mixed-cellularity, LP = lymphocyte predominant, LD = lymphocyte depleted, NOS = not otherwise specified. No significant differences; Histology X^2*=3.61, p=0.31; Stage* X^2*=5.99, p=0.11*

Blood samples, obtained by venous puncture, were processed within 2 hours after withdrawal and stored at -20˚C until assay. Blood samples were tested for FSH, LH, and sex hormone binding globulin (SHBG) using fluorescence-based immunometric methods (Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA), for inhibin B using an enzyme-immunometric method (Serotec, Oxford, UK) and testosterone, using a coated tube radioimmunoassay (Diagnostic Products Corporation). Bioavailable testosterone was calculated according to the method of Södergard et al. [31].

Andrological examination

Semen analysis was performed in 21 patients. Fresh sperm samples were produced by masturbation after 3-5 days of abstinence. After liquefaction, sperm concentration, motility and morphology were assessed within one hour according to WHO guidelines [22]. An aliquot of the semen sample was stored at -80°C for later analysis of sperm DNA damage.

Further andrological examination, including scrotal ultrasound was performed in 18 of these patients (three patients refused participation for this part of the study). Testicular volume was measured using a Prader orchidometer. Scrotal ultrasound was performed using a Toshiba Nemio 20 with a 12 Hz transducer. The ultrasonic volume of each testis was calculated from 3 perpendicular measurements in the equation V (ml) = π x length x with x depth (all in cm)/6. The mean bilateral testicular volume was calculated and used for further analysis. Epididymal caput diameter was recorded as indicative for obstruction whenever a diameter above 12 mm was found. A varicocele was diagnosed when at least 2 venous vessels with a diameter of at least 3 mm were present, in addition to reflux or diameter increase during Valsalva's maneuver. The testicular parenchyma was scored as normal texture, infertile texture, low grade microlithiasis (<5 per testis), medium grade microlithiasis (5-10 per testis), high grade microlithiasis (>10) and landscape texture microlithiasis (25).

Sperm DNA integrity

Sperm DNA integrity was measured using the Sperm Chromatin Structure Assay (SCSA) as previously described by Evenson [24]. In short, the assay is based on the higher susceptibility of defective sperm chromatin for acid DNA denaturation. Following acid denaturation sperm DNA is stained with a fluorescent dye, acridine orange (AO), which emits green fluorescence when bound to double stranded DNA but shifts to red fluorescence when bound to single stranded DNA. Sperm DNA damage is quantified by flow cytometric measurements of green and red fluorescence in each sperm cell nucleus. The extent of DNA fragmentation is then calculated as the ratio of red to total (red plus green) fluorescence and is expressed as the DNA Fragmentation Index (DFI). SCSA was performed in 7 out of the 21 sperm samples. In 10 patients SCSA could not be performed due to azoospermia, while in 4 patients no material for SCSA was available after semen analysis.

Statistical Analysis

Statistical analysis was performed using SPSS 12.01 software (SPSS, Chicago, IL). Differences in histology and disease stage between the included patients and those not included was tested using X^2-test. Differences between treatment groups were tested using Mann-Whitney U tests and correlations were tested using Spearman's correlation. Kruskal-Wallis tests were used to test for trends.

Regression analysis was performed to test the effect of different parameters on laboratory measurements (LH, FSH, inhibin B and sperm concentration). P-values <0.05 were considered significant.

Results

A total of 37 patients were pubertal at diagnosis, whereas 15 patients were prepubertal. Of four patients (ages 12.3 - 13.7 yrs. respectively), no data were available on pubertal status at start of therapy. No data on Tanner stage during therapy were available. Patients with B-symptoms (n=16) had a significantly higher disease stage (12.5% stage I; 43.8% stage II; 33.5% stage III; 12.5% stage IV) as compared to patients without B-symptoms (55.2% stage I; 31.0% stage II; 13.8% stage III; 0% stage IV; X^2=10.4; p<0.05).

Table 2 shows the median values of serum hormone and sperm concentration of patients either treated with (MOPP+) or without MOPP (MOPP-) and normal reference values as used in the Erasmus MC. Median luteinizing hormone (LH) and follicle stimulating hormone (FSH) values were significantly increased in MOPP+ patients as compared to MOPP- patients (p<0.01), who all had normal to marginally increased LH and FSH levels. Median inhibin B levels were significantly lower in MOPP+ patients as compared to MOPP- patients (p<0.01). Levels of SHBG were normal in all patients, whereas concentrations of testosterone and bio-available testosterone were normal to marginally decreased and not different between MOPP+ and MOPP- patients.

The median sperm concentrations were significantly lower in MOPP+ patients as compared to MOPP- patients (Table 2). Compromised sperm concentrations were found in 77% of the men treated with MOPP. In contrast, normospermia was found in all 4 men treated without MOPP. Azoospermia was found in 9 out of 17 (53%) MOPP+, oligozoospermia (< 20 x 10^6/ml) in 1 out of 17 (6%) and severe oligozoospermia (< 5 x 10^6/ml) in 3 out of 17 (18%) MOPP+ patients. In 4 out of 17 MOPP+ patients (23%) normospermia was found, three of these men were treated with 3 MOPP cycles, one was treated with 6 MOPP cycles (age at diagnosis 6.8 yrs., follow-up time 27.7 yrs.).

Table 2. Laboratory measurements of patients treated with and without MOPP

	MOPP – (n=16)	MOPP+ (n=40)	Normal values[$]
LH (U/l)	**2.5** (1.2-9.0)	**5.9**** (1.68-15.0)	**1.5-8.0**
FSH (U/l)	**3.0** (1.7-6.0)	**16.8**** (1.3-51.0)	**2.0-7.0**
Inhibin B (ng/l)	**144.0** (93.0-274.0)	**16.5**** (0.0-173.0)	**150-400**
Testosterone (nmol/l)	12.0 (8.8-22.0)	13.6 (6.2-21.9)	10-30
SHBG (nmol/l)	23.0 (11.1-32.2)	23.1 (8.3-59.8)	10-70
Bio-available Testosterone (nmol/l)	8.5 (5.9-12.7)	9.3 (4.5-11.0)	5-18
Sperm concentration (*10^6 /ml) [#]	**49.1** (28-63)	**1.1*** (0-72)	**> 20 *10^6**
Testicular volume (ml) [##]	18.0 (9.0-25.0)	12.9 (9.0-30.0)	15-20

*Median values (range). MOPP- = treated without MOPP, MOPP+ = treated with MOPP. LH = luteinizing hormone, FSH = follicle stimulating hormone, SHBG = sex hormone binding globulin. $ = reference values as used in the Erasmus MC. * = p<0.05; ** = p<0.01 MOPP- vs. MOPP+. #: Data on sperm concentration were available for 4 patients treated without MOPP and 17 patients treated with MOPP. ##: Data on testicular volume were available for 8 patients treated without MOPP and 26 patients treated with MOPP*

Figure 1 shows LH, FSH, inhibin B and sperm concentration in nrelation to the number of MOPP cycles. LH and FSH increased, and inhibin B levels and sperm concentration decreased significantly with an increasing number of MOPP cycles (Kruskal-Wallis for trend p<0.001 for all). MOPP- patients had significantly lower LH and FSH and significantly higher inhibin B and sperm concentration as

Table 3. Known studies on gonadal damage in males treated for childhood Hodgkin's lymphoma

Author	*N* [a]	*Age (range)* [b]	*Median F-up*	Therapy[c]		*Gonadal evaluation*[d]						
						Semen analysis			*Serum markers*			
				CT	RT	N	Azo	Oligo	N	LH	FSH	Inh B
Heikens et al. [15]	19	11 yrs. (5-15 yrs.)	10 yr	MOPP	No	18	67%	33%	19	↑	↑	-
Ortin et al. [16]	148	13 yrs. (2-15 yrs.)	9 yr.	MOPP	12	15	60%	15%	148	↑	↑	-
Shafford et al. [18]	30	10 yrs. (4-15 yrs.)	11.8 yrs.	ChlVPP	10	13	91%	9%	30	↑	↑	-
Papadakis et al. [17]	36	13 yrs. (2-22 yrs.)	6.8 yrs.	MDP	6	2	100%	-	36	↑	↑	-
Ben Arush et al. [12]	20	2-16 yrs.		MOPP/ABVD COMP MOPP	4	?	40%	40%	20	↑	↑	-
Cicogagnani et al. [13]	11	10.3 yrs. (3-15 yrs.)	5.5 yrs.	ABVD MOPP/ ABVD	0	-	-	-	11	↑	↑	↓[f]
Van den Berg et al. [1]	14	14 yrs. (5-18 yrs.)	5.1 yrs.	MOPP/ ABVD	No	-	-	-	14	=[e]	=[e]	-
Gerres et al. [14]	46	14.9 yrs.	1.9 yrs.	OEPA	0	-	-	-	46	↑[f]	↑[f]	-
This study	56	11.4 yrs. (3.7-15.9 yrs.)	15.5 yrs.	OEPA/ COPP A(or E)BVD MOPP/A(or E)BVD	0	21	53%[f]	6%[f]	56	↑[f]	↑[f]	↓[f]

Legend to table 3 ? = unknown, - = not studied; a) number of male patients; b) age at diagnosis (median and range); c)CT = chemotherapy: MOPP = mechlorethamine, vincristine, procarbazine, prednisolone; COPP = MOPP cyclophosphamide replaces mustine; COMP = COPP, methotrexate replaces procarbazine; ABVD = adriamycine, bleomycine, vinbalstine, dacarbazine; MDP = doxorubicin, procarbazine, prednisolone, vincristine, cyclophosphamide; ChlVPP = chlorambucil, vinblastine, procarbazine, prednisolone; OEPA = vincristine, VP-16, prednisolone, doxyrubicin; OPPA = OEPA procarbazine replaces VP-16. RT = number of patients with gonadal radiotherapy; d) N= number of patients tested, azo = azoospermia, oligo = oligospermia, LH = luteinizing hormone, FSH = follicle stimulating hormone, inh B = inhibin B. Arrows indicate increased (↑), decreased (↓) or normal values (=); e)Only 2 patients with increased levels of FSH and 1 with an increased level of LH; f)Only in patients treated with MOPP or COPP

compared to MOPP+ patients. No significant differences in LH, FSH, inhibin B or sperm concentration were found between men treated with 3 MOPP courses and men treated with 6 MOPP courses.

Laboratory parameters were compared with semen concentrations (fig. 2). Both LH and testosterone were not significantly related with sperm concentrations. Significant correlations were found between sperm concentration and FSH (r= -0.67 95% CI: -0.85 – -0.30; p<0.001) and inhibin B (r=0.86, 95% CI 0.94 - 0.65; p<0.001). In a multivariate analysis with inhibin B and FSH as determinants of sperm concentration, inhibin B was the only significant determinant (β = 1.61; p<0.001).

The median DFI in the studied men was 10% (5.67% - 25.55%) and did not differ from 20 healthy controls with normospermia: 12% (6.85% - 34.45%). All participants with normospermia had DFI levels in the normal, fertile range (<15%), while the 2 men with oligospermia had moderate DFI levels (15-30%) of 24% and 26% respectively.

History and andrological examination revealed several confounders for fertility in this Hodgkin's lymphoma survivor group. Two patients reported a history of bilateral orchidopexy (1 MOPP-, 1 MOPP+) and in one patient, physical examination and scrotal ultrasound revealed a left sided varicocele grade I. The latter patient was treated with 8 MOPP cycles and oligospermia was found. Furthermore, scrotal ultrasound revealed low-grade testicular microlithiasis defined as <5 per testis in 5 out of 18 patients. In 2 patients unilateral inhomogeneous, infertile parenchyma was found. These men were diagnosed with respectively azoospermia and severe oligospermia. Bilateral infertile

testicular parenchyma was found in 3 out of 18 patients of whom two had azoospermia and one was diagnosed with severe oligospermia (all MOPP+). None of the results of physical examinations or scrotal ultrasound indicated obstruction of the vas deferens.

