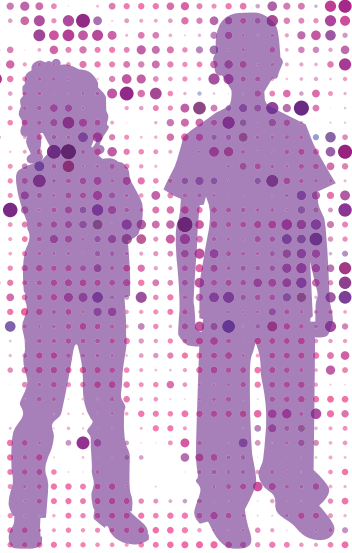
OSTEOGENIC TOXICITY IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

OSTEOGENE TOXICITEIT IN KINDEREN MET ACUTE LYMFATISCHE LEUKEMIE



MARIËL LIZET TE WINKEL

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Mariël Lizet te Winkel

Osteogenic toxicity in childhood acute lymphoblastic leukemia

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Copromotor: Dr. M.M. van den Heuvel-Eibrink

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GENERAL INTRODUCTION AND AIMS OF THE THESIS

1

HEMATOPOIESIS

The formation of blood cellular components is called hematopoiesis. Hematopoiesis starts by the third week of gestation with erythropoiesis in aggregates of blood cells in the yolk sac, called blood islands[1]. As development progresses, the primary site of blood formation migrates to the liver, where besides red blood cells also platelets and leucocytes are synthesized[2,3]. When bone marrow develops by 5-6 months' gestation, it eventually assumes the task of forming most of the blood cells. In children, virtually all marrow cavities are actively hematopoietic. Maturation, activation, and some proliferation of lymphoid cells occurs in secondary lymphoid organs (spleen, thymus, and lymph nodes).

The hematopoietic cells consist of pluripotent hematopoietic stem cells, multipotent progenitor cells, oligolineage progenitor cells and the mature blood cells (Figure 1). The mature blood cells can be divided into three groups of cells: the erythrocytes, the leucocytes and the thrombocytes. Erythrocytes or red blood cells contain hemoglobin which is used to bind oxygen and transport it throughout the body. Leukocytes or white blood cells (lymphocytes, granulocytes, and monocytes) are the cornerstones of the immune system, protecting the body against infections. Thrombocytes or platelets are derived from fragmentation of precursor megakaryocytes and are involved in hemostasis, leading to the formation of blood clots.

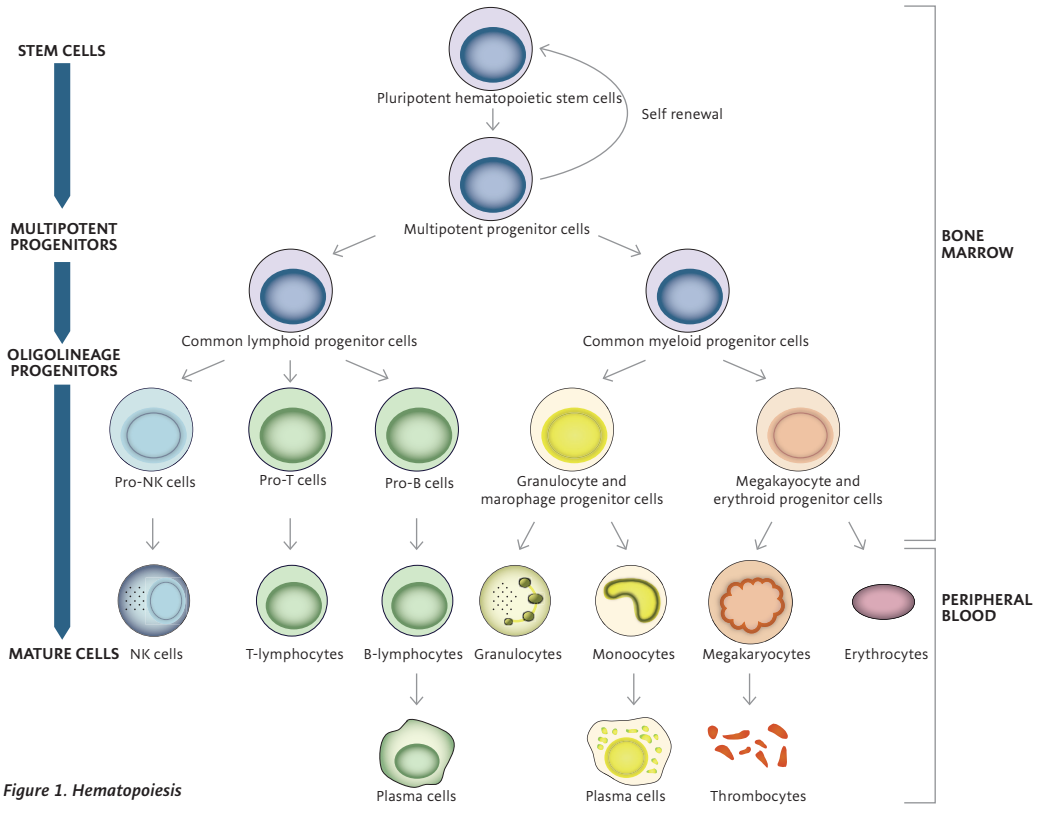


Figure 1. Hematopoiesis

ACUTE LYMPHOBLASTIC LEUKEMIA

Acute lymphoblastic leukemia (ALL) is a malignant disease (cancer) in which immature cells of the lymphoid lineage (lymphoblasts) in the bone marrow continuously multiply because of a maturation arrest and hyperproliferation. The unregulated cell growth is mainly caused by genetic lesions within the lymphoblasts. ALL can be subdivided depending on which type of lymphoblasts are affected by the maturation arrest. In the most common genetic subtypes of ALL the first genetic hit occurs in utero, initiating formation of preleukemic cells[4]. Multiple genetic hits are required to develop full-blown leukemia.

ALL is the most common pediatric malignancy[5]. In the Netherlands each year approximately 120 patients are diagnosed with ALL. The peak incidence of ALL is observed in children aged between 2 and 5 years. Symptoms result from impaired numbers of normal blood cells because they are crowded out by the malignant lymphoid cells. Therefore, children with ALL experience symptoms from malfunctioning of their erythrocytes (anemia, fatigue, short of breath, paleness), leukocytes (fever, infections), and thrombocytes (bruising, petechiae, nosebleeds, other bleedings). Moreover, they may present with bone pain due to expansion of the bone marrow by leukemic infiltration. Other presenting symptoms may be swelling of lymph nodes, hepatosplenomegaly, and enlarged testes due to infiltration of leukemic cells.

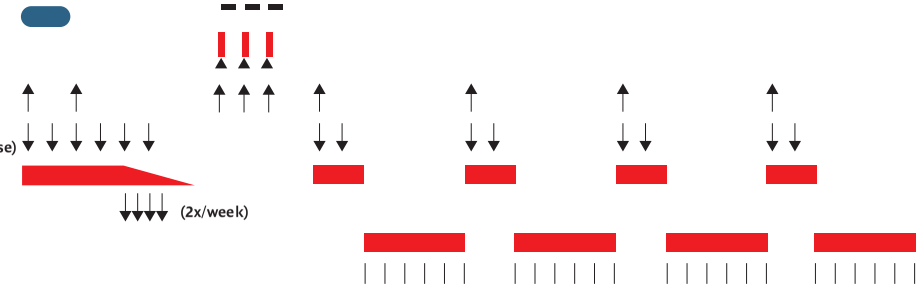
With the current 2-year treatment protocols a survival of more than 80% is reached[6-8]. Factors that account for the improvement in survival during the past 40 years include the use of effective drugs into multi-agent chemotherapy protocols, the integration of presymptomatic central nervous system (CNS) treatment, intensification of treatment using existing drugs, and risk-based stratification of treatment[9]. As increasing numbers of pediatric ALL patients are surviving, research on the complications of the disease and its treatment have gained attention[10]. Among these are skeletal complications, such as a reduced bone mineral density (BMD), an increased fracture risk as well as osteonecrosis[11,12].

STUDY POPULATION

All studies in this thesis were performed in childhood ALL patients, who were treated according to the ALL9 treatment protocol of the Dutch Childhood Oncology Group (DCOG) (Figures 2A and 2B). This protocol is a dexamethasone-based protocol which resembles the moderately intensive DCOG ALL-6 protocol (1984–1988) for non-high-risk patients. The aim of the ALL-9 protocol was to confirm the good results of the ALL-6 protocol, in which the use of dexamethasone instead of prednisone, together with medium-dose methotrexate (3×2 g/m^2) and 12 doses of triple intrathecal therapy instead of cranial irradiation not only resulted in a low incidence of meningeal relapses, but also a reduction of bone-marrow relapses. However, because concerns were raised about the skeletal toxicity of the very intensive use of dexamethasone, studies on bone toxicity were integrated.

NHR

Window		
Folinic acid (rescue)	Oral/ intravenous	3x15 mg/m²
Medium-dose methotrexate	24 h infusion	2 g/m²
Triple	Intrathecal	According to age
Vincristine	Intravenous	2 mg/m² (max 2,5 mg/dose)
Dexamethasone	Oral	6 mg/m²
Asparaginase	Intravenous	6000 IU/m²
Mercaptopurine	Oral	50 mg/m²
Methotrexate	Oral	30 mg/m²



HR

Window		
Folinic acid (rescue)	Oral/ intravenous	3x15 mg/m²
High-dose methotrexate	24 h infusion	3 g/m²
Mercaptopurine	Oral	50 mg/m²
Triple	Intrathecal	According to age
Vincristine	Intravenous	2 mg/m² (max 2,5 mg/dose)
Daunorubicin	Intravenous	25 mg/m²
Dexamethasone	Oral	6 mg/m²
Asparaginase	Intravenous	6000 IU/m²
Mercaptopurine	Oral	50 mg/m²
Asparaginase	Intravenous	10000 IU/m²
Cytarabine/cyclophosphamide	Intravenous	80/80 mg/m²
Methotrexate	Intravenous	30 mg/m²

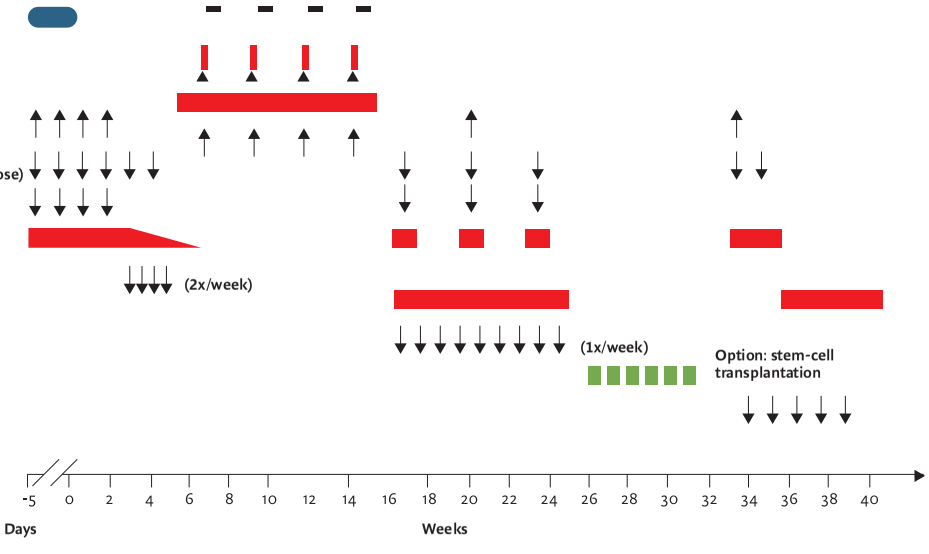


Figure 2

Figure 2: Outline of Dutch Childhood Oncology Group (DCOG) ALL-9 protocol *(Veerman et al., Lancet Oncology 20097)*

A. Non high risk (NHR) protocol. *NHR patients received a three-drug induction for 6 weeks, followed by 3 weeks of medium-dose methotrexate (2 g/m2), and maintenance therapy consisting of 2 weeks of dexamethasone treatment with two doses of vincristine and, until week 60, one intrathecal triple therapy alternated with 5 weeks 6-mercaptopurine daily and oral methotrexate weekly. Maintenance was continued until week 109. Patients received low-dose co-trimoxazole on 3 days every week except during medium-dose methotrexate therapy. Criteria for starting the medium-dose methotrexate block were white-blood-cell count >2000 cells per µL, thrombocytes >75 000 per µL, and normal liver and kidney function; treatment could be postponed for 1 week if necessary.*

B. High risk (HR) protocol. *HR patients received a four-drug induction, followed by high-dose methotrexate (3 g/m2) with oral mercaptopurine daily, and two intensification courses in week 15–22 and 24–32. Maintenance was similar to that for NHR patients, with the exception that methotrexate was given intravenously. Maintenance was continued until week 109. Patients received low dose co-trimoxazole on 3 days every week, except during treatment with high-dose methotrexate. Criteria for starting the high-dose methotrexate block were white-blood-cell count >2000 cells per µL, thrombocytes >75 000 per µL, and normal liver and kidney function; treatment could be postponed for 1 week if necessary. Red blocks denote daily medication. Arrows denote weekly or bi-weekly high-dose methotrexate or twice weekly asparaginase.*

Bone mineral density

During life time, bone mass develops as depicted in Figure 3. Bone mass in later life is determined by the peak bone mass acquired during childhood and adolescence[13].

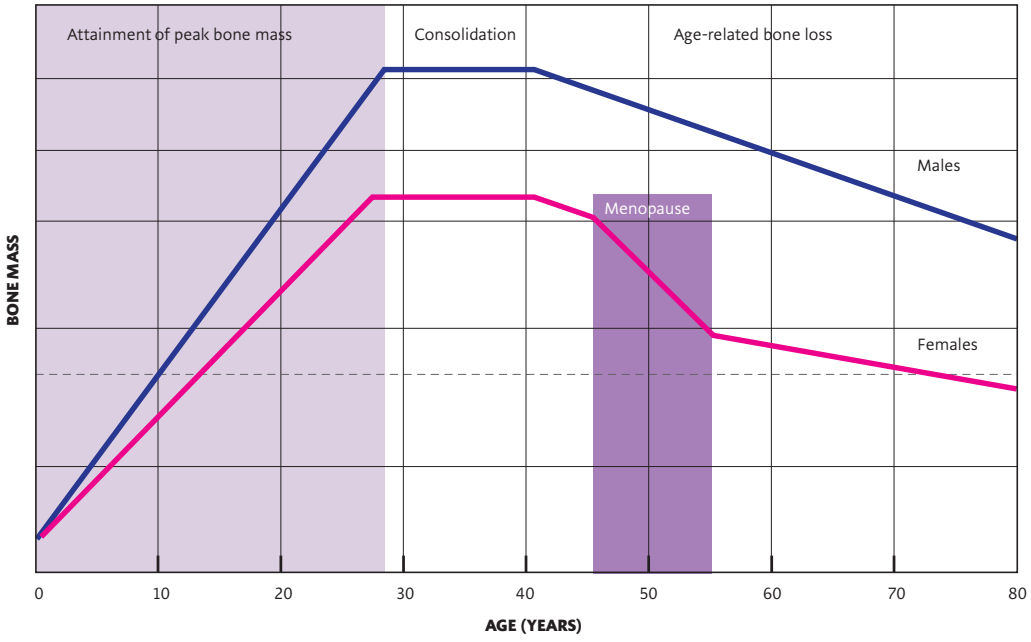


Figure 3. Development of bone mass during life

Factors that may influence the achievement of peak bone mass in children with ALL are leukemia itself, predisposing genetic factors, physical activity and chemotherapy, especially corticosteroids and methotrexate. Bone is composed of cells, minerals and organic matrix. Bone mineral density (BMD) is the ratio between the amount of minerals measured and the projected area. The golden standard for the measurement of BMD is dual energy X-ray absorptiometry (DXA)[14]. The low radiation dose and the good precision are positive features of DXA. One of the primary regions of interest to measure BMD is the lumbar spine (Figure 4A). As the spine contains mainly trabecular bone, with a high metabolic activity, changes in BMD may be detected in an early phase. Another way of evaluating skeletal health with DXA is the total body measurement, which reflects mainly cortical bone (Figure 4B). The total body DXA measurement allows evaluation of additional parameters of body composition such as body fat or lean body mass. In children BMD needs to be adjusted for age and gender and is therefore expressed as a Z-score or standard deviation score[15]. A low BMD is defined as a Z-score, adjusted for age and gender, of less than or equal to -2.0.

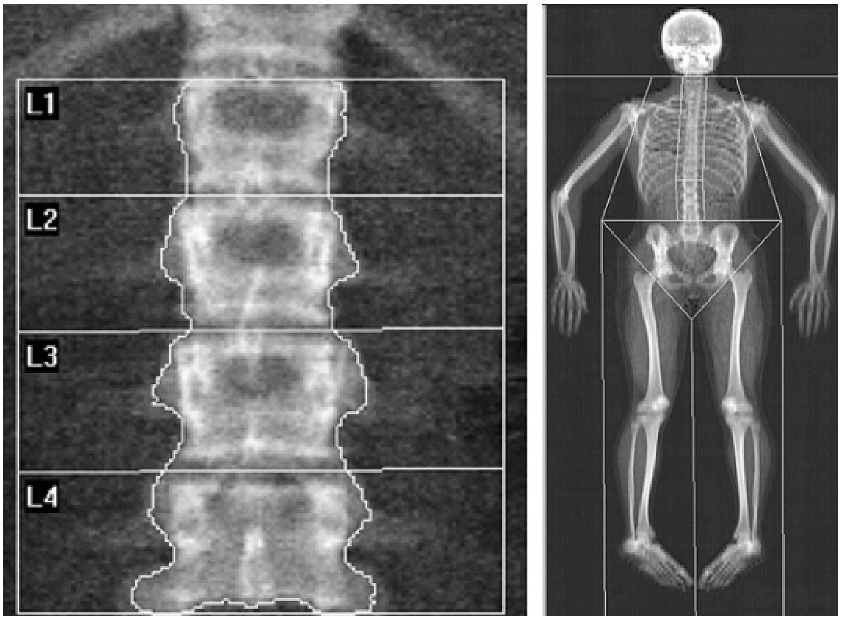


Figure 4. Pediatric dual energy X-ray absorptiometry (DXA): A. lumbar spine, B. total body *(figures adapted from van Kuijk et al., Radiol Clin North Am 2010[14])*

Osteoporosis literally means porous bones and is described as "a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk"[16]. The diagnosis of osteoporosis in children requires the presence of both a clinical significant fracture history and low BMD[15]. A clinical significant fracture history is defined as a vertebral compression fractures, fractures of long bones in the lower extremities or two or more fractures.

OSTEONECROSIS

Osteonecrosis, also called avascular necrosis of bone (AVN), aseptic necrosis of bone, or ischemic bone necrosis, results from a compromised blood circulation of the bone leading to the death of bone cells. Normally, bone remodeling is a continuous process of breaking down and rebuilding, i.e. old bone is reabsorbed and replaced with new bone to maintain a balance. In the course of osteonecrosis the bone tissue breaks down faster than the body can repair. The exact pathogenesis is not fully understood, but it is a multifactorial disease and corticosteroids have been identified as the main cause in pediatric ALL patients (Figure 5).

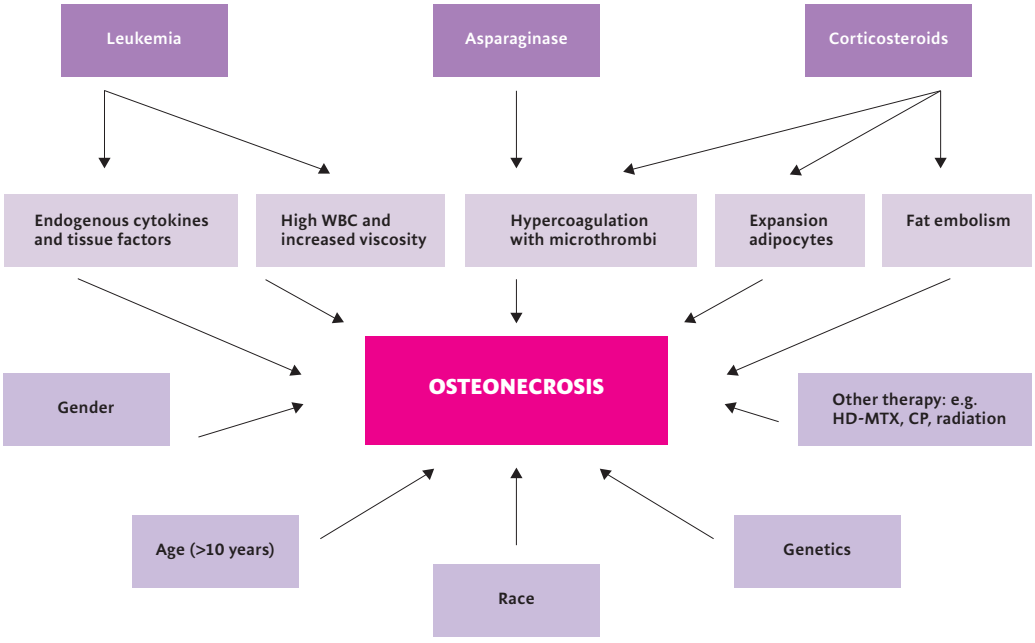


Figure 5. Multifactorial pathogenesis of osteonecrosis

Abbreviations: WBC = white blood-cell count, HD-MTX = high-dose methotrexate, CP = cyclophosphamide

Osteonecrosis causes severe bone pain, limping, limited range of motion and may eventually result in joint destruction. Osteonecrosis is most often located in the weight-bearing joints and in pediatric ALL patients often multiple joints are affected. One of the widely used clinical staging systems is the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (Table 1)[17]. These NCI criteria provide a severity scale for osteonecrosis with 1 being asymptomatic osteonecrosis diagnosed by radiologic screening, and stage 2 to 4 indicating symptomatic osteonecrosis gradually ascending from mild to disabling symptoms. MRI is the most sensitive method for detection of osteonecrosis and it is characteristically recognized by the double-line sign (Figure 6)[18,19].

COMMON TERMINOLOGY CRITERIA FOR ADVERSE EVENTS V3.0 (CTCAE) MUSCULOSKELETAL AND CONNECTIVE TISSUE DISORDERS					
Adverse Event	1	2	3	4	5
Osteonecrosis or avascular necrosis	Asymptomatic, radiographic findings only	Symptomatic and interfering with function, but not interfering with ADL	Symptomatic and interfering with ADL	Disabling	Death

Table 1. Common terminology criteria for osteonecrosis of the National Cancer Institute (NCI)[17]

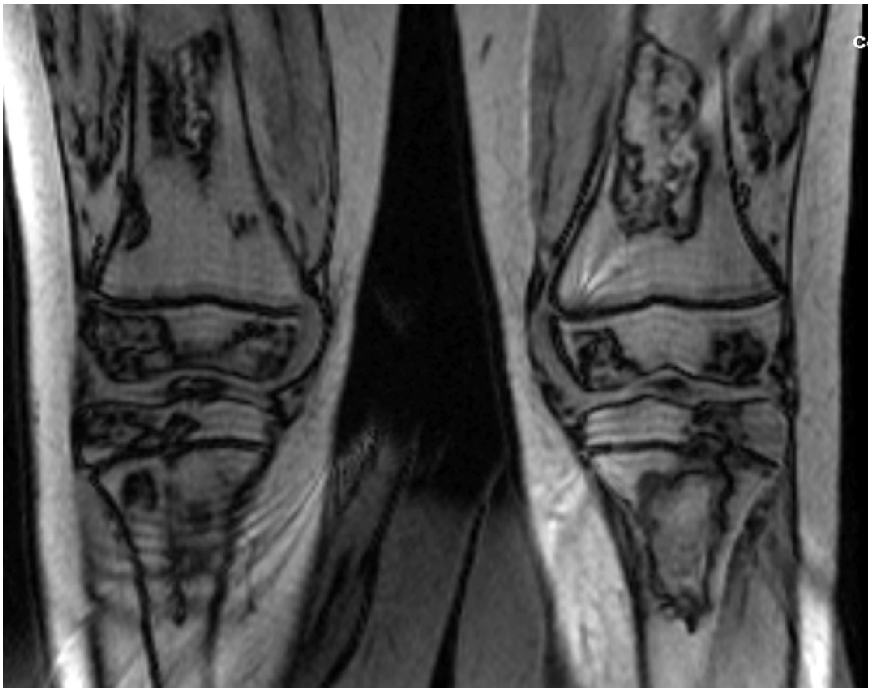


Figure 6. Magnetic resonance imaging (MRI) of bilateral osteonecrosis of the knees

The arrows indicate the classic MRI appearance of osteonecrosis with a focal serpiginous low signal intensity with fatty center.

The characteristic low signal intensity border appears as a dark line adjacent to a bright-line, the so-called "double-line sign".

GENETIC VARIATION OF OSTEOGENIC SIDE EFFECTS

Almost all cells in our body contain a nucleus in which deoxyribonucleic acid (DNA) is stored (Figure 7). DNA is organized into long structures, called chromosomes. DNA consists of nucleotides (guanine, adenine, thymine and cytosine) that encode the instructions used in the development and functioning of a person[20].

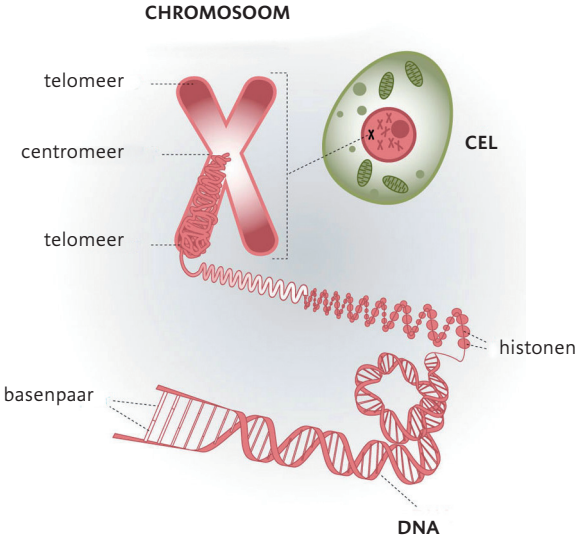


Figure 7. A schematic representation of the genetic material *(bron: www.brusselsgenetics.be).*

Although the DNA of all humans is largely identical, minor differences in the base-pair sequence are responsible for the fact that every person is unique. Moreover, these differences in DNA may account for individual differences in the risk of development of certain diseases, responses to treatment and development of side effects. There are various types of genetic variations, such as copy-number variations (CNVs), variable number tandem repeats (VNTRs), insertion/ deletion type of polymorphisms and single nucleotide polymorphisms (SNPs). CNVs are rearrangements due to deletions, duplications, inversions or translocations that result in an abnormal number of copies of a large region of the DNA (1,000 nucleotides to several megabases). VNTRs are short nucleotide sequences organized as a tandem repeat. Important characteristics of such DNA variations are their functional consequences and the frequency with which they occur in the population. For example, SNPs are DNA sequence variations of a single nucleotide that occur in more than 1% of the population, as opposed to a point mutation which is much more rare in the population but can have more dramatic consequences on the protein function. A frameshift mutation is a genetic mutation caused by insertions or deletions (indels) of a number of nucleotides in a DNA sequence that is not dividable by three, therefore leading to a change in the reading frame of the mRNA and the encoded amino acid sequence of the protein.

Because variations in DNA sequences as well as environmental factors can influence a phenotype, it is important to distinguish these factors. For complex traits and diseases, such as BMD deficits, this is analyzed by twin studies, that provide valuable information whether and to what extent a phenotype is determined by genetic factors. If identical twins show more resemblance than fraternal twins, it is likely that the studied characteristic is influenced by genes. When the environment is the only determining factor, no difference is expected between identical or fraternal twins. However, for complex diseases and traits, classical genetic linkage studies (for example those that were successfully used to clone disease genes for “simple” mono-genetic diseases like cystic fibrosis) are difficult to use. For these

complex diseases and traits association study approaches, such as candidate gene studies or genome-wide association studies (GWAS), are more suitable. These studies compare the DNA of persons with the disease (patients) with DNA of similar persons without the disease (controls). With the candidate gene studies a certain SNP or DNA region is chosen beforehand based upon the suspected biological relevance and genetic variations are then compared between patients and controls. GWAS investigate hundred thousands of such genetic variations on a genome wide scale to identify SNPs or other DNA variants that are associated with a disease in a hypothesis-free manner. After discovering an association of a SNP or DNA variation, results need to be replicated in one or more independent cohorts.

SCOPE OF THE THESIS

With this thesis we aimed to reveal the incidence of osteogenic side effects during pediatric ALL, to find risk factors influencing BMD deficits and osteonecrosis, and to evaluate therapeutic approaches for these skeletal side effects.

Chapter 1 contains an introduction to the topics described in this thesis.
In **Part I** of this thesis the studies on BMD are described.
First, **Chapter 2** describes a large prospective nation-wide study in children treated for ALL according to the Dutch Childhood Oncology Group (DCOG)-ALL9 protocol, which was performed to elucidate incidence and risk factors of skeletal toxicity such as BMD deficits and fractures.
As uniformly treated children show large variations in BMD reduction and fractures, we studied genetic risk factors for altered BMD and body composition in pediatric ALL in **Chapter 3** and **Chapter 4**.
Chapter 5 describes the results of a randomized trial investigating an exercise program to prevent reduction of BMD and motor performance during treatment for childhood ALL.

In **Part II** of this thesis we focus on osteonecrosis.
Chapter 6 presents the result of a prospective nation-wide study on incidence, risk factors and long-term outcome of osteonecrosis in pediatric ALL.
Coagulation alterations may be involved in osteonecrosis in childhood ALL. In **Chapter 7** we showed that an impaired dexamethasone-related increase of anticoagulants is associated with the development of osteonecrosis.
Finally, in **Chapter 8** we reviewed the literature on the management and treatment of osteonecrosis in children and adolescents with ALL. As there is no consensus regarding to the optimal management and treatment of osteonecrosis in pediatric ALL patients, we compose a tool for clinical decision making regarding the management of osteonecrosis in pediatric ALL patients.
Chapter 9 and 10 contain a general discussion and a summary of the work in this thesis. In addition we give our future perspectives on minimizing bone density reduction and osteonecrosis in pediatric ALL.