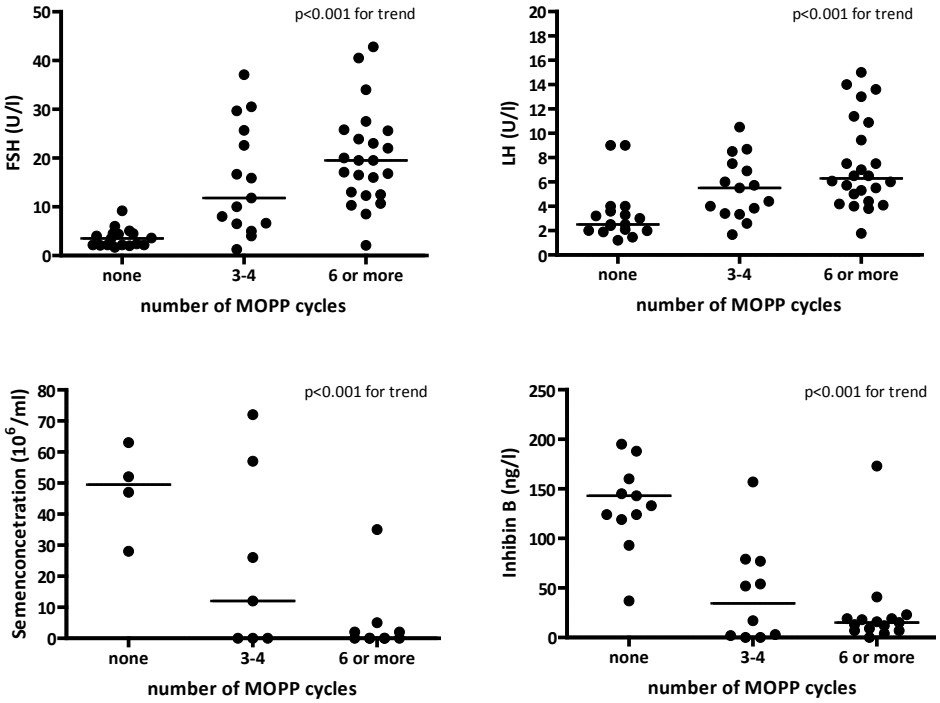


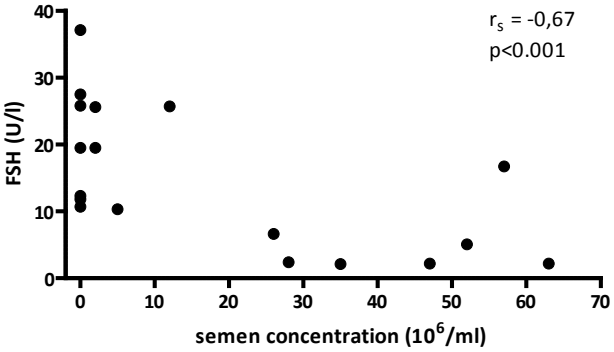
Figure 1. hormone levels and semen concentration in relation to number of MOPP cycles. *Lines indicate median values. Kruskal-Wallis test for trend between the three groups showed significance (p<0.001 for all parameters).*

The 8 MOPP- patients and 26 MOPP+ patients for whom data on testicular volume was available, did not significantly differ in their testicular volume (table 2). One of eight MOPP- patient had a testicular volume below the normal reference value, whereas 16 of 26 MOPP+ patients had testicular volumes below the normal reference value. None of the fertility-related parameters assessed in this study, i.e. FSH, inhibin B levels and sperm concentration were associated with testicular volume measurements.

The outcome of fertility parameters assessed at follow-up did not differ between patients treated during puberty and patients treated before puberty.

Patients with B-symptoms at diagnosis had significantly lower inhibin B levels ($p<0.05$) and sperm concentration ($p=0.05$) as compared to patients who did not present with B-symptoms. A multiple regression analysis was performed on the different hormonal measurements and sperm concentration considering the number of MOPP cycles, number of EBVD/ABVD cycles, puberty at start of diagnosis, age at diagnosis, disease stage at diagnosis, the presence of B-symptoms and duration of follow-up. Age at diagnosis was a significant determinant of FSH ($\beta= 1.4$; $p<0.05$) and sperm concentration ($\beta = -6.18$; $p<0.05$). The number of MOPP cycles significantly determined LH levels ($\beta= 0.87$; $p<0.01$), FSH levels ($\beta= 2.57$; $p<0.01$), inhibin B levels ($\beta= -21.59$; $p<0.05$) and sperm concentration ($\beta= -6.25$; $p<0.05$).

a.



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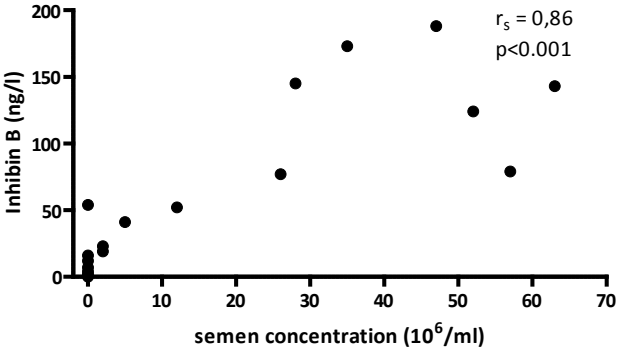

Figure 2. Spearman correlation between sperm concentration and FSH (a) and inhibin B

Alkylating agents are known to cause severe gonadal damage in both adults and children [6, 7]. It is well documented that adult men treated with

MOPP combination chemotherapy suffer from permanent gonadal damage [8, 18, 32]. Several earlier studies reported on gonadal function after treatment for pediatric Hodgkin's lymphoma (table 3), but these were relatively small and did not include up-to-date markers like inhibin B. The present study shows that inhibin B is a better serum marker for spermatogenesis than FSH in male survivors of childhood Hodgkin's lymphoma.

In the questionnaire, all men reported normal pubertal development. Five men reported 7 pregnancies. Two of the reported pregnancies were conceived by donor sperm (both in men treated with 6 MOPP cycles). Of the five spontaneous conceived pregnancies 2 men treated without MOPP each fathered one healthy child and the man treated with 6 MOPP cycles fathered one healthy child and reported two spontaneous abortions.

Discussion

Previous studies primarily used FSH levels as a marker for fertility. The frequency of elevated FSH levels after chemotherapy with alkylating agents varies from 35-100% and increases with increasing cumulative doses of alkylating agents [14, 33, 34]. The correlation between FSH levels and the number of MOPP courses was confirmed in our study. Patients treated without MOPP, had normal FSH levels whereas after treatment with MOPP 75% of the patients showed increased FSH levels.

In addition, we analyzed whether inhibin B would better predict spermatogenesis than FSH. Literature data suggest that the suppression of spermatogenesis by chemotherapy is accompanied by a decrease in serum levels inhibin B [35]. Studies relating testicular histology with spermatogenesis support this assumption [19, 36]. Our results show that male survivors of Hodgkin's lymphoma treated at childhood age with alkylating agents have significantly lower levels of inhibin B when compared to men not treated with these agents. In addition, we showed that inhibin B level is a stronger and more independent determinant of sperm concentration compared with FSH level. Inhibin B level therefore appears to be the better fertility marker, which can be used as a screening method to detect gonadal damage in men treated for childhood Hodgkin's lymphoma. Our findings in this long term pediatric Hodgkin's lymphoma follow-up cohort confirm earlier studies in adults which showed that inhibin B concentrations are correlated with sperm concentrations in both healthy men

[20] as well as in men with fertility problems [19]. Pierik *et al*. showed that in men with fertility problems inhibin B is a better marker of spermatogenesis than FSH [19]. So far, apart from a study of Cicognani *et al.* who showed similar results in a small group (n=11) of Hodgkin's lymphoma patients [13], little data exists on inhibin B levels in men after treatment for Hodgkin's lymphoma. Finally, it is noteworthy that in our study, all male survivors with inhibin B levels above 75 ng/ml had normospermia.

Some studies suggested that the prepubertal testis may be less susceptible to germinal damage from chemotherapy than pubertal or post-pubertal testes [37], whereas others did not [15]. In our study, pubertal stage at diagnosis and start of treatment did not influence gonadal function. However, since older age at diagnosis was one of the independent determinants of sperm concentration and FSH levels, maturational status of the testis might still be of some influence.

Adult studies showed that 75-100% of the patients treated with alkylating agents have a irreversibly compromised spermatogenesis [8, 18, 32]. Recovery of spermatogenesis after chemotherapy in adulthood has been reported, but only after chemotherapy without or with low doses of alkylating agents [10, 38, 39]. In the present pediatric study, two patients had normal inhibin B and FSH levels and normospermia despite a high number of MOPP courses. These two men had the longest follow-up times of respectively 27 and 28 years. This might indicate that late recovery of spermatogenesis might occur following MOPP chemotherapy. Late recovery has been described earlier in a cohort of 47 men treated in adulthood after a follow-up of more than 10 years [11].

One could raise some concern that cytotoxic chemotherapy or the disease itself might result in transmissible genetic damage. Although Kobayashi *et al*. found more pronounced sperm DNA damage in 11 Hodgkin's lymphoma patients prior to treatment as compared to controls [40], little information is known about sperm DNA damage after chemotherapy [41]. After chemotherapy for (adult) testicular cancer sperm DNA damage has been observed [42]. Thomson et al. found that sperm concentration was reduced, but the sperm DNA integrity was comparable to that in healthy controls in survivors of childhood cancer [43]. Our study confirms these results in survivors of childhood Hodgkin's lymphoma. Although the sperm chromatin structure assay cannot assess chromosomal abnormalities like aneuploidy or more subtle chromosomal damage or mutations, we can assume that men who regain spermatogenesis long after

chemotherapeutic treatment for Hodgkin's lymphoma have a normal chromatin structure. Moreover, studies in other cancer survivors, treated with high dose radiotherapy, did not show an increased risk of birth defects in offspring [28, 44]

In our study all testosterone levels as well as levels of bio-available testosterone were within the normal range or marginally decreased. However, LH levels were significantly elevated in patients treated with MOPP as compared to patients treated without MOPP. This increase of LH levels indicates that, although testosterone levels are normal, some Leydig cell damage has occurred [34, 45]. Although these men might be at risk of androgen deficiency in later life, we also showed that LH is inferior to FSH and inhibin B in detecting testicular damage.

Patients presenting with B-symptoms at diagnosis had significantly lower fertility parameters compared to patients who did not present with B-symptoms at diagnosis. This might be related to a lower semen quality before start of therapy associated with B-symptoms [46-49], or to the higher number of MOPP courses that these patients received because of higher disease stages. When correcting for other factors in the multivariate analysis, the presence of B-symptoms was not an independent factor determining a lower fertility on the long term.

We conclude that this is the first study in which both spermatogenesis and serum inhibin B levels were assessed in a group of long-term survivors of pediatric Hodgkin's lymphoma. Inhibin B is a good serum marker, superior to FSH, for spermatogenesis in men treated for childhood Hodgkin's lymphoma with combination chemotherapy. However, since men with mildly decreased inhibin B levels might still have sufficient sperm concentration for assisted reproduction or even natural conception, we recommend that semen analysis should be performed to determine treatment modalities in men with wish for paternity.

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Chapter 7

Anti-Müllerian Hormone is the Most Sensitive Serum Marker for Gonadal Function in Women Treated for Hodgkin's Lymphoma During Childhood

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Abstract

PURPOSE In recent years, anti-Müllerian hormone (AMH) and inhibin B became available as markers for ovarian reserve. Aim of this study is to evaluate the long-term effects of combination chemotherapy treatment for girls with Hodgkin's lymphoma on gonadal function using these ovarian reserve parameters.
PATIENTS AND METHODS Luteinizing hormone (LH), Follicle-stimulating hormone (FSH), inhibin B and AMH were measured in 32 women treated from 1974-1998 for pediatric Hodgkin's lymphoma (HL) with chemotherapy only. All patients (median age 25.0 yr (range 19.2-40.4 yr.)) were in complete remission with a median follow-up time of 14.0 yr. (range 5,7-24.5 yr.) after therapy. All patients were treated with combination chemotherapy ABVD or EBVD (adriamycine or epirubicin, bleomycin, vinblastine and dacarbazine) with or without MOPP (mechlorethamine, vincristine, procarbazine and predisolone). Because of incomplete remission or relapse, involved field radiotherapy was needed in seven of 32 women. Results were compared with a healthy control group.
RESULTS Patients treated with 6 or more cycles of MOPP combination chemotherapy had significantly higher levels of FSH and lower serum levels of inhibin B and AMH as compared to healthy women (FSH 17 U/l vs. 5.95 U/l ($p<0.05$); inhibin B 23 ng/l vs. 112.5 ng/l ($p<0.01$); AMH 0.39 μg/l vs. 2.10 μg/l ($p<0.01$)). AMH was also significantly lower as compared to women treated without MOPP (median 0.39 μg/l vs. 1.40 μg/l; $p=0.01$).
CONCLUSIONS Women treated during childhood for HL with MOPP seem to have a distinctly lower ovarian reserve as measured by lower AMH values at early adulthood, compared to healthy women. Moreover, AMH seems to be the only predictor that is sufficiently sensitive to detect this decrease in ovarian reserve.

Introduction

The majority of treatment protocols for Hodgkin's lymphoma (HL) consist of a combination of chemotherapy and radiotherapy. Using this strategy, childhood HL currently has an event free survival of about 90-95% and an overall survival of up to 96% [1-4]. Because of the improved survival rates, long-term side effects are an important issue [5]. Most of the studies report on late effects in adult HL patients, and little is known on the endocrine long-term effects in children treated for HL. In adults, an important long-term effect of both radiotherapy and chemotherapy

is decreased ovarian function, especially after chemotherapy protocols containing high cumulative doses of alkylating agents (e.g. MOPP or COPP) [6-9].

Usually, gonadal function is measured in follow-up studies of long-term survivors of childhood cancer by analysis of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). However, neither LH nor FSH correctly reflects the ovarian reserve [10]. In recent years, two new markers for ovarian function became available. Inhibin B, which is solely produced by granulosa cells of small antral follicles, is decreased in women with known fertility problems (e.g. premature ovarian failure) and undetectable in postmenopausal women [11-13]. Inhibin B is one of the first endocrine markers to change in perimenopausal women, even before changes in FSH levels can be detected [14]. The second new marker is anti-Müllerian hormone (AMH). This hormone is produced by granulosa cells of early developing (pre-)antral follicles of the ovary, and levels decrease when the number of follicles decreases with age [15]. A recent study showed a strong correlation between age at menopause and AMH levels randomly measured during the reproductive lifespan (ages 20-36 yr.) in a group of healthy women [16].

The aim of this study was to evaluate the gonadal long-term effects in women after treatment for childhood HL with combination chemotherapy without radiotherapy using inhibin B and AMH as predictors of ovarian reserve.