REFERENCES

1. Nelson WE: Essentials of Pediatrics (ed 4th), 2002 pp. 605
2. Thomas DB, Yoffey JM: Human Foetal Haematopoiesis. Ii. Hepatic Haematopoiesis in the Human Foetus. Br J Haematol 10:193-7, 1964
3. Tavian M, Peault B: The changing cellular environments of hematopoiesis in human development in utero. Exp Hematol 33:1062-9, 2005
4. Greaves M: Infection, immune responses and the aetiology of childhood leukaemia. Nat Rev Cancer 6:193-203, 2006
5. Jemal A, Siegel R, Xu J, et al: Cancer statistics, 2010. CA Cancer J Clin 60:277-300, 2010
6. Pui CH, Pei D, Sandlund JT, et al: Long-term results of St Jude Total Therapy Studies 11, 12, 13A, 13B, and 14 for childhood acute lymphoblastic leukemia. Leukemia 24:371-82, 2010
7. Veerman AJ, Kamps WA, van den Berg H, et al: Dexamethasone-based therapy for childhood acute lymphoblastic leukaemia: results of the prospective Dutch Childhood Oncology Group (DCOG) protocol ALL-9 (1997-2004). Lancet Oncol 10:957-66, 2009
8. Schrappe M, Nachman J, Hunger S, et al: 'Educational symposium on long-term results of large prospective clinical trials for childhood acute lymphoblastic leukemia (1985-2000)'. Leukemia 24:253-4, 2010
9. Pieters R, Carroll WL: Biology and treatment of acute lymphoblastic leukemia. Pediatr Clin North Am 55:1-20, ix, 2008
10. Geenen MM, Cardous-Ubbink MC, Kremer LC, et al: Medical assessment of adverse health outcomes in long-term survivors of childhood cancer. JAMA 297:2705-15, 2007
11. van der Sluis IM, van den Heuvel-Eibrink MM: Osteoporosis in children with cancer. Pediatr Blood Cancer 50:474-8; discussion 486, 2008
12. Barr RD, Sala A: Osteonecrosis in children and adolescents with cancer. Pediatr Blood Cancer 50:483-5; discussion 486, 2008
13. Hansen MA, Overgaard K, Riis BJ, et al: Role of peak bone mass and bone loss in postmenopausal osteoporosis: 12 year study. BMJ 303:961-4, 1991
14. van Kuijk C: Pediatric bone densitometry. Radiol Clin North Am 48:623-7, 2010
15. Official positions of the International Society for Clinical Densitometry (ISCD). 2007. www.iscd.org
16. Kanis JA, Melton LJ, 3rd, Christiansen C, et al: The diagnosis of osteoporosis. J Bone Miner Res 9:1137-41, 1994
17. National Cancer Institute Common Terminology Criteria for adverse events, version 3.0. Aug. 2006. http://ctep.cancer.gov/protocolDevelopment.
18. Mitchell MD, Kundel HL, Steinberg ME, et al: Avascular necrosis of the hip: comparison of MR, CT, and scintigraphy. AJR Am J Roentgenol 147:67-71, 1986
19. Sansgiri RK, Neel MD, Soto-Fourier M, et al: Unique MRI findings as an early predictor of osteonecrosis in pediatric acute lymphoblastic leukemia. AJR Am J Roentgenol 198:W432-9, 2012
20. Biemans ALBM, Jochems AAF, Sprangers JAP: DNA een blauwdruk, (ed 2nd), 2005

NATIONAL BONE MINERAL DENSITY STUDY: BONE MINERAL DENSITY AT DIAGNOSIS DETERMINES FRACTURE RATE IN CHILDREN TREATED ACCORDING TO THE DCOG-ALL9 PROTOCOL

Mariël L. te Winkel, Rob Pieters, Wim C.J. Hop, Jan C. Roos, Jos P.M. Bökkerink, Jan A. Leeuw, Marrie C.A. Bruin, Wouter J.W.Kollen, Anjo J.P. Veerman, Hester A. de Groot-Kruseman, Inge M. van der Sluis, Marry M. van den Heuvel-Eibrink

(Acknowledgement: M. Pruissen (assistance in collecting data))

Submitted 2013

PHARMACOGENETIC RISK FACTORS FOR ALTERED BONE MINERAL DENSITY AND BODY COMPOSITION IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

Mariël L. te Winkel, Robert D. van Beek, Sabine M.P.F. de Muinck Keizer-Schrama, André G. Uitterlinden, Wim C.J. Hop, Rob Pieters, and Marry M. van den Heuvel-Eibrink

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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 significantly 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 ≥50x10^9/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: 1,244 mg/m^2 (high risk) and 1,370 mg/m^2 (non-high risk). Total cumulative dose of methotrexate was 13,650 mg/m^2 in

the high-risk protocol and 8,100 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).

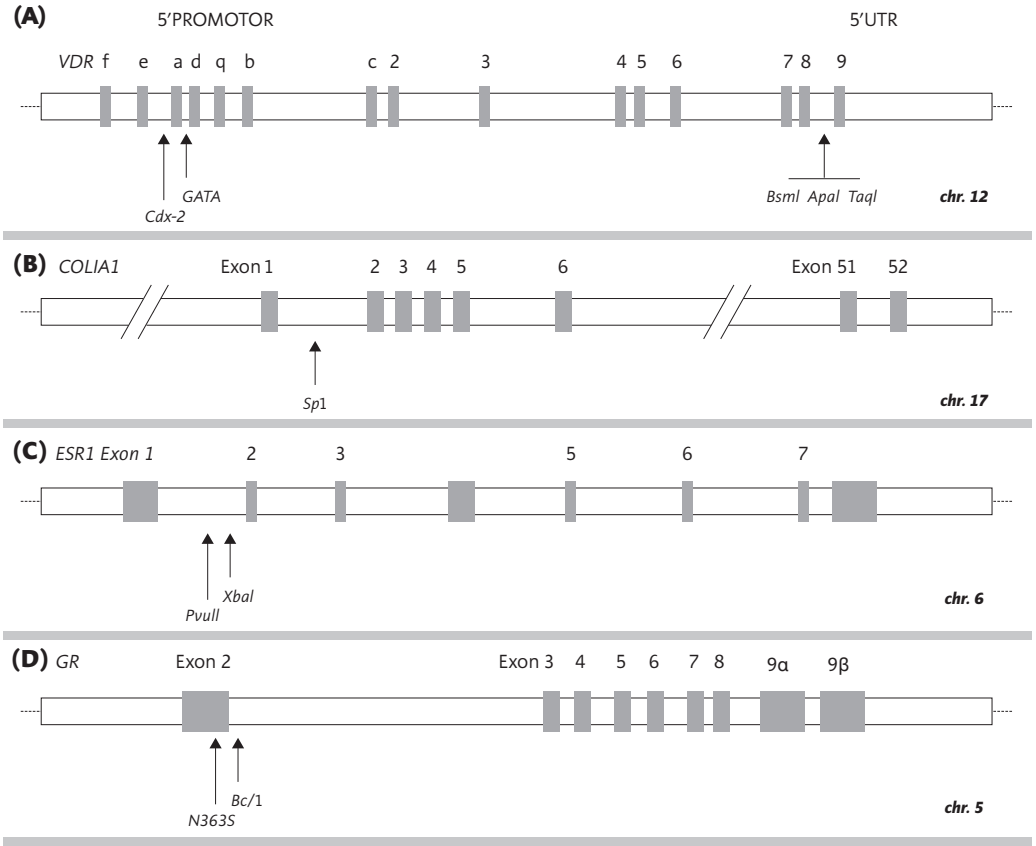

Figure 1. Genomic structure and positions of the single nucleotide polymorphisms investigated in current study. (A) The vitamin-D receptor gene (VDR). (B) The collagen type I alpha 1 gene (COLIA1). (C) The estrogen receptor alpha gene (ESR1). (D) The glucocorticoid receptor gene (GR). *Abbreviations: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

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/(πxwidth)). '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].

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 ≤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)* polymorphism were determined (data not shown).

VDR 3′-END (BSMI/APAI/TAQI)				VDR 5′-END (CDX-2/GATA)				COLIA1 (SP1)			ESR1 (PVUII/XBAI)				GR (BCLI)		
Geno-type	Haplo-type code	N	%	Geno-type	Haplo-type code	N	%	Geno-type	N	%	Geno-type	Haplo-type code	N	%	Geno-type	N	%
baT-baT	1/1	17	25	GA-GA	1/1	13	20	GG	49	73	px-px	1/1	20	29	CC	20	41
baT-BAt	1/2	16	23	GA-GG	1/2	19	30	GT	17	25	px-PX	1/2	29	43	CG	22	45
baT-bAT	1/3	8	12	GA-AG	1/3	15	23	TT	1	2	px-Px	1/3	4	6	GG	7	14
baT-BAT	1/4	2	3	GG-GG	2/2	5	8				PX-PX	2/2	10	15			
BAt-BAt	2/2	14	21	GG-AG	2/3	9	14				PX-Px	2/3	4	6			
BAt-bAT	2/3	5	7	AG-AG	3/3	3	5				Px-Px	3/3	1	1			
Bat-BAT	2/4	4	6														
bAT-bAT	3/3	2	3														
Total		68				64			67				68			49	
HWE p-value		0.30				0.72			0.73				0.84			0.36	

Table 1. Genotype distribution in pediatric ALL patients.

Abbreviations: VDR=vitamin D receptor gene, COLIA1=collagen type I alpha 1 gene, ESR1=estrogen receptor alpha gene, GR=glucocorticoid receptor gene, 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, $p=0.25$).

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 $\%fat_{TB}$ were not different between the various carrier groups during treatment, or in the year after treatment.

During treatment, the whole group of patients had a lower LBM than healthy peers (area under the curve during treatment: $p<0.001$) and it showed no significant change during treatment. In addition, non-carriers showed the same development of LBM during treatment as carriers of the different risk alleles (Table 2). Although LBM of the whole group increased in the year after completion of treatment (Δ_2LBM=+0.23SDS, $p<0.01$), LBM remained lower than in healthy peers (LBM=-0.69SDS, $p<0.001$). The first year after treatment was completed, LBM increased in non-carriers of *ESR1 (PvuII/XbaI)* haplotype 3, but not in carriers (non-carriers: Δ_2LBM=+0.28SDS, carriers: Δ_2LBM=-0.55SDS; $p<0.01$). The Δ_2LBM was not different for non-carriers compared to carriers of the other 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 $\Delta_1BM(A)D_{LS}$ and $\Delta_2BM(A)D_{LS}$ 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.

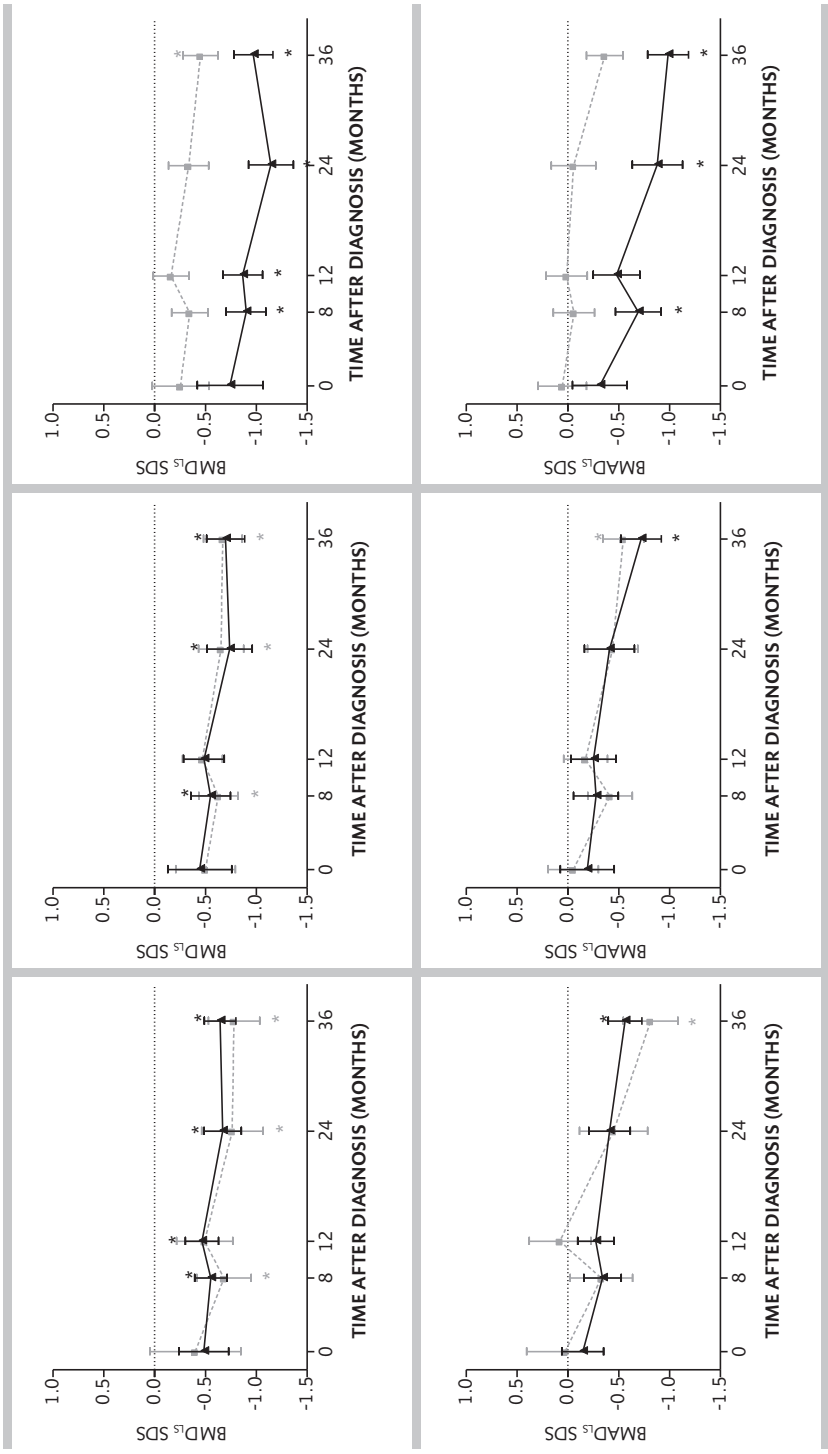

Figure 2. Bone mineral (apparent) density of the lumbar spine in non-carriers versus carriers of haplotypes of the VDR 5'-end (Cdx-2/ GATA)
(mean ± SEM) *Abbreviations: BM(A)D$_{LS}$=bone mineral (apparent) density of the lumbar spine, SDS=standard deviation score, non-carrier=—■—, carrier=--▲--, *=significant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

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).

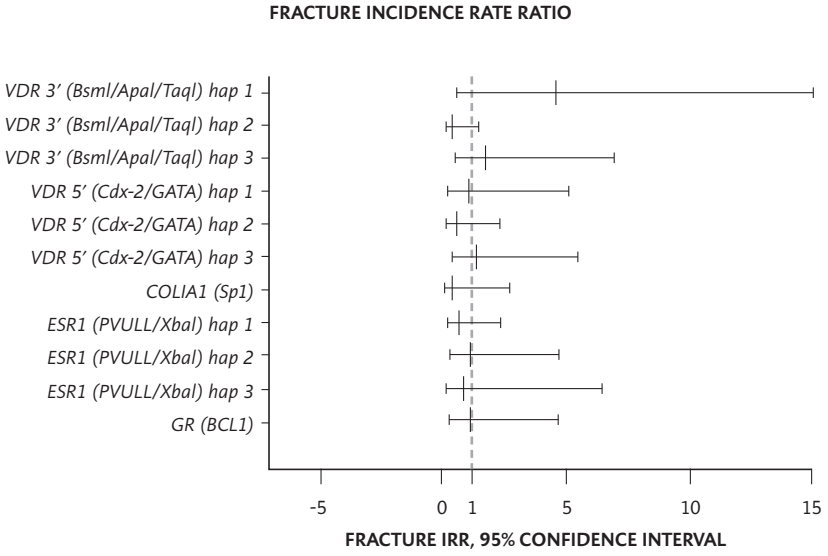

Figure 3. Fracture incidence-rate ratios for non-carriers versus carriers of the different allelic variants

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.

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.

		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	Δ1: 0.84 Δ2: 0.81
	Cessation of treatment	2.36	2.36	0.93	
	1 Year after cessation of treatment	2.38	2.37	0.78	
PTH (SD above upper limit)	Diagnosis	-2.93	-2.05	0.17	Δ1: 0.92 Δ2: 0.27
	Cessation of treatment	-1.90	-1.09	0.26	
	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	Δ1: 0.86 Δ2: 0.30
	Cessation of treatment	134.4	128.3	0.70	
	1 Year after cessation of treatment	134.5	145.9	0.25	

Table 3. Biochemical markers in non-carriers and carriers of the VDR 5'-end haplotype 3. *Abbreviations: VDR=vitamin-D receptor gene, Δ1=change during treatment, Δ2=change after treatment discontinuation, SD=standard deviation.*

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.

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.

		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
Δ1	Height (SDS)	*-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 (SDS)	*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} (SDS)	*0.17*	*-0.32*	*-0.33*	*-0.03*	*-0.08*	*-0.44*	*-0.36*	*-0.19*	*-0.15*	*-0.29*	*-0.08*	*-0.40*
	$BMAD_{LS}$ (SDS)	*-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} (SDS)	*-0.68*	*-1.20*	*-1.33*	*-0.78*	*-0.97*	*-1.15*	*-1.06*	*-1.08*	*-0.99*	*-1.17*	*-0.96*	*-1.23*
	$\%fat_{TB}$ (SDS)	*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 (SDS)	*0.09*	*-0.40*	*-0.46*	*-0.06*	*-0.20*	*-0.34*	*-0.34*	*-0.20*	*-0.07*	*-0.45*	*-0.25*	*0.24*
Δ2	**Height (SDS)**	*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 (SDS)	*-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} (SDS)	*-0.01*	*0.02*	*-0.02*	*0.02*	*-0.06*	*0.24*	*-0.02*	*0.02*	*-0.02*	*0.04*	*-0.12*	*0.17*
	$BMAD_{LS}$ (SDS)	*-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} (SDS)	*0.44*	*0.22*	*0.21*	*0.37*	*0.25*	*0.45*	*0.54*	*0.23*	*0.32*	*0.30*	*0.18*	*0.46*
	$\%fat_{TB}$ (SDS)	*-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 (SDS)	*0.23*	*0.22*	*0.26*	*0.18*	*0.12*	*0.57***	*0.61*	*0.10***	*0.03*	*0.42***	*0.11*	*0.38*

Table 2. Change of anthropometry, bone mineral density and body composition for the investigated genetic variations of the VDR, COLIA1, ESR1 and GR genes. *Abbreviations: VDR=vitamin-D receptor gene, COLIA1=collagen type I alpha 1 gene, ESR1=estrogen receptor alpha gene, GR=glucocorticoid receptor gene, Δ1=change during treatment, Δ2=change after treatment discontinuation, SDS=standard deviation score, 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).*

	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
	-0.60	*-0.68*	*-0.55*	*-0.64*	*-0.65*	*-0.60*	*-0.62*	*-0.64*	*-0.44*	*-0.62*
	1.50	*1.36*	*1.23*	*1.50*	*1.78*	*1.24*	*1.38*	*1.81*	*1.11*	*1.48*
	-0.04	*-0.44*	*-0.57*	*-0.03*	*-0.14*	*-0.16*	*-0.02*	*-1.09*	*-0.18*	*0.06*
	-0.26	*-0.28*	*-0.50*	*-0.19*	*-0.29*	*-0.25*	*-0.20*	*-0.64*	*-0.24*	*0.03*
	-0.96	*-1.13*	*-1.62*	*-0.82*	*-0.76*	*-1.13*	*-0.91*	*-1.72*	*-1.40*	*-0.80*
	1.44	*1.13*	*1.13*	*1.44*	*1.61*	*1.22*	*1.36*	*1.40*	*1.47*	*1.15*
	-0.19	*-0.30*	*-0.32*	*-0.20*	*-0.12*	*-0.28*	*-0.22*	*-0.36*	*-0.33*	*-0.10*
	0.14	*0.30*	*0.00*	*0.32*	*0.19*	*0.29*	*0.28*	*0.04*	*0.29*	*0.27*
	-0.35	*-0.26*	*-0.29*	*-0.35*	*-0.42*	*-0.28*	*-0.28*	*-0.76*	*-0.01*	*-0.19*
	0.09	*-0.17*	*0.37*	*-0.09***	*-0.11*	*0.07*	*0.00*	*0.02*	*-0.09*	*0.11*
	-0.06	*-0.55***	*-0.11*	*-0.24*	*-0.25*	*-0.20*	*-0.23*	*0.12*	*-0.24*	*-0.23*
	0.36	*0.15*	*0.50*	*0.24*	*0.27*	*0.31*	*0.27*	*0.55*	*0.15*	*0.36*
	-0.54	*-0.84*	*-0.32*	*-0.73*	*-0.81*	*-0.55*	*-0.61*	*-0.88*	*-0.55*	*-0.46*
	0.16	*0.37*	*0.22*	*0.23*	*0.11*	*0.29*	*0.28*	*-0.55**	*0.38*	*0.28*

REFERENCES

1. Veerman AJ, Hahlen K, Kamps WA, Van Leeuwen EF, De Vaan GA, Solbu G, et al. High cure rate with a moderately intensive treatment regimen in non-high-risk childhood acute lymphoblastic leukemia. Results of protocol ALL VI from the Dutch Childhood Leukemia Study Group. J Clin Oncol. 1996 Mar;14(3):911-8.
2. Boot AM, van den Heuvel-Eibrink MM, Hahlen K, Krenning EP, de Muinck Keizer-Schrama SM. Bone mineral density in children with acute lymphoblastic leukaemia. Eur J Cancer. 1999 Nov;35(12):1693-7.
3. Crofton PM, Ahmed SF, Wade JC, Stephen R, Elmlinger MW, Ranke MB, et al. Effects of intensive chemotherapy on bone and collagen turnover and the growth hormone axis in children with acute lymphoblastic leukemia. J Clin Endocrinol Metab. 1998 Sep;83(9):3121-9.
4. van der Sluis IM, van den Heuvel-Eibrink MM, Hahlen K, Krenning EP, de Muinck Keizer-Schrama SM. Altered bone mineral density and body composition, and increased fracture risk in childhood acute lymphoblastic leukemia. J Pediatr. 2002 Aug;141(2):204-10.
5. Reid IR. Glucocorticoid osteoporosis--mechanisms and management. Eur J Endocrinol. 1997 Sep;137(3):209-17.
6. Ragab AH, Frech RS, Vietti TJ. Osteoporotic fractures secondary to methotrexate therapy of acute leukemia in remission. Cancer. 1970 Mar;25(3):580-5.
7. Alvarez-Hernandez D, Naves M, Diaz-Lopez JB, Gomez C, Santamaria I, Cannata-Andia JB. Influence of polymorphisms in VDR and COLIA1 genes on the risk of osteoporotic fractures in aged men. Kidney Int Suppl. 2003 Jun(85):S14-8.
8. Fang Y, van Meurs JB, Bergink AP, Hofman A, van Duijn CM, van Leeuwen JP, et al. Cdx-2 polymorphism in the promoter region of the human vitamin D receptor gene determines susceptibility to fracture in the elderly. J Bone Miner Res. 2003 Sep;18(9):1632-41.
9. Fang Y, van Meurs JB, d'Alesio A, Jhamai M, Zhao H, Rivadeneira F, et al. Promoter and 3'-untranslated-region haplotypes in the vitamin d receptor gene predispose to osteoporotic fracture: the rotterdam study. Am J Hum Genet. 2005 Nov;77(5):807-23.
10. Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, et al. Prediction of bone density from vitamin D receptor alleles. Nature. 1994 Jan 20;367(6460):284-7.
11. Tokita A, Matsumoto H, Morrison NA, Tawa T, Miura Y, Fukamauchi K, et al. Vitamin D receptor alleles, bone mineral density and turnover in premenopausal Japanese women. J Bone Miner Res. 1996 Jul;11(7):1003-9.
12. Uitterlinden AG, Pols HA, Burger H, Huang Q, Van Daele PL, Van Duijn CM, et al. A large-scale population-based study of the association of vitamin D receptor gene polymorphisms with bone mineral density. J Bone Miner Res. 1996 Sep;11(9):1241-8.
13. Gong G, Stern HS, Cheng SC, Fong N, Mordeson J, Deng HW, et al. The association of bone mineral density with vitamin D receptor gene polymorphisms. Osteoporos Int. 1999;9(1):55-64.
14. Lorentzon M, Lorentzon R, Nordstrom P. Vitamin D receptor gene polymorphism is related to bone density, circulating osteocalcin, and parathyroid hormone in healthy adolescent girls. J Bone Miner Metab. 2001;19(5):302-7.
15. Sainz J, Van Tornout JM, Loro ML, Sayre J, Roe TF, Gilsanz V. Vitamin D-receptor gene polymorphisms and bone density in

prepubertal American girls of Mexican descent. N Engl J Med. 1997 Jul 10;337(2):77-82.

16. Baroncelli GI, Federico G, Bertelloni S, Ceccarelli C, Cupelli D, Saggese G. Vitamin-D receptor genotype does not predict bone mineral density, bone turnover, and growth in prepubertal children. Horm Res. 1999;51(3): 150-6.
17. van der Sluis IM, de Muinck Keizer-Schrama SM, Krenning EP, Pols HA, Uitterlinden AG. Vitamin D receptor gene polymorphism predicts height and bone size, rather than bone density in children and young adults. Calcif Tissue Int. 2003 Oct;73(4):332-8.
18. Grundberg E, Brandstrom H, Ribom EL, Ljunggren O, Mallmin H, Kindmark A. Genetic variation in the human vitamin D receptor is associated with muscle strength, fat mass and body weight in Swedish women. Eur J Endocrinol. 2004 Mar;150(3):323-8.
19. Garnero P, Borel O, Grant SF, Ralston SH, Delmas PD. Collagen Ialpha1 Sp1 polymorphism, bone mass, and bone turnover in healthy French premenopausal women: the OFELY study. J Bone Miner Res. 1998 May;13 (5):813-7.
20. Mann V, Hobson EE, Li B, Stewart TL, Grant SF, Robins SP, et al. A COL1A1 Sp1 binding site polymorphism predisposes to osteoporotic fracture by affecting bone density and quality. J Clin Invest. 2001 Apr;107(7):899-907.
21. Uitterlinden AG, Burger H, Huang Q, Yue F, McGuigan FE, Grant SF, et al. Relation of alleles of the collagen type Ialpha1 gene to bone density and the risk of osteoporotic fractures in postmenopausal women. N Engl J Med. 1998 Apr 9;338(15):1016-21.
22. Suuriniemi M, Kovanen V, Mahonen A, Alen M, Wang Q, Lyytikainen A, et al. COL1A1 Sp1 polymorphism associates with bone density in early puberty. Bone. 2006 Sep;39(3):591-7.
23. van der Sluis IM, de Muinck Keizer-Schrama SM, Pols HA, Lequin MH, Krenning EP, Uitterlinden AG. Collagen Ialpha1 polymorphism is associated with bone characteristics in Caucasian children and young adults. Calcif Tissue Int. 2002 Nov;71(5):393-9.
24. Berg JP, Lehmann EH, Stakkestad JA, Haug E, Halse J. The Sp1 binding site polymorphism in the collagen type I alpha 1 (COLIA1) gene is not associated with bone mineral density in healthy children, adolescents, and young adults. Eur J Endocrinol. 2000 Aug;143(2):261-5.
25. Willing MC, Torner JC, Burns TL, Janz KF, Marshall T, Gilmore J, et al. Gene polymorphisms, bone mineral density and bone mineral content in young children: the Iowa Bone Development Study. Osteoporos Int. 2003 Aug;14(8):650-8.
26. Albagha OM, McGuigan FE, Reid DM, Ralston SH. Estrogen receptor alpha gene polymorphisms and bone mineral density: haplotype analysis in women from the United Kingdom. J Bone Miner Res. 2001 Jan;16(1):128-34.
27. Ioannidis JP, Ralston SH, Bennett ST, Brandi ML, Grinberg D, Karassa FB, et al. Differential genetic effects of ESR1 gene polymorphisms on osteoporosis outcomes. Jama. 2004 Nov 3;292(17):2105-14.
28. van Meurs JB, Schuit SC, Weel AE, van der Klift M, Bergink AP, Arp PP, et al. Association of 5′ estrogen receptor alpha gene polymorphisms with bone mineral density, vertebral bone area and fracture risk. Hum Mol Genet. 2003 Jul 15;12(14):1745-54.
29. Boot AM, van der Sluis IM, de Muinck Keizer-Schrama SM, van Meurs JB, Krenning EP, Pols HA, et al. Estrogen receptor alpha gene polymorphisms and bone mineral density in

healthy children and young adults. Calcif Tissue Int. 2004 Jun;74(6):495-500.
30. Tobias JH, Steer CD, Vilarino-Guell C, Brown MA. Effect of an estrogen receptor-alpha intron 4 polymorphism on fat mass in 11-year-old children. J Clin Endocrinol Metab. 2007 Jun;92(6):2286-91.
31. van Rossum EF, Koper JW, van den Beld AW, Uitterlinden AG, Arp P, Ester W, et al. Identification of the BclI polymorphism in the glucocorticoid receptor gene: association with sensitivity to glucocorticoids in vivo and body mass index. Clin Endocrinol (Oxf). 2003 Nov;59(5):585-92.
32. Huizenga NA, Koper JW, De Lange P, Pols HA, Stolk RP, Burger H, et al. A polymorphism in the glucocorticoid receptor gene may be associated with and increased sensitivity to glucocorticoids in vivo. J Clin Endocrinol Metab. 1998 Jan;83(1):144-51.
33. van Schoor NM, Dennison E, Lips P, Uitterlinden AG, Cooper C. Serum fasting cortisol in relation to bone, and the role of genetic variations in the glucocorticoid receptor. Clin Endocrinol (Oxf). 2007 Dec;67(6):871-8.
34. Rosmond R, Chagnon YC, Holm G, Chagnon M, Perusse L, Lindell K, et al. A glucocorticoid receptor gene marker is associated with abdominal obesity, leptin, and dysregulation of the hypothalamic-pituitary-adrenal axis. Obes Res. 2000 May;8(3):211-8.
35 Veerman AJP, Kamps WA, van den Berg-de Ruiter E, van den Berg H, Bökkerink JPM, Bruin MCA, et al. Dexamethasone based therapy in childhood acute lymphoblastic leukaemia with favourable event-free survival: results of the Dutch Childhood Oncology Group (DCOG) ALL-9 protocol (1997-2004). Submitted. 2009.
36. Arai H, Miyamoto KI, Yoshida M, Yamamoto H, Taketani Y, Morita K, et al. The polymorphism in the caudal-related homeodomain protein Cdx-2 binding element in the human vitamin D receptor gene. J Bone Miner Res. 2001 Jul;16(7):1256-64.
37. Grant SF, Reid DM, Blake G, Herd R, Fogelman I, Ralston SH. Reduced bone density and osteoporosis associated with a polymorphic Sp1 binding site in the collagen type I alpha 1 gene. Nat Genet. 1996 Oct;14(2):203-5.
38. Tissing WJ, Meijerink JP, den Boer ML, Brinkhof B, van Rossum EF, van Wering ER, et al. Genetic variations in the glucocorticoid receptor gene are not related to glucocorticoid resistance in childhood acute lymphoblastic leukemia. Clin Cancer Res. 2005 Aug 15;11(16):6050-6.
39. Fredriks AM, van Buuren S, Wit JM, Verloove-Vanhorick SP. Body index measurements in 1996-7 compared with 1980. Arch Dis Child. 2000 Feb;82(2):107-12.
40. Fredriks AM, van Buuren S, Burgmeijer RJ, Meulmeester JF, Beuker RJ, Brugman E, et al. Continuing positive secular growth change in The Netherlands 1955-1997. Pediatr Res. 2000 Mar;47(3):316-23.
41. Kroger H, Vainio P, Nieminen J, Kotaniemi A. Comparison of different models for interpreting bone mineral density measurements using DXA and MRI technology. Bone. 1995 Aug;17(2):157-9.
42. van der Sluis IM, de Ridder MA, Boot AM, Krenning EP, de Muinck Keizer-Schrama SM. Reference data for bone density and body composition measured with dual energy x ray absorptiometry in white children and young adults. Arch Dis Child. 2002 Oct;87(4):341-7; discussion -7.
43. Kemper HC, Bakker I, Twisk JW, van Mechelen W. Validation of a physical activity questionnaire to measure the effect of

mechanical strain on bone mass. Bone. 2002 May;30(5):799-804.