Patients and methods

Patients and treatment protocols

From a total group of 151 pediatric HL patients, 51 female long-term survivors treated from 1974-1998 in Erasmus MC-Sophia Children's Hospital and Emma Children's Hospital-AMC were identified. From this group 11 patients refused participation (21.5%) and eight patients were lost to follow-up (15.7%). There were no significant differences in age and disease characteristics between the included 32 female survivors and those not included (table 1).

Two of the 32 included women were pregnant at time of study. Because pregnancy is known to affect serum concentrations of the hormones involved in the pituitary-ovarian axis, no laboratory parameters were included of these women. All patients were in complete remission with a median duration of 14.0 years (range 5.7-24.5 yr.). The median age at follow-up was 25.0 yr (range 19.2-40.4 yr.). All patients were treated with combination chemotherapy ABVD

(adriamycine, bleomycin, vinblastine and dacarbazine) or EBVD (epirubicin, bleomycin, vinblastine and dacarbazine) with or without MOPP (mechlorethamine, vincristine (oncovin), procarbazine and prednisone) as previously described[1,4]. ABVD included adriamycine 25 mg/m^2, (epirubicin 30 mg/m^2 in EBVD), bleomycin 10 mg/m^2, vinblastine 6 mg/m^2 and dacarbazine (DTIC) 250 mg/m^2 on day 1 and 8. MOPP included mechlorethamine 6 mg/m^2 and vincristine 2 mg/m^2 (max. 2.5 mg/dose) on day 1 and 8, prednisone 40 mg/m^2/day and procarbazine 100 mg/m^2/day on days 1-14 [1, 2]. The primary aim of this protocol was to maintain good survival rates but to avoid radiotherapy. However, seven of 32 patients received additional involved field radiotherapy because of incomplete remission with chemotherapy only (four treated with MOPP and one treated without MOPP), or relapse (two treated with MOPP). None of the patients received irradiation below the diaphragm. Scatter irradiation to the gonads is approximately 0.5% of the dose. Patients were categorized into 3 treatment groups according to the number of MOPP cycles: no MOPP (n=7), 3-4 MOPP (n=14) and ≥6 MOPP cycles (n=9). Only one patient was treated with 4 MOPP courses and none of the patients were treated with 1,2 or 5 MOPP cycles. The medical ethical committee approved this study and informed consent was obtained from all patients according to the Helsinki agreement.

Levels of FSH, LH, estradiol, inhibin B and AMH were compared to normal values from a group of 41 healthy normo-ovalatory women (age 20-35 years; median age 29 years) not using oral contraceptives (OCPs) or any other hormonal treatment at least 3 months before start of the study, as described previously [15]. Samples were drawn on the third day of the menstrual cycle in all control subjects.

Laboratory measurements

Blood samples, obtained by vena puncture, were processed within 2 hours after withdrawal and stored at -20˚C until assay. All blood samples were drawn on the 3rd-5th day of the menstrual cycle, or when women used oral contraceptives on the last day of the pill-free interval [17]. Endocrine screening included serum assays for FSH, LH (both fluorescence-based immunometric assays on the Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA), estradiol (coated tube radioimmunoassay, Diagnostic Products Corporation), inhibin B (enzyme-immunometric assay, Serotec, Oxford, UK) and anti-müllerian hormone (AMH, Diagnostic Systems Laboratories, Webster, TX). Intra- and interassay

coefficients of variation were less than 5% and 15% for LH, less than 3% and 8% for FSH, less than 5% and 7% for estradiol, less than 9% and 15% for inhibin B and less than 5% and 8% for AMH, respectively [15].

Table 1. Comparison between included and excluded female patients

		Included	Not included
Number		32	19
Histology	NS	84.4%	68.4%
	MC	6.3%	5.3%
	LP	6.3%	5.3%
	LD	-	-
	Unknown/NOS	3.1%	21.1%
Stage	I	15.6%	15.8%
	II	62.5%	42.1%
	III	18.8%	21.1%
	IV	3.1%	5.3%
	Unknown	-	15.8%
Radiotherapy		18.8%	15.8%
Relapse		9.4%	10.5%
Age at diagnosis		14.0 y	13.4 y
		(5.0-17.2)	(7.9-14.4)

NS = nodular sclerosing, MC = mixed-cellularity, LP = lymphocyte predominant, LD = lymphocyte depleted, NOS = not otherwise specified.

Questionnaires

All patients completed a questionnaire regarding previous pregnancies, menarche, menstrual cycle and the use of OCP. In addition, information was obtained on smoking, alcohol consumption, exposure to toxic agents or radiation and use of medication.

Statistics

Statistical analysis was performed using SPSS 12.0.1. Kruskal-Wallis tests were used to check for trends in the treatment groups and healthy controls. Differences between the separate treatment groups were tested using Mann-Whitney U tests. Differences between the groups in the data of the questionnaires were also tested using a Kruskal-Wallis test. A p-value <0.05 (two-tailed) was considered significant.

Results

Clinical characteristics

Patient characteristics at time of study are shown in table 2. Median values for body mass index were 23.6 kg/m^2 in patients treated without MOPP (MOPP-) and 24.0 kg/m^2 in patients treated with MOPP (MOPP+). Eighteen women used oral contraceptives and one woman used hormone replacement therapy. Three women treated with MOPP, currently using OCPs reported previous irregular menses. None of the 12 women, who were not using OCPs, had irregular menses. A total of 17 pregnancies were reported in 11 women, resulting in 10 healthy children; one pregnancy was ended electively, four pregnancies (of which three in one woman) ended in spontaneous abortion (23.5%) and two women were pregnant at time of the study. Two pregnancies (both MOPP+; 10 and 6 cycles respectively) were achieved using assisted reproductive techniques (1 intrauterine insemination, 1 *in vitro* fertilization with egg donation), both resulting in the delivery of a healthy live-born child. One woman used hormone replacement therapy because of overt premature ovarian failure. There were no significant differences in the use of alcohol, smoking or exposure to toxic agents between MOPP+ patients and MOPP- patients.

Hormone levels

Figure 1 shows the hormone levels for the different treatment groups and the healthy controls. No differences in LH levels between any of the treatment groups and healthy controls were found. FSH levels were significantly higher in patients treated with 6 or more MOPP cycles as compared to healthy controls ($p<0.05$), and as compared to those treated without MOPP ($p<0.05$). Trend analysis for higher FSH with increasing number of MOPP courses was not significant. Inhibin B levels were significantly lower in all treatment groups as compared to healthy controls ($p<0.05$ for 0 MOPP; $p<0.01$ for 3-4 MOPP; $p<0.01$ for 6 or more MOPP). There was a significant trend for lower inhibin B levels with increasing number of MOPP cycles ($p<0.001$). Estradiol levels were significantly lower in ($p<0.01$) as compared to healthy controls. AMH levels were significantly lower in both patients treated with 3-4 MOPP cycles ($p<0.05$) and patients treated with 6 or more MOPP cycles ($p<0.01$) as compared to healthy controls and as compared to MOPP- patients($p<0.05$ for 3-4 MOPP as well as for 6 or more MOPP; trend analysis $p<0.001$).

Table 2. Patient characteristics of included women

	MOPP- N=8	MOPP+ N=24	Total N=32
Median age at diagnosis (yr)	15	13.4	14.0
(range)	(8.3-16.4)	(5.0-17.2)	(5.0-17.2)
Median age at follow-up (yr)	25.5	24.4	25.0
(range)	(19.9-32.1)	(19.2-40.4)	(19.2-40.4)
Median Follow-up period (yr)	11.4	11.6	11.6
(range)	(5.7-17.7)	(6.2-24.5)	(5.7-24.5)
Pregnancies	2/2	15/9	17/11
(total number/number of women)		(2 assisted)	
Children (n)	2	8	10
Abortions	0	5/3	5/3
(total number/ number of women)			
OCP /HRT	5/0	13/1	18/1

MOPP- = no MOPP therapy, MOPP+ = MOPP therapy. OCP = oral contraceptive pill, HRT = hormone replacement therapy.

All women with increased FSH levels had decreased AMH levels, but three women with normal FSH levels had decreased AMH levels adjusted for age. No significant correlations were found between FSH and inhibin B (p=0.42). However AMH and FSH levels (r_s=-0.52; p=0.01) and AMH and inhibin B (r_s=0.43; p=0.04) were significantly correlated. Five women with decreased AMH levels had inhibin B levels within normal limits.

There were no significant differences in AMH or inhibin B levels between patients who received additional radiotherapy as compared to patients treated without additional radiotherapy (p=0.08 and p=0.31 respectively). LH and FSH levels were significantly higher in patients treated with additional radiotherapy as compared to those without (LH 21.1 vs. 12.3 U/l; p<0.05 and FSH 20.3 vs. 12.6 U/l; p<0.05).

Women using OCPs had significantly lower LH levels as compared to women not on OCPs (2.2 vs. 5.9 U/l; p<0.05), although both median values where within normal range. There were no significant differences in FSH, inhibin B and AMH between women on OCPs and women not on OCPs. The woman on HRT had lower inhibin B and AMH in concordance with the reported ovarian failure in the questionnaire.

Hormone levels and age

Hormone levels were not influenced by age at treatment (data not shown) and did not differ between women treated before puberty and women treated during puberty (table 3). Figure 2 shows the AMH values according to age at follow-up. All MOPP- patients had normal AMH levels for age, whereas 10 out of 17 MOPP+ patients had AMH-levels below the 95% confidence interval for healthy controls. FSH serum levels were increased in nine of 21 MOPP+ women; in all but one MOPP- patient, levels were below the upper 95% confidence interval and in one on the upper 95% confidence interval. The use of OCPs did not differ between women with normal *vs.* decreased levels of AMH or increased levels of FSH.

Table 3. Hormone levels and puberty at time of diagnosis

	Pubertal (n=20)	Prepubertal (n=5)
LH (U/l)	3.8 (02.1-63.2)	2.3 (0.48-6.5)
FSH (U/l)	7.2 (0.1-46.9)	6.0 (0.8-18.3)
Inhibin B (ng/l)	47 (0-119)	39 (0-68)
AMH (µg/l)	1.1 (0-3.6)	1.7 (0.6-1.8)
Estradiol (pmol/l)	56 (5-296)	52 (17-99)

Median values (range). No significant differences.

Discussion

Because HL in childhood has become a curable disease, long-term effects of the treatment become increasingly important. We investigated gonadal function in women treated for HL during childhood and compared the effects of treatment with (MOPP+) and without (MOPP-) alkylating agents. This study provides the first systematic, very long-term outcome study (>12 yrs of follow-up) for gonadal function in women treated for childhood HL without radiotherapy, using up-to-date fertility markers like AMH and inhibin B. So far only two studies reported on long-term effects after childhood HL treatment with chemotherapy; however, with shorter follow-up and using only FSH and LH as markers for ovarian function [18, 19].

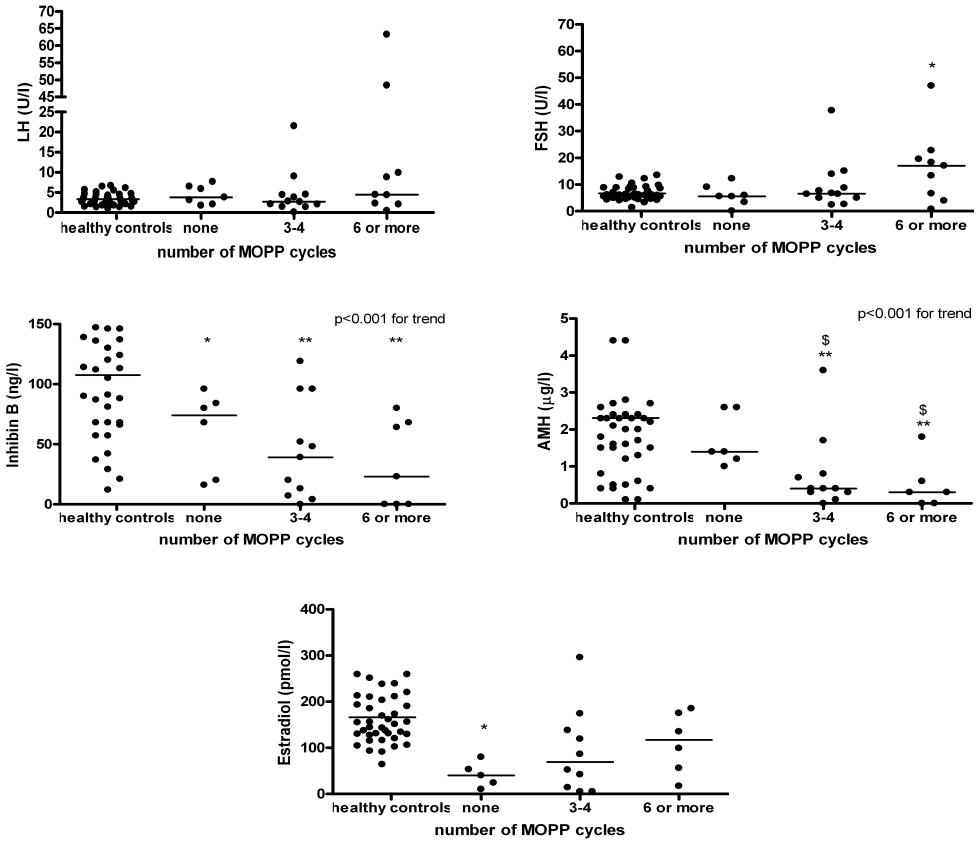

Figure 1. Serum levels of LH, FSH, inhibin B, AMH and estradiol in patients with various numbers of MOPP cycles. *Lines indicate medians. * p<0.05, ** p<0.01 as compared to healthy controls, $ p<0.05 as compared to no MOPP.*

In this study, FSH serum levels were increased and inhibin B concentrations were generally lower in HL survivors. However, AMH levels were decreased even in those patients that exhibited normal FSH values, indicating that AMH is an early and more sensitive marker to detect gonadal damage. This suggests that AMH can be useful to counsel young women who survived childhood cancer on their fertility status and family planning. Subsequently, further loss of ovarian reserve in these women could be monitored on basis of AMH serum levels.