44. Angus RM, Sambrook PN, Pocock NA, Eisman JA. A simple method for assessing calcium intake in Caucasian women. J Am Diet Assoc. 1989 Feb;89(2):209-14.
45. Roodnat JI, van Gurp EA, Mulder PG, van Gelder T, de Rijke YB, de Herder WW, et al. High pretransplant parathyroid hormone levels increase the risk for graft failure after renal transplantation. Transplantation. 2006 Aug 15;82(3):362-7.
46. McCarty MF, Thomas CA. PTH excess may promote weight gain by impeding catecholamine-induced lipolysis-implications for the impact of calcium, vitamin D, and alcohol on body weight. Med Hypotheses. 2003 Nov-Dec;61(5-6):535-42.
47. Fox CS, Yang Q, Cupples LA, Guo CY, Atwood LD, Murabito JM, et al. Sex-specific association between estrogen receptor-alpha gene variation and measures of adiposity: the Framingham Heart Study. J Clin Endocrinol Metab. 2005 Nov;90(11):6257-62.
48. Ferrari SL, Rizzoli R, Slosman DO, Bonjour JP. Do dietary calcium and age explain the controversy surrounding the relationship between bone mineral density and vitamin D receptor gene polymorphisms? J Bone Miner Res. 1998 Mar;13(3):363-70.
49. Ho YV, Briganti EM, Duan Y, Buchanan R, Hall S, Seeman E. Polymorphism of the vitamin D receptor gene and corticosteroid-related osteoporosis. Osteoporos Int. 1999;9(2):134-8.
50. Salamone LM, Glynn NW, Black DM, Ferrell RE, Palermo L, Epstein RS, et al. Determinants of premenopausal bone mineral density: the interplay of genetic and lifestyle factors. J Bone Miner Res. 1996 Oct;11(10):1557-65.

GERMLINE VARIATION IN THE MTHFR AND MTRR GENES DETERMINES THE NADIR OF BONE DENSITY IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA; A PROSPECTIVE STUDY

M. L. te Winkel, S. M. P. F. de Muinck Keizer-Schrama, R. de Jonge, R. D. van Beek, I.M. van der Sluis, W. C. J. Hop, R. Pieters, M. M. van den Heuvel-Eibrink

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ABSTRACT

Background

This study aims to identify folate-metabolism-related genetic risk factors for low bone mineral density (BMD) during/after pediatric acute lymphoblastic leukemia (ALL) treatment.

Patients and methods

We investigated the influence of methylenetetrahydrofolate reductase (*MTHFR* 677C>T and 1298A>C) and methionine synthase reductase (*MTRR* 66A>G) single nucleotide polymorphisms (SNPs) on total-body BMD (BMD_{TB}) and lumbar-spine BMD (BMD_{LS}) in 83 patients. Homocysteine, folate and vitamin-B12 were determined. BMD was measured repeatedly using dual-energy x-ray absorptiometry in patients ≥ 4 years (n=68).

Results

Carriers of the *MTHFR* 677 T-allele showed a lower baseline BMD_{TB} than non-carriers (-0.38 SDS vs. +0.55 SDS, p=0.01) and BMD_{TB} remained lower during/after treatment. *MTHFR* 677C>T did not influence treatment-related loss of BMD_{TB} (p=0.39). The *MTRR* 66G-allele carriers showed a trend towards a lower BMDTB compared with non-carriers. Combining these two SNPs, patients carrying ≥2 risk alleles had a significantly lower BMD_{TB} (-1.40 SDS) than patients with one (-0.80 SDS) or no risk alleles (-0.31 SDS). Although carriers of the *MTHFR* 1298A>C had higher homocysteine levels, this SNP was not related to BMD_{TB}. BMD_{LS} of carriers was similar to non-carriers of the investigated SNPs.

Conclusions

The *MTHFR* 677C>T SNP and the *MTRR* 66A>G SNP were identified as determinants of impaired BMD_{TB} in childhood ALL patients.

INTRODUCTION

Impairment of bone mineral density (BMD) and increased fracture risk are known complications of acute lymphoblastic leukemia (ALL) and its treatment[1-6]. Diminished BMD in childhood ALL may be caused by the disease itself as well as its treatment in which corticosteroids and the folate antagonist methotrexate play an important role[7-8]. The fact that uniformly treated children show a large variation in reduction of BMD and subsequent problems such as fractures[4], suggests a role for genetic variation in the host.

A meta-analysis of the data concerning a possible association between the methylenetetrahydrofolate reductase (*MTHFR*) 677C>T single nucleotide polymorphism (SNP) and BMD showed that healthy adult patients with a TT genotype had a slightly but significant lower BMD than adults carrying TC and CC genotypes[9]. Although the underlying pathophysiological mechanism is not fully understood, it is hypothesized that reduced MTHFR enzyme activity raises plasma homocysteine concentration, which disturbs collagen cross-linking[10-13]. Adult studies found a relation between elevated homocysteine levels and low BMD[14] and fracture risk[15-16]. The enzyme MTHFR synthesizes 5-methyltetrahydrofolate, the primary circulatory form of folate, which serves as a methyl donor for homocysteine remethylation to methionine (Figure 1). Both the *MTHFR* 677C>T and the *MTHFR* 1298A>C SNPs are associated with a reduction of catalytic activity of the MTHFR enzyme[17], resulting in an increased homocysteine level[18]. In young female adults, coexistence of the *MTHFR* 677C>T with the methionine synthase reductase (*MTRR*) 66A>G has been described to exacerbate the enhancement of plasma homocysteine[19]. Other factors that may decrease MTHFR activity and increase plasma homocysteine concentrations are folate or vitamin B12 deficiency.

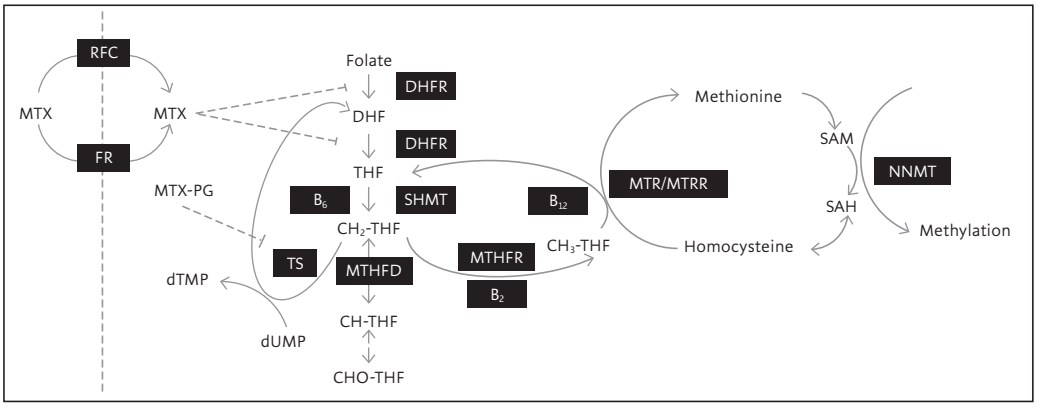


Figure 1. Folate and homocysteine metabolism.

The folate metabolism is depicted on the left and the homocysteine metabolism on the right. The enzymes are presented in boxes. Abbreviations: MTX = methotrexate, MTX-PG = methotrexate-polyglutamates, RFC = reduced folate carrier, FR = folate receptor, DHFR = dihydrofolate reductase, DHF = dihydrofolate, THF = tetrahydrofolate, SHMT = serine hydroxylmethyltransferase, B6 = vitamin B6, CH2-THF = 5,10-methylene-tetrahydrofolate, TS = thymidylate synthase, dUMP = 2'-deoxyuridine-5'-monophosphate, dTMP = 2'-deoxythymidine-5'-monophosphate, MTHFD = methylenetetrahydrofolate dehydrogenase, CH-THF = 5,10-methenyl-tetrahydrofolate, CHO-THF = 10-formyl-tetrahydrofolate, MTHFR = methyltetrahydrofolate reductase, B2 = vitamin B2, CH3-THF = 5'-methyl-tetrahydrofolate, MTR = methionine synthase, MTRR = methionine synthase reductase, B12 = vitamin B12, SAM = S-adenosylmethionine, SAH = S-adenosylhomocysteine, NNMT = nicotinamide N-methyl transferase.

Only one study describes the influence of the *MTHFR* 677C>T on BMD in healthy children[20]; carriers of the T-allele were at risk for a lower BMD_{LS}. Studies of the effect of *MTRR* SNPs on BMD in children are not available. The *MTHFR* and *MTRR* SNPs, may be of particular interest in children treated for ALL, since these children receive high dosages of methotrexate, which is known to increase plasma homocysteine levels (Figure 1)[21-22]. The present study is the first report investigating whether *MTHFR* and *MTRR* SNPs as well as homocysteine, folate and vitamin B12 levels influence therapy-induced variation in BMD during and after treatment of pediatric ALL. The aim of this study is to elucidate folate-metabolism-related genetic risk factors for low BMD and fractures, to identify which pediatric ALL patients have an increased risk of these skeletal complications.

DESIGN AND METHODS

Patients

In this study children with newly diagnosed ALL treated according to the dexamethasone-based protocol of the Dutch Childhood Oncology Group (DCOG-ALL9) at the Erasmus MC-Sophia Children's Hospital in Rotterdam were eligible[23]. High risk criteria were white blood cell count ≥50x10^9/L, T-cell immunophenotype, mediastinal mass, central nervous system (CNS) involvement, testes infiltration, t(9;22) and 11q23/*MLL* gene rearrangements. The treatment schedules included dexamethasone administered in repetitive pulses (cumulative dose: 1,244mg/m^2 (high risk) and 1,370mg/m^2 (non-high risk)[23]. Cumulative dose of methotrexate was 13,650mg/m^2 in the high risk and 8,100mg/m^2 in the non-high risk protocol[23]. No patient received CNS irradiation. The Medical Ethical Committee of the Erasmus MC-University Medical Center in Rotterdam approved the study. Written informed consent according to the Helsinki agreement was obtained from all patients. As a control group we used healthy Dutch children (403 Caucasian) from the Rotterdam region who participated previously in a study to obtain reference values for dual-energy X-ray absorptiometry (DXA) measurements. In a subset of these healthy children and young adults (n=148, 57 boys and 91 girls) we determined the *MTHFR* and *MTRR* SNPs. The general characteristics of these controls were previously described[24]. For the data on weight, height and body mass index (BMI) the Dutch reference charts were used[25-26].

SNP analysis

After reaching complete remission, germline genomic DNA was extracted from peripheral blood mononuclear cells using TRIzol reagent (Gibco BRL, Life technologies) according to the manufacturer's protocol. The DNA was quantified using spectrophotometry. From isolated DNA, the 677C>T in the *MTHFR* gene (rs1801133) on chromosome 1 was determined using PCR and Hinf1 digestion, as previously described[27]. PCR-restriction fragment length polymorphism was also used to detect the *MTHFR* 1298A>C (rs1801131)[17]. The *MTRR* 66A>G (rs1801394) on chromosome 5 was determined with real-time PCR (forward primer: 5'CAGTTTCACTGTTAC ATGCCTTG-3'; reverse primer: 5'-CAATTTTTGAGACCATTTAGTCT-3') using hybridization probes and Lightcycler Technology (Roche, Mannheim, Germany). The anchor probe (5'-CTGCGATGGCCTTTGCCTGTCCCT-3') labeled with a LC Red fluorophore hybridized to the part of the target sequence on the amplified DNA fragment that is not mutated. The sensor probe (5'-CCACAGCTTGCTCACACATTTC-3') was labeled with fluorescein and spans the polymorphic site. Samples

were heated for 10min at 95°C and amplified in 60 cycles of 3s at 95°C, 10s at 57°C and 25s at 57°C. Hereafter, the melting curve followed: 20s at 95°C, 5min at 40°C, subsequent slow heating to 85°C (at 0.1°C/s), and a final melting step to 40°C for 1min. After hybridization to the template DNA, the two probes come into close proximity, resulting in energy transfer between the two fluorophores. During melting curve analysis, the sensor probe is released earlier when a SNP is present resulting in loss of the fluorescence signal.

Bone mineral density measurements

BMD was measured at diagnosis, after 32 weeks, after one year, two years (at cessation of therapy) and three years (one year after cessation of therapy). Bone mineral density of the lumbar spine (BMDLS) and of the total body including the head (BMD_{TB}) were measured by DXA (Lunar DPX-L, Madison, WI, USA). The coefficient of variation was 1.04% for BMD_{LS} and 0.64% for the BMD_{TB}[28]. To correct for bone size we calculated apparent BMD of the lumbar spine ($BMAD_{LS}$, grams/m^3) with the model $BMAD_{LS}=BMD_{LS} * [4/(\pi * width)]$. 'Width' is the mean width of the second to the fourth lumbar vertebral body. This model was validated by in vivo volumetric data obtained from magnetic resonance imaging of lumbar vertebrae[29]. DXA results of the patients were compared with data of healthy Dutch children and expressed as age-matched and sex-matched standard deviation scores (SDS)[30]. DXA was only performed in children aged four years or older, because of availability of normal values. Pediatric software was used for children with a weight below 30 kilogram. All reported fractures were symptomatic, and confirmed by X-ray.

Body composition, physical activity and calcium intake

Pubertal stage was evaluated according to the method of Tanner[31], and we classified Tanner stage 1 as prepubertal, Tanner stages 2, 3, 4 as pubertal, and Tanner stage 5 as postpubertal. Body composition parameters (lean body mass and percentage fat) were measured using total body DXA. DXA results were compared with data of healthy Dutch children and expressed as age-matched and sex-matched SDS[30]. Height was measured with a Harpenden stadiometer and weight with a standard clinical balance. The BMI was calculated as BMI=weight/height^2. Values of height, weight and BMI of the patients were compared with age-matched and sex-matched reference values[25-26] and expressed as SDS. Habitual physical activity measured in minutes/week included physical education classes, organized sports, recreational activities, habitual walking/cycling. Calcium intake was determined by a detailed food-frequency questionnaire.

Biochemical markers

At diagnosis, after 32 weeks and after one year of therapy, serum total homocysteine levels were determined using high-performance liquid chromatography method (HPLC) with fluorescence detection. Serum folate and vitamin B12 levels were measured using electrochemiluminescence immunoassay (Elecsys 2010, Roche GmbH, Mannheim, Germany). The inter-assay coefficients of variation for homocysteine were: 3.8% (level 16 µmol/L) and 2.8% (level 46.6 µmol/L), for folate: 12% (level 4.16 nmol/L) and 4.9% (level 24.70 nmol/L), and for vitamin B12: 7.2% (level 107 nmol/L) and 2% (level 744 pmol/L). The intra-assay coefficients of variation for homocysteine were: 2.32% (level 5.99 µmol/L), 1.37% (13.69 µmol/L) and 1.28% (level 43.9 µmol/L), for folate: 2.5% (level 17.3 nmol/L) and 3.2% (level 38.6 nmol/L), and for vitamin B12: 3.5% (level 368 nmol/L) and 1.5% (level 734 pmol/L). Homocysteine, folate and vitamin B12 levels were compared with age-matched reference values and expressed as SDS.

Statistical analysis

The SNPs were tested for deviation from the Hardy-Weinberg equilibrium by comparing the observed and expected genotype frequencies using the chi-square test. Baseline characteristics were compared between groups with the chi-square test for categorical variables or the Mann-Whitney U-test for continuous variables. Baseline values of the endpoints were compared with normal references (0 SDS) with the one-sample T-test. Because distributions of plasma concentrations of homocysteine, folate and vitamin B12 were skewed, logarithmic transformations were applied to normalize these distributions. Statistical analyses were carried out with SPSS for Windows version 15.0 (Chicago, IL, USA). Differences in change of BM(A)D during the two-year treatment period (Δ_1) and during the first year after cessation of chemotherapy (Δ_2) between non-carriers and carriers of the three SNPs were calculated. Differences between carriers and non-carriers of the three SNPs in BM(A)D at the different time points and in changes of BM(A)D (Δ_1 and Δ_2) were analyzed using repeated measurements analysis (SAS 9.2 PROC MIXED, Cary, NC, USA), with an unstructured repeated covariance type. We pooled heterozygous and homozygous carriers of the risk alleles under a dominant inheritance model. In the same way differences in biomarkers between carriers and non-carriers of the investigated SNPs were analyzed. Moreover, patients were categorized into tertiles based on homocysteine, folate and vitamin B12 values at diagnosis. Differences in development of BM(A)D between groups of patients divided according to these tertiles of the biomarkers were compared using repeated measurements analysis. In view of the multiple comparisons, P-values of ≤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 (for example due to death or relapse) data prior to going off study were included.