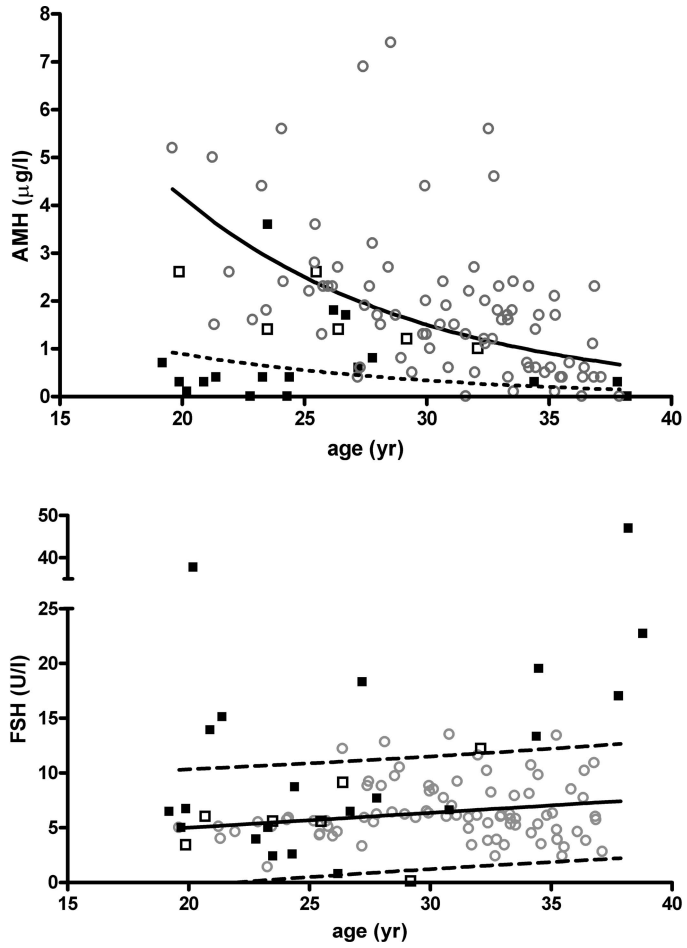

Figure 2. AMH levels and FSH levels in Hodgkin lymphoma patients treated with or without MOPP *vs.* normal controls in relation to age. *—– Mean healthy controls, ---- lower limit 95% confidence interval of healthy controls, ○ healthy controls,* ■ *MOPP+,* □ *MOPP-*

In the current study AMH levels were significantly lower in MOPP+ women, compared with MOPP- women. One of them was treated for premature ovarian failure with hormone replacement therapy, whereas three women reported irregular menses after MOPP. Interestingly, already at a very young age (20-25 yr.), women treated with MOPP had AMH levels comparable to those found in peri-menopausal women, indicating loss of ovarian reserves and increased risk of premature ovarian failure. This suggests that premature ovarian failure is a disguised long-term follow-up problem, for which AMH is the only reliably marker, not only in women with a regular menstrual cycle, but also in

those using OCPs [20, 21]. AMH is produced not only by small antral follicles, but also by all smaller follicles from primary follicles onwards [22], which may not be seen on an ultrasound of the ovaries. Therefore, AMH constitutes a more sensitive marker for ovarian reserve than the determination of the antral follicle count by ultrasound which is more time consuming and not always possible in young adolescents [23]. AMH is a good predictor of the number of oocytes and a marker for ovarian aging [15, 24, 25]. In healthy controls AMH decreases with age as the number of follicles in the ovary decreases[26], and AMH levels can be used as a predictor for menopause [16]. Another advantage of using AMH as a predictive marker for ovarian failure is that AMH is not influenced by confounding factors, such as OCP use, day of menstrual cycle, and pregnancy [20]. Therefore, it is a promising marker for ovarian reserve in all patients, although it is not yet fully established in clinical care and more research is needed.

The use of MOPP has major consequences on ovarian reserve. AMH levels in the MOPP+ group were lower, compared with the MOPP- group, but also AMH levels decreased significantly with an increasing number of MOPP courses. This suggest that the used treatment regimen and the number of MOPP courses are the main factors that affect ovarian reserve. To our knowledge no studies exist on the effect of the disease itself on gonadal function in female patients. Because there is no data available on pretreatment values of the hormonal measurements, no conclusions on that matter can be drawn.

There are few data on inhibin B levels in women after treatment for childhood cancer. Bath *et al.* [27] reported no differences in inhibin B levels between long-term survivors and healthy controls; however, the majority of patients were treated with chemotherapy without alkylating agents and only two women treated for HL were included. Larsen *et al*. [28] reported lower inhibin B levels in women (among which seven patients treated for childhood HL) treated with alkylating agents and radiotherapy. The present study is the first study to describe inhibin B levels as a marker for ovarian reserve in women in which the majority (25 of 32) was treated for childhood HL with chemotherapy only. Although inhibin B serum levels were slightly lower in MOPP+ women, this difference did not reach significance. Apparently, inhibin B and FSH levels constitute late markers of depletion of the primordial follicle pool whereas AMH levels are directly proportional with the number of primordial follicles *i.e.* the ovarian reserve. Moreover, AMH is superior to inhibin B in detecting depletion of the ovarian reserve as demonstrated by the patients with low AMH levels and

normal inhibin B levels in our study. Furthermore, AMH levels are at least an order of magnitude larger than those of inhibin B, making it easier to estimate the former parameter, which is reflected in the lower intra- and interassay coefficients of variation for AMH. Finally, assay costs for AMH and inhibin B are comparable.

In the current study LH levels did not differ between the treatment groups and healthy controls. However, FSH levels in patients treated with 6 or more MOPP cycles were significantly higher as compared to those treated without MOPP or healthy controls. The lower estradiol levels in both women treated without MOPP and those with 3-4 MOPP cycles as compared to the healthy control group might be explained by the use of oral contraceptives in our study group in contrast to the control group. It is known that LH remains normal in a large majority of patients after chemotherapy with alkylating agents [29-31], whereas FSH is increased in 15% to 50% of the women [30, 31]. This so called monotropic rise in FSH, without an increase in LH is characteristic for premature ovarian failure as well as women with an abnormal menstrual cycle [23]. FSH itself is not sensitive enough to detect early loss of ovarian reserve, whereas AMH has been shown to be a more reliable predictor in this respect [22].

The abortion rate of 23.5% appears to be very high in our study, compared with other studies on long-term effects of treatment [32]. However, of the four pregnancies that ended in a spontaneous miscarriage, three were reported by one and the same patient treated with MOPP. Most women that reported one or more pregnancies, had normal AMH levels for age at the time of study. However, AMH levels at time of pregnancies were not available and since AMH rapidly decreases with age, those women with low AMH levels at time of study, might very well have had normal AMH levels at time of pregnancy.

The number of pregnancies is difficult to compare with other studies because many different confounders are involved such as fertility of the partner and time to pregnancy. The percentage of women becoming pregnant after treatment for HL in previous studies varies from less than 10% [18] to almost 30% [33]. It might be that a considerable group of women in the current study was not yet thinking of family planning. The median age in our study group is 25 years, whereas the mean age at which women in the Netherlands have their first child is 29.3 years [34]. Moreover, this study was not designed, or powered to assess fertility or pregnancy outcome, and the pregnancy data therefore cannot be

extrapolated. Unfortunately, data on time to pregnancy were not available in our study.

In conclusion, the present study shows that women treated with chemotherapy during childhood for HL do show a distinct decrease in ovarian reserve. AMH constitutes the most sensitive predictor, superior over FSH, and inhibin B for ovarian reserve in these women. Hence, AMH serum levels can be used during follow-up of these childhood cancer survivors in order to predict incipient ovarian failure. Consequently, AMH may be a valuable predictive marker in patients for fertility counseling and future family planning.

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Chapter 8

Long-Term Endocrine Side Effects of Childhood Hodgkin's Lymphoma Treatment; a Review

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Submitted

Abstract

Childhood Hodgkin's lymphoma (HL) has a very good prognosis. Consequently, long term side effects of the treatment gain importance. This review provides an overview of the literature on long term endocrine effects of the treatment of childhood HL. We discuss growth retardation, osteoporosis and altered body composition, as well as thyroid problems and gonadal failure. The severity of the endocrine toxicity after childhood HL depends on the type of treatment. The knowledge obtained in specific follow-up programs for pediatric cancer survivors will help finding the optimal balance between cure and late effects in the treatment for childhood and adolescent HL.

Introduction

Hodgkin's lymphoma (HL) has two incidence peaks in age distribution. The first peak is between the ages of 15 and 30 years and the second is between 45 and 55 years. HL is very rare in children under 15 years of age (incidence $0.6/10^6$) [1].
The treatment of childhood HL consists of chemotherapy, radiotherapy or a combination of both. Currently, most pediatric oncology centres use a risk adapted treatment schedule consisting of both chemotherapy and radiotherapy. Using this strategy survival of childhood HL increased over the last decades [2], identical as for many other types of childhood cancer [3]. Pediatric HL has a very good prognosis: an event free survival (EFS) up to 93% and an overall survival of even 96% has been reported [4-9]. Therefore, long term side effects after treatment have gained increasing importance.

Both chemotherapy and radiotherapy have serious potential side effects, especially when used in children. In general, long term side effects of chemotherapy are related to cumulative dose and to the kind of chemotherapy (e.g. alkylating agents, anthracyclines), whereas the toxicity of radiotherapy is related to dose, fractionating and extent of the irradiation field.
As so far an overview of the literature on the endocrine late effects of the therapy of childhood HL is lacking. We hereby summarize all available reports with a special focus on late effects on growth, bone mineral density, body composition, thyroid and gonadal function after childhood HL treatment.

Growth, bones and body composition

Growth. Reduced growth in children during treatment for malignancies is caused by the disease itself, as well as by treatment related morbidity, such as recurring infections, malnutrition during treatment and treatment itself (both chemotherapy and radiotherapy) [10-12].
Irradiation of parts of the spine in children contributes to poor growth by decreasing the growth of individual bones of the spine. Chemotherapy induced growth impairment might be caused by disturbance in growth hormone secretion [12] or by direct interference with bone turnover [13]. This may result in impaired final height, but also in disproportional growth. Most of the loss in height after radiotherapy or chemotherapy affects the upper part of the body, reflected by loss in sitting height [14]. This is not surprising, considering the fact that the spine contains a total of 48 growth plates [15].

Most of the data regarding the negative effects of childhood HL treatment on growth are collected from survivors treated with combined modality treatment (table 1). In a study of 124 childhood HL survivors, treated at the age of 9 to 16 years, a height loss of 13 cm (-2 standard deviations) was described, after a median of more than 2 years after cessation of therapy [16]. This loss was most severe in pre-pubertal treated children who had received high dose radiotherapy (≥33 Gy) to the entire spine [16]. In 80 childhood HL survivors, aged 14 years and younger, Papadakis *et al.* showed a small, but significant, loss of final height of 0.4 SD after radiotherapy (n=69), that was correlated with younger age at diagnosis [17]. Nysom *et al.* also showed a reduced height after radiotherapy to the spine [18]. Summarizing all studies, it appears that radiotherapy in a dose of more than 30 Gy including the spine (with or without chemotherapy) results in loss of height, especially in pre-pubertal children [16-18]. However, when lower doses of radiotherapy are used the impairment of skeletal growth appears to be minimal (table 1).

Only scarce data are available on growth in childhood HL survivors after chemotherapy only. The 11 children reported by Papadakis *et al.* treated with chemotherapy only had normal final height [17]. We recently showed that of a unique cohort of 88 Dutch childhood HL survivors treated with chemotherapy only, only male survivors after treatment with MOPP had reduced height [19].

Osteoporosis and osteopenia. As bone mass is acquired during childhood and adolescence, disturbance of this process can result in a lower peak bone mass

which in later life can result in osteoporosis. In general, bone mineral density (BMD) is determined by several factors, like gender, race, physical activity, calcium intake, smoking and alcohol consumption [20]. In girls, pubertal stage is the most important determinant of BMD, whereas in boys weight is the most important determinant [21].

Several treatment schedules for HL involve the use of considerable doses of corticosteroids, which can cause osteopenia and osteoporosis [22-24]. Corticosteroids interfere with both osteoblast and osteoclast function, resulting in increased in bone resorption. Apart from these direct effects, also indirect effects of chemotherapy may affect bone turnover. First, gonadal damage which can be caused by cancer therapy has a negative effect on BMD [25-27]. Gonadal damage may cause impaired estrogen production necessary for starting growth and acquiring bone mass during puberty, in females, but also in males [28-30]. Secondly, some chemotherapeutic agents, like cyclophosphamide and cisplatin, can cause renal damage. This may cause deregulation of the calcium and vitamin D metabolism resulting in lower BMD [31]. In addition, physical activity is an important factor for acquiring bone mass during childhood [32]. It has been described that patients during therapy, but also cancer survivors, are generally less physically active in comparison to healthy controls [33]. In children with cancer, the lack of physical activity can potentially cause decreased BMD [34].

Studies in survivors of adult HL reported decreased BMD in female patients with premature ovarian failure (POF) [25-27, 35, 36]. Three studies reported a slightly reduced BMD in survivors of childhood HL [18, 19, 37]. A cross-sectional study of 23 HL survivors showed that total body BMD was lower in patients treated with lumbar spine irradiation (table 1). However, after correcting for height, BMD no longer differed from that of a control group [18]. We have shown that female childhood HL survivors treated with alkylating chemotherapy (MOPP) without radiotherapy had a slightly, but significantly reduced bone mineral density of the total body and after correction for bone size, also of the lumbar spine [19]. Sala *et al*. described a reduced BMD of the lumbar spine in 9 out of 22 childhood HL survivors, which was correlated with cumulative dose of corticosteroids [37]. Another study in childhood HL survivors reported normal BMD, however male patients had a 3.5 times increased risk of BMD SDS < -1.5, also after correction for BMI [38].

Overall, the effects of childhood HL therapy on BMD appear to be small and longer follow-up studies are needed to assess the consequences, especially whether or not the incidence of osteoporosis increases, later in life.

Body composition. We and others reported that treatment with prednisolone is a risk factor for increased percentage body fat in different groups of childhood cancer survivors [39, 40]. Only scarce data are available on body composition in survivors of childhood HL. Nevertheless, such studies are important as higher fat mass and BMI increases the risk of cardiovascular incidents and metabolic syndrome in later life [41, 42].

Nysom *et al.* showed an increased percentage body fat in 23 survivors of childhood HL who were treated with combined modality treatment [43]. Our study in 88 childhood HL survivors also revealed a higher percentage body fat in survivors that had not received radiotherapy [19]. However, the percentage body fat of patients treated with MOPP (a prednisolone containing regimen) was comparable to that of patients treated without MOPP. This may indicate that prednisolone is not an important determinant of increased percentage body fat in patients treated for HL as it is in ALL. The different influences of steroids in HL survivors as compared to ALL survivors may be due to the fact that the total cumulative dose prednisolone is substantially lower in HL than in ALL [23]. In childhood HL survivors Nysom *et al*. reported normal BMI, despite an increased percentage fat and explained this by a decreased lean body mass, although this was not directly measured [43]. In our childhood HL survivor study median BMI was increased, while lean body mass was normal, indicating that increased fat mass plays indeed an important role [19]. This is important as increased BMI and body fat increase the risk of the development of metabolic syndrome [42] and cardiovascular problems later in life [41].

Thyroid

After radiotherapy of the cervical region a large proportion of the childhood HL survivors show thyroid disorders, like hypothyroidism, thyroid nodules and thyroid cancer [44-52] (Table 2). In most of the protocols used in these studies, the mean radiation dose on the thyroid region was ≥35 Gy.

Hypothyroidism is the most common thyroid problem after treatment. Up to 40% of the childhood HL patients treated with radiotherapy had impaired thyroid function [46-48]. In contrast, in two studies including respectively 41 and

Table 1. Studies on bone mineral density and growth in childhood Hodgkin's lymphoma survivors

Author	*N* [a]	*Age (range)* [b]	*Median F-up*	*Therapy* [c]		*Outcome*		
				CT	RT	Growth	Bone mineral density	Body composition
Willman *et al.* [16]	124	9-16 yr	> 2 yr	MOPP MOPP/ ABVD	124[2]	↓≥ 33 Gy radiotherapy in pre-pubertal children	-	-
Papadakis *et al.* [17]	80	9.7 yr (2.4-14.0 yrs)	> 3 yr	MDP	11[1] 58[4]	↓ final height after RT in children treated youngest	-	-
Nysom *et al.* [18]	44 (23 HL)	11,1 yr (3,9-15,0 yr)	10.5 yr	Prednisone Methotrexate	1[1] 10[2] 6[3] 27[4]	↓	=	↑ % fat
Sala *et al.* [37]	22	14.7 yr (5.6-17.4 yr)	> 1 yr	-	17[4]	-	↓ BMD related to cumulative dose corticosteroids	-
Kaste *et al. [38]*	109	15.1 yr (3.1-20.7 yr)	7.5 yr	Procarbazine, cyclophosphamide methotrexate predisone	39	-	=, males increased risk for SDS < -1.5	-
Van Beek *et al.* [19]	88	11.6 yr 3.7-17.2 yr	15.5 yr	A(or E)BVD MOPP/ A(or E)BVD	18[4]	↓in male MOPP+	↓ female MOPP+	↑ % fat (female MOPP-) = lean body mass

- = information not available, HL = Hodgkin's lymphoma, Arrows indicate increased (↑), decreased (↓) or normal values (=); a) number of survivors; b) age at diagnosis (median and range); c) CT = chemotherapy: MOPP = mustine, vincristine, procarbazine, prednisone; ABVD = adriamycin, bleomycin, vinblastine, dacarbazine, MDP = doxorubicin, procarbazine, prednisone, vincristine, cyclofosfamide; EBVD = ABVD, epiadriamycin replaces adriamycin. RT = number of patients with radiation to [1] = gonads, [2] = lumbar spine, [3] = cranial, [4] = other fields

Table 2. Studies on thyroid complications in childhood Hodgkin's lymphoma survivors

Author	*N* [a]	*Age (range)* [b]	*Median F-up*	*Therapy* [c]		*Outcome*			
				CT	RT	Hypo thyroidism	Hyper thyroidism	Carcinoma	Ultrasound abnormalities
Van den Berg *et al.* [5]	21	14 yr (5-18 yr)	5.0 yr	MOPP/ ABVD	1	1/21	0/21	0/21	-
Soberman *et al.* [45]	18	14 yr	6.4 yr	-	18	7/18	-	1/18	16/18
Hancock *et al.* [46]	1787[d]	28 yr (2-82 yr)	9.9 yr	MOPP MVP ABVD	1677	513/1677	32/1677	6/1677	44/1671
Healy *et al.* [47]	46	12.5 yr (4-16 yr)	10.3 yr	None	46	28/46	-	2/46	46/46
Sklar *et al.* [48]	1791	14 yr (2-20 yr)	> 5 yr	-	1210	456/1791	82/1791	20/1791	146/1791
Atahan *et al.* [49]	46	8.5 yr (2-18 yr)	10.5 yr	COPP COPP/ ABVD	46	22/46	1/46	-	-
Hudson *et al.* [50]	79	14.6 yr (4.3-20.1 yr)	4.1 yr	COP/ABVD	79	19/79	0/79	2/79	0/79
Solt *et al.[51]*	26	10.8 yr (2.1-16.4 yr)	11.3 yr	MOPP MOPP/ABVD	26	14/26	0/26	0/26	14/26
Van Beek *et al.* [19]	88	11.6 yr 3.7-17.2 yr	15.5 yr	A(or E)BVD MOPP/ A(or E)BVD	18	5/88	0/88	0/88	-

- = information not available; a) number of survivors; b) age at diagnosis (median and range); c) CT = chemotherapy: MOPP = mustine, vincristine, procarbazine, prednisone; COPP = cyclofosfamide, vincristine, procarbazine, prednisone; COP = cyclofosfamide, vincristine, procarbazine; ABVD = adriamycin, bleomycin, vinblastine, dacarbazine; EBVD = ABVD, epiadriamycin replaces adriamycin; MVP = melphalan, procarbazine, vinblastine. RT = number of patients with radiotherapy to the neck; d) pediatric patients (<17 yr): n=272

20 children treated with chemotherapy only, no hypothyroidism was reported [4, 5]. Alternatively, in our Dutch HL survivor cohort all but one of the patients with thyroid dysfunction had received radiotherapy to the neck [19]. In addition, hyperthyroidism (mainly Graves' disease) may occur after radiotherapy (>35 Gy), although much less frequently than hypothyroidism [46, 48, 49]. In patients under the age of 17, radiation dose was the most important risk factor for developing hypothyroidism [46], but also female sex and older age at diagnosis are reported as independent risk factors [48]. Van Santen *et al.* showed that addition of chemotherapy did not alter the damage to the thyroid axis already caused by radiotherapy in a cohort of 205 childhood cancer survivors (of which 28.5% had either HL or Non-Hodgkin's lymphoma) [53].

The risk of thyroid cancer after radiotherapy is markedly higher than in the normal population. An American study of more than 1700 childhood HL survivors reported 20 cases of thyroid carcinoma (RR 18.3) after a median follow-up of 15 years [48]. All these 20 patients received radiotherapy to the neck (table 2). Hancock *et al.* reported 1677 HL survivors (both children and adults) after combined modality treatment of whom 6 developed thyroid cancer (RR 15.6) after a mean follow-up of 10 years. [46, 54]. In childhood HL survivors treated with chemotherapy only, no cases of thyroid cancer have been reported so far, however these studies contained small cohorts and follow-up was relatively short [4, 5, 19]. In our larger study, with markedly longer follow-up (median 15 yrs.), no association with thyroid cancer was found [19]. However, it should be appreciated that the risk of thyroid cancer increases after even longer follow-up of more than 20 years [55].

Gonads

An important side effect of both radiotherapy and chemotherapy is gonadal dysfunction. This can result in reduced fertility and in women also to subsequent loss of bone mass.

Male survivors. Azoospermia or oligospermia are common long-term side effects in male childhood HL patients after radiotherapy and chemotherapy, especially when alkylating agents, e.g. mustine or procarbazine, are used (table 3).

Endocrine markers for male fertility. Serum luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels are generally higher after alkylating chemotherapy as compared to treatment without alkylating chemotherapy [6, 56,

57]. No LH/FSH changes were found after chemotherapy with low dosages of alkylating agents [56, 57]. In general, the levels of LH and FSH are inversely correlated to the cumulative dose of alkylating agents [6, 58, 59].

Another marker for testicular function is inhibin B. This hormone is produced by Sertoli cells of the testis, and inhibits the production of FSH in the pituitary. Inhibin B is strongly correlated with sperm counts in both healthy and sub fertile men [60-62]. Only two studies in childhood HL survivors used inhibin B as a marker for male gonadal function. Cicognani *et al*. reported decreased inhibin B levels in childhood HL survivors treated with COPP compared to healthy controls [63]. We showed in 56 male survivors of childhood HL that inhibin B was decreased after treatment with MOPP, and that inhibin B was the best indicator for spermatogenesis, superior to FSH [59].

Radiotherapy. Pelvic radiotherapy in adults causes azoospermia that is reversible in most cases within 2 years after cessation of therapy [64, 65]. Ortin *et al.* reported a small group of boys treated with pelvic radiotherapy (30-45 Gy). Recovery of spermatogenesis occurred after longer follow-up, and recovering to normal levels was less frequent than reported in patients treated during adulthood [66].

Chemotherapy. Nearly all male HL survivors treated with alkylating chemotherapy, suffer from azoospermia. This is the case in both adult HL survivors [67-70], and childhood HL survivors [71, 72]. Three small series of childhood HL survivors treated with alkylating chemotherapy alone are reported (procarbazine and mustine/procarbazine) [56, 71, 72]. All these young men had either azoospermia or oligospermia. After 10 years of follow-up in 7 patients, no recovery of spermatogenesis was seen [71] (table 3). We reported on 56 male survivors of childhood HL, and showed that 60% of the survivors treated with MOPP had azoospermia or severe oligospermia, whereas those treated without alkylating chemotherapy all had normospermia [59]. Nevertheless, recovery of spermatogenesis after alkylating chemotherapy is reported in a small proportion of adult HL survivors [66, 73, 74]. It has been suggested that recovery of spermatogenesis is related pubertal state during treatment. However, recent studies showed that there is no difference in the severity of the gonadal damage or the chance of recovery of spermatogenesis between boys treated before puberty and boys treated during or after puberty [59, 66, 75]. Reduction of alkylating agents reduces the risk of gonadal damage, as detected by increased serum FSH levels and decreased inhibin B levels [56]. However, semen analysis

studies are scarce. We recently reported, using sperm analysis combined with fertility markers like inhibin B, that gonadal damage was significantly related to the cumulative doses of alkylating agents in childhood HL survivors [59].

Combined modality treatment. In childhood HL survivors in which radiotherapy and chemotherapy were combined or in whom therapy was not described in detail, spermatogenesis was disturbed in 75-100% of the male patients, similar to studies with chemotherapy containing alkylating agents only [27, 66, 72, 75-77] (table 3).

Female survivors. In female HL survivors both alkylating agents and abdominal radiotherapy can cause severe ovarian damage, eventually leading to premature ovarian failure (POF) [78-80]. In addition radiotherapy may cause damage to the uterus, which may lead to premature labour and low birth weight [80-82]. The studies that describe the reproductive status of women after chemotherapy and/or radiotherapy for HL during childhood are reported in table 4.

Endocrine markers for female fertility. Usually, gonadal function is measured in follow-up studies of female long-term survivors of childhood cancer by analysis of LH and FSH. However, neither LH nor FSH are predictive for the ovarian reserve [83]. In recent years, two new markers for ovarian function became available. Inhibin B, which in females is solely produced by granulosa cells of small antral follicles, is decreased in women with known fertility problems (e.g. POF) and undetectable in postmenopausal women [84-86]. Inhibin B is one of the first endocrine markers to change in perimenopausal women, even before changes in FSH levels can be detected [87]. The second new marker is anti-Müllerian hormone (AMH). This hormone is produced by granulosa cells of early developing (pre-)antral follicles of the ovary, and levels decrease when the number of follicles decreases with age [88]. A recent study showed a strong correlation between age at menopause and AMH levels randomly measured during the reproductive lifespan in a group of healthy women [89]. In addition, recently AMH was shown to be a good predictor for the success of artificial reproductive technology [90]. We measured inhibin B and AMH in female HL survivors and showed that AMH is a good early marker for ovarian dysfunction, even when LH and FSH are still within normal ranges [78]. These results were confirmed in a larger cohort of survivors of other types of childhood cancer in our institute [79].