RESULTS

Study population

In this longitudinal study, SNPs were determined in all 83 included pediatric ALL patients (47 male). The median age at diagnosis was 6.4 (range: 1.5-16.8) years. Fifty-eight patients were treated according to the non-high risk protocol and 25 patients according to the high risk protocol. In 68 patients DXA-scans were performed; of the 15 patients without DXA-scans, 12 were younger than four years of age at diagnosis, and of three children the parents refused a DXA-scan.

Distributions of baseline characteristic like age, gender and pubertal stage were not significantly different between carriers and non-carriers of the *MTHFR* and *MTRR* SNPs (Table1, at the end of this chapter). In addition, there were no significant differences regarding calcium intake and physical activity at diagnosis between non-carriers and carriers of the three investigated SNPs (Table 1). Moreover, parameters of body composition were not different at diagnosis (Table 1), nor during or after treatment, between non-carriers and carriers of the *MTHFR* and *MTRR* SNPs.

Genotype distribution

The genotype distributions of the ALL patients were in Hardy-Weinberg equilibrium (*MTHFR* 677C>T: p=0.54, *MTHFR* 1298A>C: p=0.28, *MTRR* 66A>G: p=0.64; Table 2). The genotype distribution of the 68 patients who underwent the DXA-scans was equal to that of the total group of ALL patients.

MTHFR 677C>T							*MTHFR* 1298 A>C							*MTRR* 66 A>G						
	Study population, ALL patients				Healthy controls			Study population, ALL patients				Healthy controls			Study population, ALL patients				Healthy controls	
Geno-type	Boys	Girls	N	%	N	%	Geno-type	Boys	Girls	N	%	N	%	Geno-type	Boys	Girls	N	%	N	%
CC	20	23	43	52	64	43	AA	23	18	41	50	63	43	AA	9	8	17	21	15	26
CT	24	11	35	42	73	50	AC	19	12	31	38	69	46	AG	21	21	42	53	26	45
TT	3	2	5	6	10	7	CC	4	6	10	12	16	11	GG	15	6	21	26	17	29
Total	**47**	**37**	**83**		**147**			**46**	**36**	**82**		**148**			**45**	**35**	**80**		**58**	

Table 2. Genotype distribution in pediatric ALL patients and healthy controls. *Abbreviations: MTHFR=methylenetetrahydrofolate reductase gene, MTRR=methionine synthase reductase gene.*

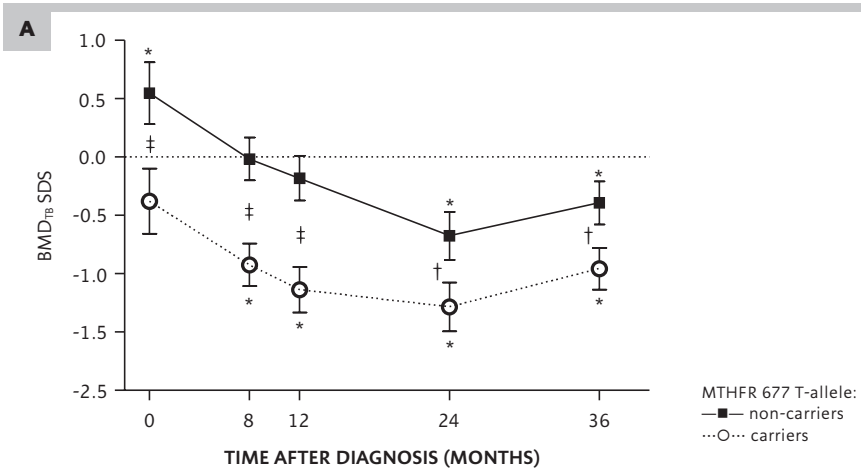
Bone mineral density and fractures

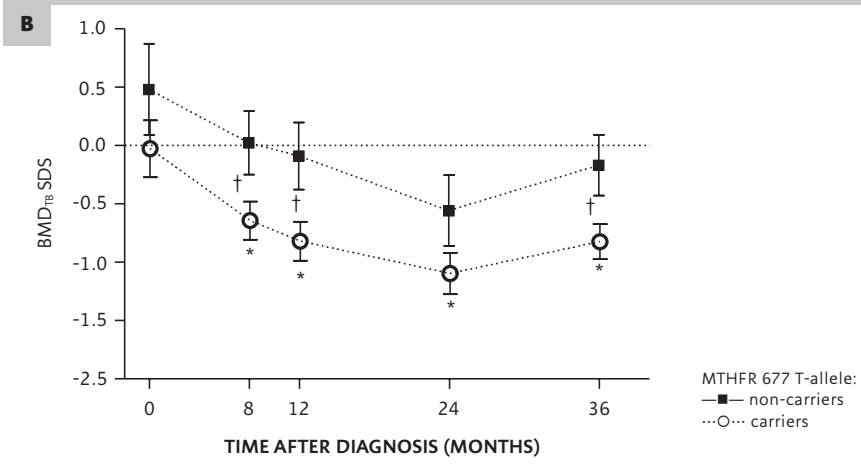
At diagnosis, mean BMD_{TB} of ALL patients was not different from mean BMD_{TB} of healthy references (mean BMD_{TB}=0.09SDS vs. test-value=0SDS, p=0.63). Baseline BMD_{TB} of carriers as well as non-carriers of the *MTHFR* 677C>T was not significantly different from healthy controls (Figure 2A). Carriers of the *MTHFR* 677 T-allele had a lower mean BMD_{TB} at diagnosis than non-carriers (carriers: -0.38SDS and non-carriers: 0.55SDS, p=0.01). This lower BMD_{TB} in carriers remained significant during treatment and the year thereafter (Figure 2A). During therapy, BMD_{TB} decreased but treatment-related bone loss was similar in carriers vs. non-carriers of the *MTHFR* 677 T-allele (carriers: $\Delta_1 BMD_{TB}$=-1.22SDS vs. non-carriers: Δ_1BMDTB=-0.91SDS, p=0.39). The profiles of non-carriers and carriers did not deviate from parallelism (interaction group*time, p=0.37). Hence, during the whole treatment period, carriers showed significantly lower BMD_{TB} levels than healthy controls (the nadir of BMD_{TB}=-1.28SDS vs. test-value=0SDS, p<0.01). In contrast, the difference between non-carriers and healthy controls only reached significance after two years of treatment (moment of treatment discontinuation) (BMD_{TB}=-0.67SDS vs. test-value=0SDS, p<0.01).

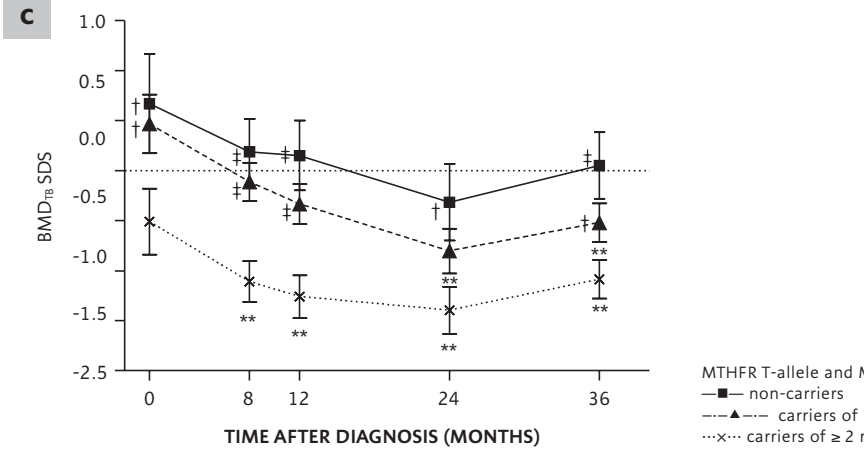
No differences in BMD_{TB} were found between carriers and non-carriers of the *MTHFR* 1298A>C SNP. Moreover, no differences were shown for $\Delta_1 BMD_{TB}$ and $\Delta_2 BMD_{TB}$ between carriers and non-carriers of the *MTHFR* 1298A>C SNP.

Figure 2: Bone mineral density of the total body according to carriage of A. the MTHFR 677C>T SNP, B. the MTRR 66A>G SNP and C. the combination of these two SNPs.

*Data shown are ANOVA results (mean (+/- standard error of the mean)). * p≤0.05 and ** p≤0.01 compared with zero SDS, e.g. mean for healthy controls. † p≤0.05 and ‡ p≤0.01 compared with A. carriers of the MTHFR T-allele, B. carriers of the MTRR 66 G-allele or C. carriers of ≥ 2 risk alleles. Abbreviations: BMD_{TB}=bone mineral density of the total body, SDS=standard deviation score, MTHFR=methylenetetrahydrofolate reductase gene, MTRR=methionine synthase reductase gene.*


A
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.5
BMD$_{TB}$ SDS
0
8
12
24
36
TIME AFTER DIAGNOSIS (MONTHS)
MTHFR 677 T-allele:
non-carriers
carriers


B
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.5
BMD$_{TB}$ SDS
0
8
12
24
36
TIME AFTER DIAGNOSIS (MONTHS)
MTHFR 677 T-allele:
non-carriers
carriers


C
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.5
BMD$_{TB}$ SDS
0
8
12
24
36
TIME AFTER DIAGNOSIS (MONTHS)
MTHFR T-allele and MTRR G-allele:
non-carriers
carriers of 1 risk allele
carriers of ≥ 2 risk alleles

The *MTRR* 66 G-allele carriers showed a trend for a lower BMD_{TB} than non-carriers (Figure 2B). At diagnosis, BMD_{TB} of carriers as well as non-carriers of the *MTRR* 66A>G SNP was not different from that of healthy controls (carriers: BMD_{TB}=-0.03SDS vs. test-value=0SDS, p=0.91 and non-carriers: BMD_{TB}=0.48SDS vs. test-value=0SDS, p=0.22). Like for the *MTHFR* 677C>T, treatment-related bone loss was similar in carriers and non-carriers (carriers: Δ_1BMD_{TB}=-1.07SDS vs. non-carriers: Δ_1BMD_{TB}=-1.04SDS, p=0.95); the two profiles (Figure 2B) did not deviate from parallelism (interaction group*time, p=0.63). However, during treatment, BMD_{TB} of carriers of the *MTRR* 66 G-allele was lower than that of healthy peers (the nadir of BMD_{TB}=-1.10SDS vs. test-value=0SDS, p≤0.01), whereas there was no difference in BMD_{TB} between non-carriers and healthy peers (the nadir of BMD_{TB}=-0.56SDS vs. test-value=0SDS, p=0.07; Figure 2B).

Simultaneous evaluation of the *MTHFR* 677C>T and the *MTRR* 66A>G SNPs showed that both allelic variants that were found to enhance the risk of low BMD, had a significant effect on BMD_{TB} and the effect of one of them was not modified by the other (interaction: p=0.47). When combining these risk alleles of the *MTHFR* 677C>T and the *MTRR* 66A>G SNPs, patients carrying at least two risk alleles had lower BMD_{TB} than patients with only one or no risk allele (Figure 2C). The three profiles (Figure 2C) did not deviate from parallelism (interaction group*time, p=0.65).

Baseline $BM(A)D_{LS}$ was lower in ALL patients than in healthy peers (BMD_{LS}=-0.68SDS, p≤0.01 and $BMAD_{LS}$=-0.34SDS, p=0.05). There was no significant difference in $BM(A)D_{LS}$ at diagnosis nor during treatment between carriers and non-carriers of the *MTHFR* 677 T-allele, the *MTHFR* 1298 C-allele or the *MTRR* 66 G-allele. Furthermore, no significant differences were observed for $\Delta_1BM(A)D_{LS}$ and $\Delta_2BM(A)D_{LS}$ between carriers and non-carriers of the two *MTHFR* SNPs or the *MTRR* SNP.

Eleven patients sustained a total of 12 fractures during therapy (n=7) or within one year after treatment discontinuation (n=5), none were present at diagnosis. Fractures involved the forearm (n=4), the tibia (n=5), the clavicle (n=1) and vertebra (n=2). Except for the vertebral compression fractures, all fractures were preceded by minor trauma. Using Kaplan-Meier analysis, fractures were not found to occur only in one of the carrier groups of the three investigated SNPs.

Biochemical markers

There were no significant baseline or therapy-induced differences in homocysteine, folate or vitamin B12 concentrations between carriers and non-carriers of the *MTHFR* 677C>T or the *MTRR* 66A>G SNPs (Table 3). In addition, when combining the allelic variants of the *MTHFR* 677C>T and the *MTRR* 66A>G SNPs, no differences in homocysteine, folate and vitamin B12 levels were observed. Carriers of the *MTHFR* 1298 C-allele did not have different homocysteine levels at baseline (p=0.67), but they had higher homocysteine levels than non-carriers after 32 weeks of treatment (p<0.01). Folate and vitamin B12 levels of carriers and non-carriers of the *MTHFR* 1298 C-allele were not significantly different. Moreover, there were no differences in BMD_{TB} and $BM(A)D_{LS}$ when patients were divided in tertiles of high, intermediate and low levels of homocysteine, folate or vitamin B12.

	MTHFR 677C>T			*MTHFR* 1298A>C			*MTRR* 66A>G		
	CC	**CT /TT**	**P**	**AA**	**AC/CC**	**P**	**AA**	**AG/GG**	**P**
Diagnosis									
Homocysteine (SDS)	1.65 (0.36)	2.55 (0.36)	0.09	2.04 (0.38)	2.27 (0.38)	0.67	2.08 (0.48)	2.17 (0.33)	0.89
Folate (SDS)	-2.04 (0.27)	-2.44 (0.31)	0.33	-2.42 (0.28)	-1.99 (0.29)	0.29	-2.25 (0.45)	-2.20 (0.23)	0.92
Vitamin B12 (SDS)	0.16 (0.24)	-0.28 (0.27)	0.22	-0.08 (0.25)	-0.03 (0.25)	0.89	-0.43 (0.39)	0.04 (0.20)	0.28
32 Weeks after diagnosis									
Homocysteine (SDS)	1.29 (0.27)	1.18 (0.27)	0.77	0.58 (0.27)	1.78 (0.23)	<0.01	1.29 (0.41)	1.30 (0.23)	0.98
Folate (SDS)	-1.28 (0.26)	-1.30 (0.26)	0.95	-1.08 (0.28)	-1.51 (0.24)	0.25	-1.57 (0.39)	-1.26 (0.21)	0.49
Vitamin B12 (SDS)	-0.24 (0.27)	-0.27 (0.27)	0.95	-0.02 (0.28)	-0.46 (0.24)	0.25	0.12 (0.39)	-0.38 (0.21)	0.27
1 Year after diagnosis									
Homocysteine (SDS)	1.51 (0.24)	1.77 (0.27)	0.48	1.53 (0.28)	1.77 (0.24)	0.52	1.41 (0.34)	1.75 (0.22)	0.40
Folate (SDS)	-1.89 (0.21)	-1.89 (0.24)	0.98	-1.85 (0.24)	-1.97 (0.21)	0.71	-1.99 (0.29)	-1.89 (0.19)	0.77
Vitamin B12 (SDS)	0.06 (0.25)	-0.32 (0.29)	0.33	0.15 (0.28)	-0.38 (0.25)	0.17	-0.13 (0.37)	-0.16 (0.23)	0.93

Table 3. Values of homocysteine, folate and vitamin B12 according to carriage of the MTHFR SNPs and the MTRR SNP

Data shown are ANOVA results. Values are expressed as mean (standard error of the mean). Abbreviation: SDS=standard deviation score, MTHFR=methylenetetrahydro-folate reductase gene, MTRR=methionine synthase reductase gene.