Radiotherapy. Ortin *et al.* reported 10 girls with HL treated with pelvic irradiation of whom 8 retained normal ovarian function. None of the girls treated with radiotherapy to other sites than the pelvis suffered from POF [66]. However, AMH was not measured in this study and abdominal radiotherapy can cause decreased levels of AMH, indicating diminished ovarian reserve [79].

Chemotherapy. Mackie *et al.* reported that one third of the pediatric HL survivors treated during childhood with procarbazine containing therapy suffered from POF. Recovery of ovarian function was rare [72] (table 4). Ortin *et al.* reported <10% POF after chemotherapy only [66]. Our study showed ovarian damage in the majority of women treated with MOPP as reflected by severely decreased anti-Müllerian hormone (AMH) serum levels, with one woman having clinically overt ovarian failure [78]. These childhood HL survivors had significantly reduced AMH levels as compared to 250 survivors of other types of childhood cancer [79]. Also age at treatment might play a role. De Bruin *et al*. showed that the time to premature menopause was much longer in women treated with MOPP at early ages. However, cumulative risk of menopause at the age of 40 did not differ between patients treated at younger age as compared to those treated at older age [91].

Combined modality treatment. Several studies reported up to 40% POF in female HL survivors treated during childhood with both pelvic irradiation and alkylating chemotherapy (mustine and procarbazine) [50, 66], while none of the women treated without pelvic irradiation (n=17) had POF [50]. Some studies in adult female HL survivors suggest less POF when patients were treated before the age of 25 years as compared to those patients treated after that age [92] and a better chance of recovery of ovarian function [44]. Two large childhood cancer survivor cohorts, both reported increased risks of premature menopause or acute ovarian failure (ovarian failure within 5 years after cessation of therapy) after exposure to procarbazine and pelvic irradiation (≥20 Gy) [93, 94].

Different responses in male and female survivors Gonadal damage seems to be less frequent in female childhood HL survivors than in male survivors [66]. This might be due to the fact that the primordial follicles in the ovary are much more resistant to the cytotoxic effects of alkylating agents than the rapidly dividing sperm cells in male patients. Although animal studies suggest a protection against the cytotoxic effects of chemotherapy before puberty in both males and females [95], clinical studies in male patients treated for HL before puberty report azoospermia, comparable to male patients treated during or after

Table 3. Studies on testicular function in male childhood Hodgkin's lymphoma survivors

Author	*N* [a]	*Age (range)* [b]	*Median F-up*	Therapy [c]		*Gonadal evaluation* [d]						
						Semen analysis			*Serum markers*			
				CT	RT	N	Azo	Oligo	N	LH	FSH	Inh B
Heikens et al. [71]	19	11 yr (5-15 yr)	10 yr	MOPP	0	18	67%	33%	19	↑	↑	-
Ortin et al. [66]	148	13 yr (2-15 yr)	9 yr	MOPP	12	15	60%	15%	11	↑	↑	-
Mackie et al.[72]	46	12.2 yr (8.2-15.3 yr)	6 yr	ChlVPP	0	-	-	-	46	↑	↑	-
Shafford et al. [76]	30	10 yr (4-15 yr)	11.8 yr	ChlVPP	10	13	91%	9%	30	↑	↑	-
Papadakis et al. [77]	36	13 yr (2-22 yr)	6.8 yr	MDP	6	2	100%	-	36	↑	↑	-
Ben Arush et al. [75]	20	2-16 yr		MOPP/ABVD COMP MOPP	4	-	40%	40%	20	↑	↑	-
Cicogagnani et al. [63]	11	10.3 yrs. (3-15 yr)	5.5 yr	ABVD MOPP/ ABVD	0	-	-	-	11	↑	↑	↓[f]
Van den Berg et al. [56]	14	14 yr (5-18 yr)	5.1 yr	MOPP/ ABVD	0	-	-	-	14	=[e]	=[e]	-
Gerres et al.[57]	46	14.9 yr	1.9 yr	OEPA OEPA/ COPP	0	-	-	-	46	↑[f]	↑[f]	-
Van Beek et al. [59]	56	11.4 yr (3.7-15.9 yr)	15.5 yr	A(E)BVD MOPP/A(E)BVD	0	21	53%[f]	6%[f]	56	↑[f]	↑[f]	↓[f]

Table 4. Studies on ovarian function in female childhood Hodgkin's lymphoma survivors

Author	*N* [a]	*Age (range)* [b]	*Median F-up*	*Therapy* [c]		*Gonadal evaluation* [d]						
				CT	RT	Amenorrhea Irregular Cycle [g]	Pregnancies	Serum markers				
								N	LH	FSH	Inh B	AMH
Hudson *et al.* [50]	37	14.6 yr (4.3-20.1 yr)	4.1 yr	COP/ABVD	18	6/37	17 in 10 women	-	-	-	-	-
Ortin *et al.[66]*	86	13 yr (2-15 yr)	9 yr	MOPP MOPP/ABVD ABVD PAVe VBM	28	14/86	40 in 86 women	-	-	-	-	-
Van den Berg *et al.* [56]	14	14 yr (5-18 yr)	5.1 yr	MOPP ABVD MOPP/ ABVD	0	2/14	1 in 14 women	14	= [h]	= [h]	-	-
Mackie *et al.* [72]	32	13.0 yr (9.0-15.2 yr)	4.3 yr	ChlVPP	0	10/32	11 in 9 women	32	↑ [i]	↑ [i]	-	-
Papadakis *et al.[77]*	29	14.1 yr (6.1-20.0 yr)	5.1 yr	MDP	6	-	8 in 6 women	29	↑	↑	-	-
Van Beek *et al.* [78]	32	11.6 yr (5.7-24.5 yr)	15.5 yr	A(or E)BVD MOPP/A(E)BVD	0	1/32	17 in 11 women	32	=	↑ [j]	↓ [j]	↓ [j]

Legend table 3 and 4

- = information not availabl; a) number of survivors; b) age at diagnosis (median and range); c) CT = chemotherapy: MOPP = mechlorethamine, vincristine, procarbazine, prednisolone; COPP = MOPP cyclophosphamide replaces mustine; COMP = COPP, methotrexate replaces procarbazine; ABVD = adriamycine, bleomycine, vinbalstine, dacarbazine; MDP = doxorubicin, procarbazine, prednisolone, vincristine, cyclophosphamide; ChlVPP = chlorambucil, vinblastine, procarbazine, prednisolone; OEPA = vincristine, VP-16, prednisolone, doxyrubicin; OPPA = OEPA procarbazine replaces VP-16; PAVe = procarbazine, alkeran, velban; VBM = velban, bleomycin, methotrexate. RT = number of patients with gonadale radiotherapy; d) N= number of patients tested, azo = azoospermia, oligo = oligospermia, LH = luteinizing hormone, FSH = follicle stimulating hormone, inh B = inhibin B, AMH = anti-Müllerian hormone Arrows indicate increased (↑), decreased (↓) or normal values (=); e) Only 2 male survivors with increased levels of FSH and 1 with an increased level of LH; f) Only in patients treated with MOPP or COPP; g) Amenorrhea or irregular cycle only in female survivors treated with alkylating therapy or pelvic irradiation; h) Increased FSH in 17/32 and LH in 15/32 female survivors; i) Only 2 female survivors with increased levels of FSH and 1 with an increased level of LH; j) Only in women treated with MOPP

puberty. Already before puberty starts, the testes are active. Sertoli cells are dividing, and are forming the structural and functional framework over which germ cells will proliferate and differentiate in later life. Disturbance of this process might be the reason why treatment with alkylating agents before puberty can result in irreversible azoospermia [96].

Although some young women suffer from POF, female patients younger than 25 years of age seem to be protected against the toxic effects of alkylating agents [44, 79, 92]. Maybe this is because pre-antral and early antral follicles are less vulnerable to alkylating agents than follicles in a later stage of development and that the smaller reserve of the early follicles at older age diminishes the chances for recovery of the ovary. However, none of these studies used AMH and disturbance of the menstrual cycle is very late sign of POF. Therefore the damage to the ovaries might be underestimated. Early menopause might be a disguised problem in female childhood HL survivors, especially after MOPP, and therefore needs more attention. It could have a major impact on fertility counselling and family planning of female childhood HL survivors treated with alkylating agents.

Intervention In some clinical trials GnRH-antagonists (GnRH-a) were used to prevent gonadal damage in patients treated with alkylating agents, however large clinical trials are lacking. The first results from studies in adult female

patients with GnRH-a to induce a pre-pubertal stage before chemotherapy were promising [97-99]. However, GnRH-a only protects FSH-dependent follicles and not the FSH-independent early developing (pre-)antral follicles. Hence, in spite of GnRH-a treatment no new follicles will develop and fertility will probably not be preserved in the long term [79]. Trials with GnRH-a in male patients, show no protection of GnRH-a treatment against cytotoxic damage [100, 101].

Conclusion and perspectives

No consensus exists yet on how to monitor long-term endocrine side effects. Besides the use of proper history taking and complete physical examination (including height, sitting height and weight), there are several ways of detecting endocrine long-term effects. Dual energy X-ray absorptiometry (DEXA) is an easy and reliable way to assess BMD and body composition.

In male survivors evaluation of testicular function should consist of assessment ofTanner stage, testicular volume, sperm analysis and LH, FSH and inhibin B measurement. Inhibin B, produced by Sertoli cells has the advantage that it is a direct marker that correlates strongly with spermatogenesis [61, 62]. Recent data on inhibin B in male childhood HL survivors show that inhibin B is currently the best marker for spermatogenesis in male HL survivors [59, 102]. So far, semen analysis has been the standard and therefore, it is important to assess. In female patients fertility markers (LH, FSH, inhibin B and AMH) should be determined on day 2-5 of the menstrual cycle. If the patient uses oral contraceptives, these tests should be performed a minimum of 1 week after the last pill was taken to minimize the effects of the pill on the gonadal markers [103]. Using AMH ovarian dysfunction can be detected earlier and possibly more frequently than with FSH [78]. In contrast to FSH, AMH levels are constant during the menstrual cycle [104]. However, since the clinical value of AMH remains to be confirmed, ovarian ultrasound remains important. Thyroid function can easily be followed using TSH and free thyroid hormone in both male and female patients. To detect morphological changes of thyroid tissue, an ultrasound of the thyroid is useful. This is especially the case if any radiotherapy to the cervical region was administered [47, 51].

There is no world-wide consensus on the prevention of long-term endocrine side effects. Commonly used techniques to preserve gonadal function include sperm and embryo cryopreservation and are advised to use in all

applicable patients [105]. However, embryo cryopreservation can only be used in adult patients. Although the first results on the use of GnRH-a during the treatment of adult female HL patients are promising, the number of patients are small [97, 98] and GnRH-a does not protect the FSH-independent early (pre-) antral follicles [79]. The use of preventive therapeutic measures such as protective medication (e.g. GnRH-a) and other methods such as preservation oocytes, entire ovaries or oophoropexy remains subject of further studies [106]. Semen cryopreservation in boys is a feasible method to preserve spermatozoa before gonadotoxic therapy is started and should be offered to all pubertal boys despite their young age [62].

As shown in this review, the severity of the endocrine toxicity after childhood HL depends mainly on the type of treatment. Long-term follow up is necessary to assess all adverse effects of both chemotherapy and radiotherapy and to develop preventive strategies. Nowadays, many centres have specific follow-up programs to detect and, if necessary, treat the long-term side effects of cancer treatment at childhood age. The knowledge obtained in these outpatient clinics can help in the search for the optimal balance between cure and late effects in the treatment for childhood and adolescent Hodgkin's lymphoma.

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Chapter 9

General Discussion

This thesis describes studies on endocrine effects of treatment of acute lymphoblastic leukemia (ALL) and Hodgkin's lymphoma (HL) during childhood. The ***first part*** consists of studies on bone mineral density (BMD), body composition and growth, both during and after therapy for childhood ALL. In addition, the role of genetic variation in symptomatic avascular necrosis (AVN) of the bone in patients treated for childhood ALL was studied. The ***second part*** consists of studies on BMD, body composition and growth, as well as thyroid hormones and gonadal function in long-term survivors of HL.

9.1 Endocrine effects of therapy for ALL on bone and body composition in childhood ALL

Concern exists about the long-term effects of high-dose corticosteroids in children treated for ALL. Earlier studies in pediatric ALL patients showed therapy related decrease of BMD during and after treatment [1, 2]. The fact that uniformly treated children show a large variety in occurrence of osteogenic problems, such as reduction of BMD, fractures [1], and avascular necrosis suggests a genetic variation in host factors that influence these problems.

9.1.1 Bone mineral density (BMD), body composition and fracture risk

We studied the influence of 5 different risk alleles of the *VDR, COLIA1, ESR1* and *GR* genes on BMD, body composition and fracture rate in pediatric ALL patients during and after therapy.