DISCUSSION

This longitudinal study shows that carriage of the *MTHFR* 677 T-allele as well as the *MTRR* 66 G-allele, but not the *MTHFR* 1298A>C, is a risk factor for a low BMD_{TB}, starting already at diagnosis of ALL and remaining during treatment and the year thereafter. The negative effects on BMD_{TB} were reinforced by coexistence of two or more of these risk alleles. The decline in BMD_{TB} during treatment does however not differ between carriers and non-carriers of the risk alleles. This indicates that in carriers of the risk alleles, the nadir of the BMD_{TB} after treatment with chemotherapy is lower than in non-carriers because they start with a lower BMD_{TB}.

In healthy adults, the *MTHFR* 677 T-allele has previously been shown to be associated with a decreased BMD[9] and postmenopausal women carrying the T-allele had an increased fracture risk[32-33]. The underlying mechanism is suggested to be a homocysteine-related disturbance of collagen cross-linking[10-13]. Homocysteine itself is predictive for hip fractures in the elderly[15-16]. In the current study, differences in BMD_{TB} between carriers and non-carriers of the risk alleles of the *MTHFR* 677C>T and the *MTRR* 66A>G

were most striking during the first year of treatment. This is interesting, as high-dose methotrexate is administered during the first part of the anti-leukemic therapy. Methotrexate causes a decrease in plasma folate levels[34-35], which can subsequently result in an increase of homocysteine levels. In healthy adults, the *MTHFR* 677C>T SNP results in 50-60% reduction of MTHFR activity[17], which predisposes to increased homocysteine levels[18]. Moreover, the vitamin B12-dependent enzyme MTRR is needed for re-methylation of homocysteine to methionine and is therefore involved in the homocysteine metabolism. In young female adults, the presence of the *MTRR* 66A>G exacerbates the negative effect of the *MTHFR* 677C>T SNP on plasma homocysteine[19]. As carriers and non-carriers of the *MTHFR* 677 T-allele and the *MTRR* 66 G-allele showed no differences in homocysteine levels, we could not confirm the hypothesis of a homocysteine-related disturbance of collagen cross-linking in the current study.

At diagnosis, both carriers and non-carriers of the investigated SNPs had elevated homocysteine levels compared with healthy peers, which is consistent with previously published data in the literature[36]. Carriers of the *MTHFR* 1298 C-allele had higher homocysteine levels than non-carriers after 32 weeks of treatment, however after one year of treatment these differences had disappeared. Although, in this study homocysteine levels were not measured during high-dose MTX courses, high-dose methotrexate was administered during the first 32 weeks of treatment. This may suggest that homocysteine levels of carriers and non-carriers of the *MTHFR* 1298A>C respond different on administration of high-dose MTX. There were no differences in BMD between carriers and non-carriers of the *MTHFR* 1298 C-allele. An explanation may be that homocysteine may cause a qualitative disturbance of collagen cross-linking rather than a decrease of the amount of collagen. This is supported by a large study among elderly, in which homocysteine levels correlated significantly with fracture risk, independently from BMD values[15]. As the number of fractures in the current study population was relatively low, it was not possible to draw strong conclusions regarding the correlation between homocysteine levels and fracture risk.

Only BMD of the total body was different between carriers and non-carriers of the *MTHFR* 677 T-allele and the *MTRR* 66 G-allele, and not BMD measured at the lumbar spine. These differences between the two measured locations may reflect difference between types of bone. Cortical bone comprises 80 percent of the skeleton, whereas the lumbar spine mainly exists of trabecular bone. Why specifically cortical bone was involved rather than trabecular bone remains unknown and a suitable explanation could not be drawn from the literature.

In our study population, fractures did not predominantly occur in one of the carrier groups of the three investigated SNPs, but numbers were low. In addition, other factors may contribute to fracture risk, like clumsiness due to vincristine neuropathy. Regarding the possibility of gene-environment interaction, we can not rule out an effect of factors like age, gender, physical activity, calcium intake and body composition[37]. However, those factors were not significantly different between carriers and non-carriers of the three SNPs.

Finally, we want to mention some limitations of the study. First, because the number of included patients was relatively low, validation of the results in larger, independent cohorts is recommended to confirm our results and to exclude the risk of false-negative findings. Pharmacogenetics may have a significant impact on individualizing medical care, although in the future prediction models for the changes in BMD during pediatric ALL treatment need to combine both genetic factors and environmental components into one

	WHOLE COHORT	*MTHFR* 677C>T			*MTHFR* 1298A>C			*MTRR* 66A>G		
		CC	CT /TT		AA	AC/CC		AA	AG/GG	
	Mean (SD)	Mean (SD)	Mean (SD)	P (MWU)	Mean (SD)	Mean (SD)	P (MWU)	Mean (SD)	Mean (SD)	P (MWU)
Age (years)	8.3 (4.2)	8.4 (4.5)	8.3 (4.0)	0.99	8.0 (4.4)	8.6 (4.1)	0.52	8.6 (3.8)	8.0 (4.4)	0.54
Height (SDS)	-0.01 (1.05)	-0.02 (1.13)	0.00 (1.00)	0.75	-0.03 (0.94)	0.03 (1.17)	0.54	0.05 (0.94)	-0.01 (0.10)	0.92
Weight (SDS)	-0.31 (1.17)	-0.22 (1.31)	-0.41 (1.01)	0.40	-0.35 (1.05)	-0.28 (1.32)	0.70	-0.18 (0.99)	-0.36 (1.26)	0.70
BMI (SDS)	-0.42 (1.22)	-0.25 (1.22)	-0.61 (1.21)	0.24	-0.47 (1.16)	-0.39 (1.30)	0.86	-0.30 (1.34)	-0.50 (1.21)	0.57
% Fat (SDS)	-0.04 (1.19)	0.05 (1.08)	-0.16 (1.31)	0.54	-0.26 (1.28)	0.18 (1.08)	0.19	0.26 (1.09)	-0.15 (1.23)	0.26
LBM (SDS)	-0.65 (0.93)	-0.62 (0.95)	-0.68 (0.94)	0.82	-0.66 (0.99)	-0.65 (0.92)	0.70	-0.71 (0.96)	-0.62 (0.97)	0.70
Calcium intake (mg/day)	1158 (433)	1264 (452)	1034 (382)	0.10	1097 (364)	1217 (498)	0.44	1231 (395)	1115 (465)	0.26
Physical activity (min/week)	611 (305)	555 (309)	671 (296)	0.12	588 (286)	622 (330)	0.79	516 (284)	653 (314)	0.18
		Number (%)	Number (%)	(2)	Number (%)	Number (%)	(2)	Number (%)	Number (%)	(2)
Gender (M/F)	41/ 27 (60%/ 40%)	18/ 16 (53%/ 47%)	23/11 (68%/ 32%)	0.22	19/ 15 (56%/ 44%)	21/ 12 (64%/ 36%)	0.52	9/ 7 (56%/ 44%)	30/ 19 (61%/ 39%)	0.72
Pubertal stage (prepubertal/ pubertal/ mature)	51/ 12/ 4 (76%/ 18%/ 6%)	25/7/2 (73%/ 21%/ 6%)	26/ 5/ 2 (79%/ 15%/ 6%)	0.61† 0.19	26/ 6/ 1 (79%/ 18%/ 3%)	24/ 6/ 3 (73%/ 18%/ 9%)	0.57† 0.08	14/ 4/ 0 (75%/ 25%/ 0%)	38/ 7/ 3 (79%/ 15%/ 6%)	0.73
Risk group (NHR/ HR)	47/ 21 (69%/ 31%)	26/ 8 (76%/ 24%)	21/ 13 (62%/ 38%)		20/ 14 (59%/ 41%)	26/ 7 (79%/ 21%)		12/ 4 (75%/ 25%)	32/ 17 (65%/ 35%)	0.47

Table 1. Baseline characteristics of the ALL patients who underwent DXA

Abbreviations: MTHFR=methylenetetrahydrofolate reductase gene, MTRR=methionine synthase reductase gene, MWU=Mann-Whitney U-test, χ2=chi-square test, SDS=standard deviation score, BMI=body mass index, LBM=lean body mass, M=male, F=female, NHR=non-high risk, HR=high risk. † To meet the criteria of the chi-square test, pubertal and mature were pooled into one group.

model. In addition, genome-wide association studies (GWAS) have become a promising approach to unravel unknown pharmacogenetic loci that may affect BMD in pediatric ALL. Another important remark is that DXA measures an areal density and may consequently underestimate BMD in short stature. We corrected for bone size of the lumbar vertebral body, by the use of a mathematical model to calculate a volumetric density of the lumbar spine, but the BMD_{TB} measurements were not corrected for bone size. Finally, the future challenge is to study what the impact of differences in changes of BMD during and shortly after childhood ALL treatment will be on the achieved BMD in later life, as a growing number of survivors of ALL reach menopause.

In conclusion, this is the first study investigating the influence of genetic variation of the *MTHFR* and *MTRR* on BMD in pediatric ALL. We identified the *MTHFR* 677C>T and *MTRR* 66A>G SNPs as determinants of BMD in children treated for ALL. However, we did not establish that the effects of these genetic risk factors on BMD were carried out by differences in homocysteine levels. These findings may be used to develop a prediction model for low bone density in pediatric ALL patients.

REFERENCES

1. Arikoski P, Komulainen J, Riikonen P, Voutilainen R, Knip M, Kroger H. Alterations in bone turnover and impaired development of bone mineral density in newly diagnosed children with cancer: a 1-year prospective study. J Clin Endocrinol Metab l1999;84: 3174-81.
2. Vassilopoulou-Sellin R, Ramirez I. Severe osteopenia and vertebral compression fractures after complete remission in an adolescent with acute leukemia. Am J Hematol l1992;39: 142-143.
3. Boot AM, Van den Heuvel-Eibrink MM, Hählen K, Krenning EP, de Muinck Keizer-Schrama SMPF. Bone mineral density in acute lymphoblastic leukaemia. Eur J Cancer l1999;35: 1693-1697.
4. van der Sluis IM, van den Heuvel-Eibrink MM, Hahlen K, Krenning EP, de Muinck Keizer-Schrama SMPF. Altered bone mineral density and body composition and fracture risk in childhood acute lymphoblastic leukemia. Journal of Pediatrics l2002;141: 204-210.
5. Halton JM, Atkinson SA, Fraher L, Webber C, Gill GJ, Dawson S, Barr RD. Altered mineral metabolism and bone mass in children during treatment for acute lymphoblastic leukemia. J Bone Miner Res l1996;11: 1774-83.
6. Crofton PM, Ahmed SF, Wade JC, Stephen R, Elmlinger MW, Ranke MB, Kelnar CJ, Wallace WH. Effects of intensive chemotherapy on bone and collagen turnover and the growth hormone axis in children with acute lymphoblastic leukemia. J Clin Endocrinol Metab l1998;83: 3121-9.
7. Reid IR. Glucocorticoid osteoporosis--mechanisms and management. Eur J Endocrinol l1997;137: 209-17.
8. Ragab AH, Frech RS, Vietti TJ. Osteoporotic fractures secondary to methotrexate therapy of acute leukemia in remission. Cancer l1970;25: 580-5.
9. Riancho JA, Valero C, Zarrabeitia MT. MTHFR polymorphism and bone mineral density: meta-analysis of published studies. Calcif Tissue Int l2006;79: 289-93.
10. Levasseur R. Bone tissue and hyperhomocysteinemia. Joint Bone Spine l2009;76: 234-40.
11. Harris ED, Jr., Sjoerdsma A. Collagen profile in various clinical conditions. Lancet l1966;2: 707-11.
12. Kang AH, Trelstad RL. A collagen defect in homocystinuria. J Clin Invest l1973;52: 2571-8.
13. Lubec B, Fang-Kircher S, Lubec T, Blom HJ, Boers GH. Evidence for McKusick's hypothesis of deficient collagen cross-linking in patients with homocystinuria. Biochim Biophys Acta l1996;1315: 159-62.
14. Gjesdal CG, Vollset SE, Ueland PM, Refsum H, Drevon CA, Gjessing HK, Tell GS. Plasma total homocysteine level and bone mineral density: the Hordaland Homocysteine Study. Arch Intern Med l2006;166: 88-94.
15. van Meurs JB, Dhonukshe-Rutten RA, Pluijm SM, van der Klift M, de Jonge R, Lindemans J, de Groot LC, Hofman A, Witteman JC, van Leeuwen JP, Breteler MM, Lips P, Pols HA, Uitterlinden AG. Homocysteine levels and the risk of osteoporotic fracture. N Engl J Med l2004;350: 2033-41.
16. McLean RR, Jacques PF, Selhub J, Tucker KL, Samelson EJ, Broe KE, Hannan MT, Cupples LA, Kiel DP. Homocysteine as a predictive factor for hip fracture in older persons. N Engl J Med l2004;350: 2042-9.

17. Weisberg I, Tran P, Christensen B, Sibani S, Rozen R. A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. Mol Genet Metab l1998;64: 169-72.
18. Krajinovic M, Lamothe S, Labuda D, Lemieux-Blanchard E, Theoret Y, Moghrabi A, Sinnett D. Role of MTHFR genetic polymorphisms in the susceptibility to childhood acute lymphoblastic leukemia. Blood l2004;103: 252-7.
19. Vaughn JD, Bailey LB, Shelnutt KP, Dunwoody KM, Maneval DR, Davis SR, Quinlivan EP, Gregory JF, 3rd, Theriaque DW, Kauwell GP. Methionine synthase reductase 66A->G polymorphism is associated with increased plasma homocysteine concentration when combined with the homozygous methylenetetrahydrofolate reductase 677C->T variant. J Nutr l2004;134: 2985-90.
20. Steer CD, Emmett PM, Lewis SJ, Smith GD, Tobias JH. Methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism is associated with spinal BMD in 9-year-old children. J Bone Miner Res l2009;24: 117-24.
21. Broxson EH, Jr., Stork LC, Allen RH, Stabler SP, Kolhouse JF. Changes in plasma methionine and total homocysteine levels in patients receiving methotrexate infusions. Cancer Res l1989;49: 5879-83.
22. Refsum H, Wesenberg F, Ueland PM. Plasma homocysteine in children with acute lymphoblastic leukemia: changes during a chemotherapeutic regimen including methotrexate. Cancer Res l1991;51: 828-35.
23. Veerman AJ, Kamps WA, van den Berg H, van den Berg E, Bokkerink JP, Bruin MC, van den Heuvel-Eibrink MM, Korbijn CM, Korthof ET, van der Pal K, Stijnen T, van Weel Sipman MH, van Weerden JF, van Wering ER, van der Does-van den Berg A, Dutch Childhood Oncology G. Dexamethasone-based therapy for childhood acute lymphoblastic leukaemia: results of the prospective Dutch Childhood Oncology Group (DCOG) protocol ALL-9 (1997-2004). Lancet Oncol l2009;10: 957-66.
24. van der Sluis IM, de Muinck Keizer-Schrama SM, Krenning EP, Pols HA, Uitterlinden AG. Vitamin D receptor gene polymorphism predicts height and bone size, rather than bone density in children and young adults. Calcif Tissue Int l2003;73: 332-8.
25. Fredriks AM, van Buuren S, Burgmeijer RJ, Meulmeester JF, Beuker RJ, Brugman E, Roede MJ, Verloove-Vanhorick SP, Wit JM. Continuing positive secular growth change in The Netherlands 1955-1997. Pediatr Res l2000;47: 316-23.
26. Fredriks AM, van Buuren S, Wit JM, Verloove-Vanhorick SP. Body index measurements in 1996-7 compared with 1980. Arch Dis Child l2000;82: 107-12.
27. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, den Heijer M, Kluijtmans LA, van den Heuvel LP, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet l1995;10: 111-3.
28. Johnson J, Dawson-Hughes B. Precision and stability of dual-energy X-ray absorptiometry measurements. Calcif Tissue Int l1991;49: 174-8.
29. Kroger H, Vainio P, Nieminen J, Kotaniemi A. Comparison of different models for interpreting bone mineral density measurements using DXA and MRI technology. Bone l1995;17: 157-9.
30. van der Sluis IM, de Ridder MA, Boot AM, Krenning EP, de Muinck Keizer-Schrama SM. Reference data for bone density and

body composition measured with dual energy x ray absorptiometry in white children and young adults. Arch Dis Child l2002;87: 341-7; discussion 341-7.