None of the studied Single Nucleotide Polymorphisms (SNPs) influenced treatment related bone loss. However, carriers of the *VDR* 5'-end haplotype 3 had a lower BMD_{ls} at start of therapy compared with non-carriers, which resulted in lower BMD at the end of therapy.

The SNP of the *VDR* 5'-promoter region (*Cdx-2/GATA*) appeared to influence body composition during ALL treatment. Children who did not carry the *VDR* 5'-end (*Cdx-2/GATA*) haplotype 3 had a significantly larger gain of body fat from start to cessation of treatment than carriers of the *VDR* 5'-end haplotype 3 .

The mechanisms behind these effects are not yet clarified. It has been suggested that vitamin D regulates body fat, by influencing the intestinal calcium absorption and calcium controlled lipogenesis in adipocytes [3, 4]. We could however not confirm this in childhood ALL patients as serum calcium, vitamin D and PTH did not differ between carriers and non-carriers of *VDR* 5'-end haplotype 3.

We also showed that recovery of lean body mass was diminished in carriers of the *ESR1* haplotype 3. None of the other studied polymorphisms influenced therapy related changes in body composition. Furthermore, none of the studied polymorphisms were associated with fracture risk during ALL therapy.

This thesis also shows that BMD was normal in long-term ALL survivors (mean age at follow-up 21 years) treated with prednisolone as well as in those treated with dexamethasone. Of the various studies that reported BMD in survivors of childhood leukemia [5-12], most showed a reduced BMD after ALL treatment. It has become clear now that especially cranial irradiation is a very important risk factor for decreased BMD [5, 7, 8]. In patients treated with chemotherapy only, BMD recovered to normal values after cessation of therapy [6, 9].

Clinical implications and suggestions for further research:

Results in this thesis are based upon a single center study in pediatric ALL patients. Although results are consistent with results reported earlier, cohorts are relatively small. It is important to confirm these results in large prospective studies in pediatric ALL patients treated with the same protocol, such as the Dutch national protocol and using matched control groups.

We were the first to show that therapy related changes in body composition in childhood ALL may be determined by genetic variation. So far most studies on the influence of genetic polymorphisms on BMD and body composition were performed in healthy elderly. Only scarce data are available in healthy children [13-17] and no information is available in children treated for ALL. Results between these studies vary greatly, and effects are small. It is therefore very important to confirm our results in a larger study. It should be emphasized that the effects of SNPs on BMD in healthy individuals is not directly comparable with these effects on treatment related decrease of BMD in pediatric cancer patients. It is, for example, unknown how factors such as age and the use of high-dose corticosteroids alter the effects of SNPs on BMD, body composition and fracture risk.

In larger cohort studies it will also be possible to look for other possible SNPs that might influence treatment related bone loss or changes in body composition in pediatric ALL patients. Possible candidates are SNPs of the methylenetetrahydrofolate reductase gene (*MTHFR*) [18, 19], methionine

synthase reductase gene (*MTRR*) [20, 21], the low-density lipoprotein receptor-related protein 5 gene (*LRP5)* [22-25] and corticotrophin-releasing hormone receptor-1 (CHRH1) [26] .

Treatment for pediatric ALL has only minor long-term effects on growth, BMD and body composition. BMD is normal, both after prednisolone based protocols as well as after dexamethasone based protocols. It is however unknown how the treatment will affect physiological bone loss later in life. It might be that patients who suffered from decreased BMD over a period of time suffer from early, or more severe osteoporosis later in life. Therefore, further follow-up of these patients is necessary.

At this moment, no data exist on the effects of genetic variations on long-term effects of childhood ALL therapy. Identifying patients at risk at an early stage, possibly already at start of therapy by genome-wide techniques in large (inter)national cohorts is important. Increased BMI and body fat enhance the risk of the development of metabolic syndrome [27] and cardiovascular problems later in life [28]. It is therefore necessary to investigate whether the risk alleles that were identified in this thesis or maybe other SNPs influence the occurrence of metabolic syndrome or cardiovascular problems.

In the future it might be possible to use certain SNPs, like in the *VDR* or *ESR1* gene, to identify patients at risk for osteoporosis and increased body fat at an early stage. In this way, it might be possible to enter these patients into an intervention program to prevent bone loss or changes in body composition, at an early stage. Recently, we showed that an exercise program did not prevent bone loss during therapy for childhood ALL, which might have been due to compliance problems. There was however a significantly improved recovery of BMI and body fat to normal values after cessation of therapy in the patients in the program [29]. Further research should be done to improve compliance and effectiveness of such prevention programs.

Other preventive strategies could include the use of bisphosphonates. The results of a few small studies in patients treated for childhood ALL are promising [30-32], but longer follow-up and larger groups are necessary to assess the risks and benefits of bisphosphonates in pediatric ALL patients.

9.1.2 Avascular necrosis of bone

In this thesis we describe the first study on the relation between Lp(a) levels and the number of kringle IV repeats in the *LPA* gene and their effects on symptomatic

AVN in children with ALL. So far most studies that reported an association between Lp(a) levels and increased risk of idiopathic osteonecrosis, similar to AVN, were performed in adults without ALL [33-35].

We found no differences in Lp(a) serum levels or the number of kringle IV repeats in the *LPA* gene between patients with symptomatic AVN and patients without AVN. Furthermore, we could not confirm hyperlipidemia as an etiological factor for osteonecrosis. Lipid profiles showed no significant differences between pediatric ALL patients with or without symptomatic AVN.

Clinical implications and suggestions for further research:

AVN is a rare, but very serious complication of the treatment for childhood ALL. Identification of specific risk factors for AVN are needed to develop prevention strategies. The pathogenesis of AVN is still largely unknown. Several etiological factors are suggested. Most important are changes in lipid profiles and altered coagulation factors. Almost all reported data on the role of lipid profiles in corticosteroid induced AVN are derived from animal studies [36-41]. In contrast to the studies in animals, we did not assess lipids during corticosteroid treatment but five weeks later just before the next block of dexamethasone therapy, which may explain the rather normal lipid levels in our cohort of patients. Further prospective follow-up studies are to assess lipids and coagulation factors both during and after therapy [42].

Also further research of possible genetic determinants of AVN is needed. Relling *et al.* showed that polymorphisms in the vitamin D receptor and thymidylate synthase are independent predictors for osteonecrosis in a pediatric ALL [43]. In addition, polymorphisms in the folate pathway (e.g. methylenentetrahydrofolate reductase; [44]) and polymorphisms in cytochrome P_{450} [45] have been suggested to influence the development of AVN.

For now, prevention of AVN is not possible and the possibilities for treatment are limited. The use of bisphosphonates might be effective to reduce pain and slowing the progression of AVN, but bisphophonates do no prevent AVN [46, 47]. Randomized controlled trials are lacking and are needed to determine the efficacy and if effective the correct dose and the most effective way and timing to administer the drug.

9.2 Late effects of treatment for childhood Hodgkin's Lymphoma (HL)

Hodgkin's Lymphoma (HL) in childhood has become a curable disease. Hence, long-term effects of the treatment become increasingly important. In this thesis we studied several endocrine late effects in patients treated for HL during childhood and compared the effects of treatment with and without alkylating agents (MOPP). These studies are the first systematic, very long-term outcome studies (median 15.5 years of follow-up) for endocrine late effects in patients treated for childhood HL without radiotherapy, including the use of up-to-date fertility markers.

Although fracture incidence was comparable to a healthy population, female survivors of childhood HL treated with alkylating chemotherapy (MOPP) appeared to have a decreased lumbar spine bone mineral apparent density. This may be due to premature reduced ovarian function, as indicated by the low AMH levels found in women treated with MOPP. In previous studies that reported decreased BMD in female survivors of adult HL, also a relation with ovarian failure was found [48, 49]. We showed that in women treated with alkylating agents, AMH reached levels comparable to peri-menopausal women, indicating severe, gonadal damage in these women. This is a disguised problem, since these women had normal FSH levels and an normal menstrual cycle.

Male survivors treated with alkylating chemotherapy had normal BMD but increased body fat and BMI. This might be related to the diminished gonadal function, although caloric intake was not measured, a higher caloric intake could therefore not be excluded as possible cause. Inhibin B had the strongest correlation with spermatogenesis. Although concerns exist on sperm quality in men treated for childhood cancer, we showed that men who retained spermatogenesis after treatment for childhood HL had normal sperm DNA.

Both men and women had normal thyroid function. Moreover no thyroid cancers were found in this group treated with chemotherapy only, after a median follow-up of 15.5 years. Studies in which radiotherapy to the neck region was used showed up to 18 times increased risk for thyroid cancer [50, 51]. This illustrates the benefit of the treatment for childhood HL without radiotherapy.

<u>Clinical implications and suggestions for further research:</u>

We showed that both inhibin B and AMH could be very useful in the detection of late effects in survivors of childhood HL. In males inhibin B is very well correlated

with sperm count and might be used as non-invasive screening method. In female survivors, AMH was found to be an early marker for gonadal damage.

However, further research is needed to determine the predictive value of inhibin B and AMH levels for fertility, before it can be used as a screening method. In male survivors, there is need for a prospective study in which both inhibin B and semen analysis are performed before and several times after therapy. In female survivors, combining AMH measurement and ovarian ultrasounds should be used to confirm the value of AMH as a screening marker to detect disguised fertility problems in HL survivors with still a normal menstrual cycle.

As shown in this thesis treatment of childhood HL with chemotherapy only can cause several late effects. It is important to study ways to prevent these effects in future patients, but at least as important to counsel the survivors of today on their late effects and study possibilities to treat late effects that have occurred.

In comparison to ALL the extent of bone loss in HL survivors is much less and fracture incidence was comparable to a healthy population. Further research is needed to determine the effects of therapy related bone loss on physiological loss of bone mass at old age. However, our results showed no need for intervention programmes in childhood HL survivors.

For now, the methods for fertility preservation are limited. In adults, it is possible to use cryopreservation of semen [52] or embryos [53] before treatment is started, however these method are not suitable for children. Oopherexy can be used to prevent or limit radiation damage to the ovaries and is also possible in children. In literature several other preventive strategies for gonadal damage are suggested, but further research is needed to establish their value. The first results on the use of GnRH-a during the treatment of adult female HL patients are promising, but the number of patients are small [54, 55] and GnRH-a does not protect the FSH-independent early (pre-) antral follicles [56]. GnRH-a as preventive strategy in males is shown to be ineffective [57, 58]. Other experimental methods include cryopreservation of ovarian or testicular tissue and cryopreservation of oocytes [59].

An important dilemma remains, i.e. to use alkylating chemotherapy or radiotherapy in the treatment for childhood HL. Both types of treatment are associated with late effects. Radiotherapy is associated with a markedly increased risk of second tumours, especially breast and thyroid cancer and thyroid dysfunction, whereas the use of alkylating chemotherapy is associated with

gonadal damage. We did not find any second tumours in our group of HL survivors treated with chemotherapy only. However, since these second tumours usually appear after a very long follow-up, sometimes more than 20 years [60], longer follow-up is needed.

Future research should focus on the use of tailored therapy in childhood HL patients, minimizing the therapy needed and thus minimizing the effects. It is important to consider the gender of the patient in this respect. In male patients the use of low dose radiotherapy combined with non-alkylating chemotherapy could be the treatment of choice to prevent long term gonadal damage and infertility, especially in young boys for whom cryopreservation is not possible. In female patients, more chemotherapy might be possible if preventive strategies such as the preservation of oocytes are successful.

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Chapter 10

Nederlandse Samenvatting

Het aantal volwassenen dat op de kinderleeftijd behandeld is voor enige vorm van kanker neemt steeds meer toe. Dit proefschrift gaat over kinderen en volwassenen die op de kinderleeftijd behandeld zijn voor acute lymfatische leukemie (ALL) of de ziekte van Hodgkin. In de laatste decennia is de overleving van kinderen met ALL gestegen tot rond de 80% en voor de ziekte van Hodgkin zelfs tot meer dan 90%. Vanwege deze verbeterde overlevingskansen worden lange termijn effecten van de behandeling voor ALL en de ziekte van Hodgkin steeds belangrijker.

In **hoofdstuk 1** wordt ter inleiding op het proefschrift de context waarbinnen het onderzoek heeft plaatsgevonden beschreven. Het proefschrift is opgebouwd in twee delen, waarbij elk deel één aandoening beschrijft.

In het eerste deel worden kinderen en volwassenen die op de kinderleeftijd behandeld zijn voor ALL besproken. De onderzoeksvragen van het onderzoek in het eerste deel waren:

1. Hebben variaties in genen, die geassocieerd zijn met botontkalking bij gezonde volwassenen, bij kinderen met ALL invloed op de groei, botdichtheid (BMD) en lichaamssamenstelling. Zo ja, kan met behulp van deze variaties worden vastgesteld welke kinderen het hoogste risico hebben op botontkalking tijdens de behandeling voor ALL.
2. Zorgt een variatie in het gen dat codeert voor een op cholesterol lijkend eiwit (apo(a)), voor een verhoogd risico op avasculaire necrose (AVN) tijdens de behandeling voor ALL. AVN is een ernstige bijwerking waarbij de bloedtoevoer naar het bot verstoord wordt en het bot kan afsterven.
3. Wat zijn bij mensen, die als kind zijn behandeld voor ALL, de lange termijn effecten op groei, botdichtheid (BMD) en lichaamssamenstelling.

Het tweede gedeelte van het proefschrift beschrijft de lange termijn effecten bij jongvolwassenen die op de kinderleeftijd behandeld zijn voor de ziekte van Hodgkin. Onderzoeksvragen bij deze groep waren:

4. Wat zijn de lange termijn effecten op het gebied van
    a. de groei, BMD, lichaamssamenstelling en schildklier
    b. de vruchtbaarheid: het functioneren van de zaadballen (bij mannen) en de eierstokken (bij vrouwen)

Deel 1 – Endocriene effecten van de behandeling voor ALL op de kinderleeftijd.

De kalkhoudendheid van het skelet (botdichtheid) wordt voor een belangrijk deel door genetische factoren bepaald. Een lage botdichtheid verhoogd de kans op

botbreuken (fracturen). Bot is voor een groot deel opgebouwd uit het eiwit collageen type I. Bij de vorming van bot spelen vitamine D en het hormoon oestrogeen een belangrijke rol. Bij de behandeling van leukemie worden veel corticosteroïden gebruikt (prednison en dexamethason), die de botdichtheid negatief beïnvloeden. Een aantal genen die van belang zijn voor botdichtheid en botontwikkeling, coderen voor het collageen (*COLIA1*), de vitamine D receptor (*VDR*), oestrogeenreceptor (*ESR1*) en de glucocorticoïd receptor (GR). Deze genen spelen ook een rol bij lengtegroei en lichaamssamenstelling

In de studie beschreven in **hoofdstuk 2** is daarom gekeken naar de invloed van genetische variatie of polymorfismen in deze 4 genen op de groei, BMD, lichaamssamenstelling en fracturen bij 69 kinderen die werden behandeld voor ALL. Een polymorfisme is een kleine verandering in een enkel gen waarbij 1 base van het gen vervangen is door een andere base. In het *VDR* gen zijn polymorfismen in de twee uiteinden van het gen onderzocht. Aan het ene einde (de 3'-kant) betrof dit een combinatie van drie bij elkaar gelegen variaties (*BsmI/ApaI/TaqI*) en aan het andere einde (de 5'-kant) 2 bij elkaar gelegen variaties (*Cdx-2/GATA*). Verder zijn een polymorfisme in het *COLIA1* gen *(SpI),* een combinatie van twee bij elkaar gelegen polymorfismen in het *ESR1* gen (*PvuII/XbaI*)*,* één in het *GR* gen *(*BclI) onderzocht.

Een combinatie van polymorfismen, zoals bij het *VDR* gen en het *ESR1* gen wordt een haplotype genoemd. Voor het *VDR* gen werden twee haplotypes onderzocht, één voor de SNPs aan het 3'-einde(*BsmI/ApaI/TaqI*) en één voor de SNPs aan het 5'-einde (*Cdx-2/GATA*) .

Het bleek dat geen van de onderzochte polymorfismen invloed had op de lichaamssamenstelling bij kinderen tijdens de behandeling voor ALL, met uitzondering van haplotype 3 van het 5'-einde van het *VDR* gen. Bij kinderen die dit haplotype niet hadden nam het lichaamsvet tijdens behandeling meer toe dan bij kinderen die dit haplotype wel hadden. Dit suggereert een rol voor vitamine D bij de vethuishouding bij kinderen die behandeld worden voor ALL. Het mechanisme waardoor dit gebeurt is echter onduidelijk.

In het jaar na afloop van de behandeling herstelde de spiermassa bij kinderen die drager waren van haplotype 3 van het *ESR1* gen minder goed dan bij andere kinderen. De botdichtheid (BMD) bleek te worden beïnvloed door haplotype 3 van het 5'-einde van het *VDR* gen. Dragers van dit haplotype hadden een lagere BMD van de lumbale wervelkolom bij aanvang van de behandeling en dit bleef zo tijdens en na de behandeling. Geen van de andere polymorfismen

waren van invloed op de botdichtheid. Ook was er geen invloed in het optreden van fracturen tijdens de behandeling.

AVN is een ernstige bijwerking die bij 4-12% van de kinderen die worden behandeld voor ALL kan optreden. De aandoening komt vaker voor bij oudere kinderen en de oorzaak is niet duidelijk. Er is een vorm van AVN bekend die familiair voorkomt, waarbij lipoproteïne(a) (Lp(a)), een op cholesterol lijkende stof, een belangrijke rol speelt. Het is gebleken dat de hoeveelheid Lp(a) in het bloed samenhangt met een polymorfisme in het gen voor apo(a), een belangrijke bestanddeel voor Lp(a). Meer herhalingen van een bepaalde combinatie van baseparen (Kringle IV-herhalingen) in het gen voor apo(a) leiden tot een lagere concentratie Lp(a) in het bloed.

In **hoofdstuk 3** is het onderzoek beschreven naar de invloed van dit polymorfisme op het voorkomen van AVN bij kinderen die behandeld zijn voor ALL. Hoewel de eerder aangetoonde relatie tussen het aantal herhalingen en de concentratie Lp(a) bij deze kinderen ook werd gevonden, bleek er geen relatie met het voorkomen van AVN. Bij deze groep kinderen behandeld voor ALL, speelt Lp(a) en het aantal Kringle IV-herhalingen dus geen rol in het ontwikkelen van AVN. Ook in de andere lipiden werd geen verschil gevonden tussen kinderen die AVN ontwikkelden tijdens de behandeling en de kinderen waarbij dit niet het geval was.

Bij de behandeling van ALL zijn corticosteroïden een belangrijk onderdeel van de behandeling. In de verschillende protocollen die in de loop van de tijd zijn toegepast zijn prednison of dexamethason gebruikt.

In **hoofdstuk 4** zijn de lange termijn effecten op groei, BMD en lichaamssamenstelling beschreven bij 90 mensen die minimaal 5 jaar geleden gestopt zijn met hun behandeling voor ALL op de kinderleeftijd. Hieruit bleek dat er geen verschil was tussen mensen behandeld met een protocol met prednison of met dexamethason. Wel bleek dat in beide groepen de body mass index (BMI) was verhoogd, al waren er niet meer mensen met ernstig overgewicht dan in de normale bevolking.

Deel 2 – Endocriene effecten van de behandeling voor de ziekte van Hodgkin op de kinderleeftijd.

De studies beschreven in dit deel zijn uitgevoerd bij een groep van 88 volwassenen die als kind waren behandeld voor de ziekte van Hodgkin met alleen

chemotherapie in het Erasmus MC/Sophia kinderziekenhuis en het Academisch Medisch Centrum en allen minimaal 5 jaar gestopt waren met de behandeling .

In **hoofdstuk 5** worden de lange termijn effecten op het gebied van groei, BMD, lichaamssamenstelling en de schildklier beschreven. Uit dit onderzoek bleek dat vrouwen die behandeld waren met een protocol met zogenaamde alkylerende chemotherapie (MOPP) een lagere BMD van het gehele lichaam hadden, terwijl deze behandeling bij mannen leidde tot een kleinere lichaamslengte en hogere BMI. De lichaamssamenstelling en de schildklierfunctie waren voor allen normaal.

Vervolgens beschrijft **hoofdstuk 6** het onderzoek naar schade aan de zaadbal bij mannen die als kind behandeld zijn voor de ziekte van Hodgkin. Bij deze mannen zijn meerdere hormonen bepaald die samenhangen met de functie van de zaadballen (Luteïniserend hormoon (LH), follikel stimulerend hormoon (FSH), inhibine B, testosteron en sexhormoon binden eiwit (SHBG)). Ook is er bij 21 mannen aanvullend zaadonderzoek gedaan. Mannen die behandeld waren met MOPP hadden duidelijk verhoogde concentraties LH en FSH en verlaagde concentraties inhibine B in het bloed. Dit wijst op ernstige schade aan de zaadballen. Er was een significante correlatie tussen de hoeveelheid MOPP kuren waarmee een man werd behandeld en de mate van verstoring van de hormoonwaarden in het bloed. De gemiddelde concentratie van zaadcellen in het ejaculaat was significant lager in mannen die behandeld waren met MOPP kuren. Tenslotte is uit dit onderzoek gebleken dat inhibine B de beste voorspeller is voor de concentratie van zaadcellen in het ejaculaat.

In **hoofdstuk 7** wordt het onderzoek naar de schade aan de eierstokken bij vrouwen die als kind behandeld zijn voor de ziekte van Hodgkin beschreven. Er werd gekeken naar verschillende hormonen (LH, FSH, inhibine B, oestrogeen en anti-müllerisch hormoon (AMH)). In dit onderzoek bleek dat bij vrouwen behandeld met MOPP de eierstokken slechter werkten dan bij vrouwen die zonder MOPP waren behandeld. Inhibine B en AMH concentraties in het bloed waren lager en FSH concentraties hoger bij vrouwen behandeld met MOPP. In vergelijking met de mannen waren de verschillen in FSH en inhibine B tussen vrouwen behandeld met en zonder MOPP echter beduidend minder groot. Deze hormoonwaarden zijn bij vrouwen dus minder goed bruikbaar bij het opsporen van mogelijke schade aan de eierstokken. AMH bleek echter veel bruikbaarder bij het opsporen van mogelijke schade. AMH is een hormoon dat wordt aangemaakt door nog niet uitgerijpte eicellen in de eierstok. Aangezien aangenomen wordt dat AMH niet wordt beïnvloed door pilgebruik of de menstruele cyclus, in

tegenstelling tot FSH en inhibine B, is het een zeer goed bruikbare marker bij de screening op schade aan de eierstokken bij vrouwen behandeld voor M. Hodgkin, maar ook voor andere vormen van kanker. De concentraties van AMH waren bij vrouwen behandeld met MOPP vergelijkbaar met die bij vrouwen vlak voor de menopauze. Dit duidt op een groot verlies van eicellen bij deze vrouwen.

In **hoofdstuk 8** wordt een overzicht gegeven van de literatuur over lange termijn effecten van de behandeling voor de ziekte van Hodgkin op de kinderleeftijd met alleen chemotherapie. Tot slot wordt in de discussie in **hoofdstuk 9** ingegaan op de uitkomsten van de verschillende studies en worden aanbevelingen gedaan voor toekomstig onderzoek. Het onderzoek naar de effecten van de polymorfismen op de botten en lichaamssamenstelling moet worden uitgebreid naar een grotere, (inter)nationale, groep kinderen die worden behandeld voor kanker. Dit om de door ons gevonden uitkomsten op BMD en lichaamssamenstelling tijdens de behandeling voor ALL te kunnen bevestigen. Daarnaast kan in een dergelijke grote groep ook onderzoek worden gedaan naar de effecten op groei, BMD en lichaamssamenstelling van polymorfismen in andere genen die zijn gerelateerd aan bijwerkingen van de behandeling, zoals bijvoorbeeld *MTHFR* en *MTRR.* Tot slot zouden de verschillende polymorfismen ook onderzocht moeten worden bij grotere cohorten overlevenden van kinderkanker om inzicht te krijgen in de effecten van genetische variaties op de lange termijn effecten

Bij mensen die als kind behandeld zijn voor ALL of de ziekte van Hodgkin is het van belang onderzoek te doen naar mogelijkheden om het optreden van lange termijn effecten te voorkomen of te verminderen, dan wel deze te behandelen als ze toch zijn opgetreden. Botontkalking tijdens of na behandeling voor ALL kan mogelijk voorkomen of verminderd worden door meer lichamelijke activiteit of een behandeling met medicijnen die de botontkalking tegen gaan zoals calcium en vitamine D of bisfosfonaten. Om de vruchtbaarheid van kinderen en volwassenen die voor de ziekte van Hodgkin behandeld worden te beschermen moet niet alleen gezocht worden naar nieuwe methoden om eierstok- of zaadbalweefsel te bewaren, maar moet ook gezocht worden naar effectieve behandelmethoden voor de ziekte van Hodgkin die minder schadelijk zijn voor de inwendige geslachtsorganen en meer toegespitst zijn op de individuele patiënt.

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Curriculum Vitae

De auteur werd geboren op 2 augustus 1975 te Rotterdam. Na het VWO in Groningen ging hij Geneeskunde studeren aan de Erasmus Universiteit Rotterdam. Tijdens de studie werd hij lid van de studentenvereniging SSR-R en was hij penningmeester bij de Medische Faculteitsvereniging (MFVR). Na zijn arts-examen in 2000 ging hij een jaar als arts-assistent niet in opleiding werken op de kinderafdeling van de Isala klinkieken, locatie Weezenlanden. In 2001 begon hij als arts-onderzoeker op de afdeling kinderoncologie en kinderendocrinologie van het, toen nog gewoon Sophia kinderziekenhuis, bij dr. M.M. van den Heuvel-Eibrink en dr. S.M.P.F. de Muinck Keizers-Schrama aan zijn promotieonderzoek. Van maart 2005 tot augustus 2006 is hij nog werkzaam geweest als arts-assistent niet in opleiding op de kinderafdeling van het UMCG te Groningen. Inmiddels is hij na een jaar als arts-assistent psychiatrie niet in opleiding bij de GGZ Friesland in Leeuwarden, begonnen aan zijn opleiding tot psychiater bij diezelfde instelling (opleider: dr. A. Wunderink). Zijn keuzestage kinder- en jeugdpsychiatrie deed hij bij Accare in Groningen. In zijn vrije tijd doet hij aan theatersport en improvisatietheater bij theatersportvereniging Ulteam in Groningen en speelt hij piano. Daarnaast is hij vrijwilliger bij de Wielewaal, een vakantieorganisatie voor jongeren met een handicap, voor deze organisatie is hij ook lid van het Medisch Team. Hij woont samen met zijn vriendin Monique Roelofs.

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