31. Tanner JM, Whitehouse RH. Clinical longitudinal standards for height, weight, height velocity, weight velocity, and stages of puberty. Arch Dis Child l1976;51: 170-9.
32. Bathum L, Von Bornemann Hjelmborg J, Christiansen L, Madsen JS, Skytthe A, Christensen K. Evidence for an association of methylene tetrahydrofolate reductase polymorphism C677T and an increased risk of fractures: results from a population-based Danish twin study. Osteoporos Int l2004;15: 659-664.
33. Abrahamsen B, Madsen JS, Tofteng CL, Stilgren L, Bladbjerg EM, Kristensen SR, Brixen K, Mosekilde L. A common methylenetetrahydrofolate reductase (C677T) polymorphism is associated with low bone mineral density and increased fracture incidence after menopause: longitudinal data from the Danish osteoporosis prevention study. J Bone Miner Res l2003;18: 723-9.
34. Jackson RC. Biological effects of folic acid antagonists with antineoplastic activity. Pharmacol Ther l1984;25: 61-82.
35. Baram J, Allegra CJ, Fine RL, Chabner BA. Effect of methotrexate on intracellular folate pools in purified myeloid precursor cells from normal human bone marrow. J Clin Invest l1987;79: 692-7.
36. Ruud E, Holmstrom H, Brosstad F, Wesenberg F. Children with acute lymphoblastic leukaemia have high plasma levels of total homocysteine at time of diagnosis. Scand J Clin Lab Invest l2006;66: 67-78.
37. Jones TS, Kaste SC, Liu W, Cheng C, Yang W, Tantisira KG, Pui CH, Relling MV. CRHR1 polymorphisms predict bone density in survivors of acute lymphoblastic leukemia. J Clin Oncol l2008;26: 3031-7.

A RANDOMISED TRIAL INVESTIGATING AN EXERCISE PROGRAM TO PREVENT REDUCTION OF BONE MINERAL DENSITY AND MOTOR PERFORMANCE DURING TREATMENT FOR CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

M.L. te Winkel MD/ A. Hartman MSc, R.D. van Beek MD, S.M.P.F. de Muinck Keizer-Schrama MD PhD, H.C.G. Kemper PhD, W.C. J. Hop PhD, M.M. van den Heuvel-Eibrink MD PhD, R. Pieters MD PhD

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ABSTRACT

Background

Reduced bone mineral density (BMD), altered body composition, impaired motor performance and passive ankle dorsiflexion are side effects of acute lymphoblastic leukemia (ALL) treatment. We performed a randomised study investigating whether an exercise program could prevent these side effects.

Procedure

At diagnosis we randomised 51 ALL patients (median age: 5.4 years) into a group receiving a two-year exercise program or a control group receiving standard care. BMD of total body (BMD_{TB}), lumbar spine (BMD_{LS}) and body composition were measured using dual energy X-ray absorptiometry, motor performance with Bayley Scales of Infant Development or Movement-ABC, and passive ankle dorsiflexion with a goniometer. The investigator was blinded to the randomisation.

Results

Body fat increased equally during treatment in both groups. One year after cessation of therapy more rapid decline of excessive body fat was observed in the intervention group than in the controls ($p=0.01$). Lean body mass, BMD_{TB} and BMD_{LS} of both groups decreased equally during treatment and increased equally thereafter. Both groups showed a similar decrease in passive ankle dorsiflexion and motor performance during treatment. Adherence to the intervention program varied considerably. Adherence to intervention: 11% of children exercised daily, 37% > once a week, 16% once weekly, 36% < once a week.

Conclusions

The exercise program was not more beneficial than standard care in preventing reduction in BMD, motor performance and passive ankle dorsiflexion than standard care, most likely due to unsatisfactory compliance. Increased BMI and body fat in the intervention group normalised faster after cessation of chemotherapy.

INTRODUCTION

Because the survival rate of acute lymphoblastic leukaemia (ALL) has improved, preventing side effects of chemotherapy has become increasingly important. Several studies have shown that motor performance, peripheral muscle strength and passive ankle dorsiflexion in children treated for ALL was impaired during treatment and also after cessation of chemotherapy[1-4]. These problems were mainly attributed to vincristine-induced neuropathy. Moreover, treatment protocols for ALL contain a considerable amount of prednisone and/or dexamethasone. This may cause steroid-associated myopathy with weakness of proximal musculature and muscle atrophy leading to a decreased lean body mass (LBM)[5]. In addition, corticosteroids and methotrexate (MTX) are known to cause reduction of bone mineral density (BMD). Several studies have shown that BMD is already lower at diagnosis of ALL and decreases further during the two-year treatment period[6, 7]. Although a decreased BMD is unlikely to affect motor performance directly, it is associated with an increased fracture risk[8]. Studies investigating BMD in long-term survivors of childhood ALL show conflicting results[9-13]. In general, BMD of patients treated without cranial irradiation shows a tendency to improve after cessation of therapy[8, 14]. Several animal studies in which mechanical loads were applied showed that the crucial factor in stimulation of bone acquisition is the magnitude rather than the number of repetitions of the load applied[15, 16]. Therefore, short-burst high-intensity activities might be effective to enhance BMD during childhood[17-21]. However, no studies have been performed to investigate this effect of exercise on BMD during treatment of childhood ALL.

Because vincristine-related neuropathy causes weakness in the dorsiflexors of the foot, children are at risk for developing a plantigrade contracture of the ankle. Wright et al. found positive effects of preventative education and physiotherapy consisting of stretching and strengthening exercises on passive ankle dorsiflexion during treatment for ALL[22]. Another study reported positive effects of physical exercises on ankle dorsiflexion mobility and strength of knee extensors, but no improved functional outcome was found[23].

In the current prospective randomised study in childhood ALL we investigated whether an exercise program starting at onset and continued during 2-year treatment for ALL has a beneficial effect on BMD, body composition, motor performance and passive ankle dorsiflexion.

METHODS

Patients

Between April 2001 and September 2004 the study was conducted in Erasmus MC-Sophia Children's Hospital Rotterdam, the Netherlands. Children with ALL aged 1-18 years that did not have cognitive impairment and had good command of the Dutch language were eligible. Clinical data (age, gender, immunophenotype of leukaemia, fractures) were obtained from the medical records. The Medical Ethical Committee approved the study. Written informed consent according to the Helsinki agreement was obtained from all parents and from patients ≥ 12 years.

Chemotherapy treatment

Patients were treated according to the ALL-9 protocol of the Dutch Childhood Oncology Group (DCOG), which was identical to the previously used ALL-6 protocol for the non-high-risk (NHR) patients[24]. The ALL-9-NHR protocol started with induction therapy including dexamethasone and vincristine, followed by high-dose MTX courses and maintenance therapy with 6-mercaptopurine/MTX plus vincristine/dexamethasone pulses. Patients were treated according to the high-risk (HR) protocol when they met one of the following criteria: white blood cell count ≥50x10^9, T-cell immunophenotype, mediastinal mass, involvement of the central nervous system, infiltration of the testes, t(9;22) or BCR-ABL gene rearrangement, t (4;11) or translocations involving 11q23 with MLL gene-rearrangements. High-risk ALL patients received anthracyclines during induction treatment, higher doses of MTX during central-nervous-system (CNS) prophylaxis and they received two additional intensification courses before start of maintenance therapy. Patients did not receive CNS irradiation.

Randomisation

At diagnosis randomisation into the intervention or the control group was carried out in randomly permuted blocks of randomly chosen size, using sealed envelopes prepared by the statistician. The research nurse informed parents into which group their child was randomised. The investigators and treating physicians were blinded for the randomisation.

Intervention and standard care

The intervention group received an initial session and follow-up sessions that were conducted every six weeks with the hospital-based paediatric physiotherapist, throughout the two-year treatment period. The initial session comprised of education regarding possible motor problems resulting from chemotherapy. General measures to ensure an optimum level of motor functioning, for example walking or cycling to school, were discussed. In addition an exercise program was introduced, consisting of exercises to maintain hand and leg function, stretching exercises to maintain ankle dorsiflexion mobility and short-burst high-intensity exercises (e.g. jumping) to prevent reduction in BMD. Exercises to maintain hand and leg function had to be performed once a day and stretching and jumping exercises twice daily. Parents were supplied with an exercise list, enabling them to select exercises most appropriate for their child's age and also to vary exercises. They were asked to keep a daily record of the exercises, which had been carried out.

The follow-up sessions, conducted every six weeks involved an evaluation of the child's main motor skills (walking, running, jumping), discussion and adjustment of the exercise program if necessary. Passive ankle dorsiflexion was monitored and if < 5° beyond the neutral position, patients received plaster of Paris splints overnight. In case of concern regarding a child's motor ability they would be referred to a local paediatric physiotherapist for additional treatment. Follow-up sessions with the hospital-based physiotherapist always coincided with regular visits to the oncology outpatient department.

Standard care for the control group included neither an initial session nor any prescheduled follow-up sessions with the hospital-based physiotherapist. If child or parents reported motor problems to the treating physician a referral to a local paediatric physiotherapist was allowed. On cessation of

chemotherapy parents of children in both groups received a short questionnaire. All parents were asked whether they had valued testing of motor performance by the investigator. Parents of children in the intervention group were questioned about adherence to the exercise program.

End points

At diagnosis, 32 weeks after diagnosis, one year after diagnosis, on cessation of treatment (two years after diagnosis) and one year after cessation of therapy the following end points were measured: anthropometric data (height, weight, body mass index (BMI)), body composition (LBM and percentage body fat), BMD (primary end point) of the total body (BMD_{TB}), and lumbar spine (BMD_{LS}). Motor performance (primary end point) and passive ankle dorsiflexion were assessed at diagnosis, following induction therapy (six weeks after diagnosis), one year after diagnosis and on cessation of treatment.

Anthropometry, body composition and BMD

Height was measured using a Harpenden stadiometer and weight using a standard clinical balance. Body mass index (BMI) was calculated as weight/height^2. In patients aged 4-19 years body composition parameters, BMD_{TB}, and BMD_{LS} were measured using dual energy X-ray absorptiometry (DEXA; Lunar DPX-L, Madison, WI). To correct for bone size, bone mineral apparent density of the lumbar spine ($BMAD_{LS}$) was calculated as $BMAD_{LS}= BMD_{LS}*[4/(\pi*width)]$. The DEXA of total body provided estimates of body composition: lean body mass (LBM) which consists mainly of muscle mass and percentage of body fat. All results were compared with those of Dutch healthy children and expressed as standard deviation scores (SDS)[25, 26]. Fractures were registered and had to be confirmed on X-ray.

Motor performance

For children < 3.5 years motor performance was measured using the motor scale of the Dutch Bayley Scales of Infant Development (BSID-II) and for children aged ≥ 4 years the Dutch version of the Movement Assessment Battery for Children (movement-ABC) was used[27, 28]. The motor scale of the BSID-II consists of several motor tasks and the total number accomplished by the child is converted into a SDS. The movement-ABC has eight standardized tasks divided in three subsections: hand function, ball skills and balance skills. The total score of the movement-ABC is transformed by age-related norms into a percentile score.

Passive ankle dorsiflexion

To measure passive ankle dorsiflexion a standardised position was used: supine position, with the knee extended. A range of motion past neutral position had a positive notation and less than neutral was negative. The lower of the two passive ankle dorsiflexion values (preferred and non-preferred side) will determine the level of impairment and was therefore used for analysis.

Statistical analysis

The Mann-Whitney U-test/χ2-test was used to compare patient characteristics of the intervention and control groups. Motor performance of the whole study group was compared with reference values using the one-sample T-test. Within-group differences in motor performance and passive ankle dorsiflexion

mobility were analysed using a paired T-test. These statistical analyses were carried out with SPSS for Windows version 11.0.1 (SPSS Inc., Chicago, IL, USA). Differences between the two groups in changes of endpoints during the two-year treatment period (Δ1) and from cessation of chemotherapy during the first year after cessation (Δ2) were analysed using repeated measurements analysis (SAS PROC MIXED SAS Institute Inc., Cary, North Carolina, USA), with an unstructured repeated covariance type. P-values less than 0.05 (two-sided) were considered statistically significant. Power calculations resulted in entering 50 children in the study. 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

During the inclusion period 67 children were eligible of which 12 declined and 4 were not randomised. There were no significant differences in age at diagnosis (median age: 5.4 vs. 4.8 year, p=0.41), gender (50% vs. 59% male, p=0.53) and immunophenotype of ALL (80% vs. 75% B-lineage ALL, p=0.73) between patients who did or did not enter the randomised study. Figure 1 shows the study profile. Patient characteristics of the intervention and the control group at diagnosis are shown in Table I.

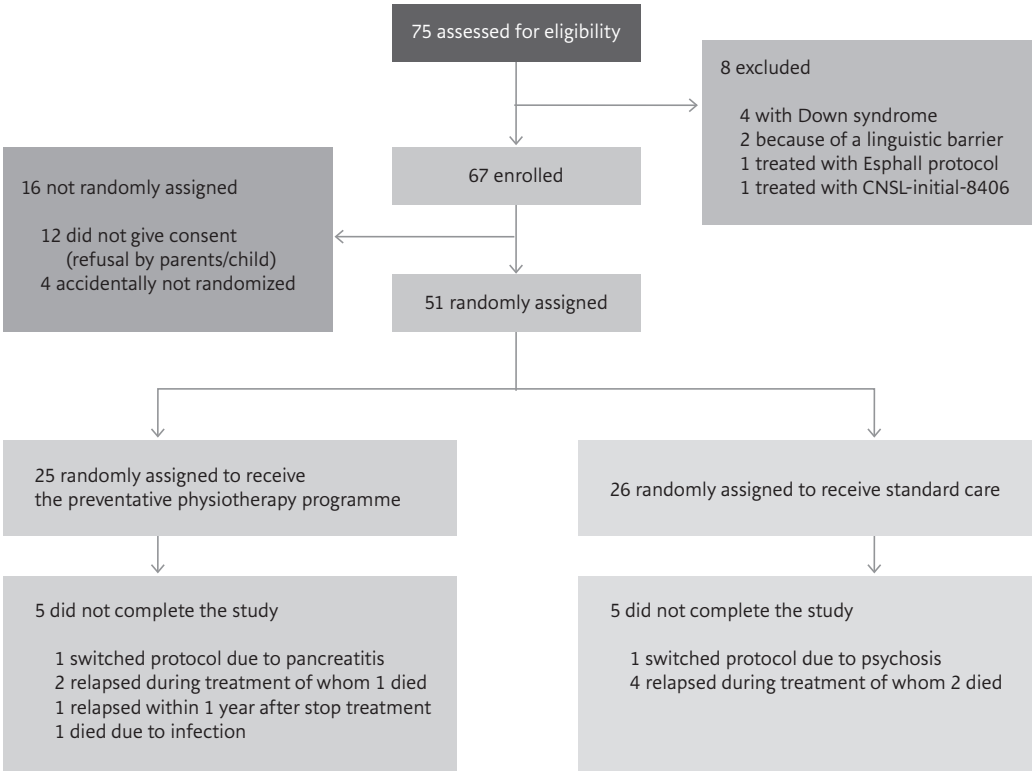


Figure 1. Study profile

	INTERVENTION GROUP	CONTROL GROUP	P-VALUE
GENDER			**0.69**
Boys	14 (56 %)	16 (62 %)	
Girls	11 (44 %)	10 (38 %)	
AGE (YEARS)			**0.52**
Median	5.3	6.2	
Minimum	1.3	1.7	
Maximum	15.6	17.1	
IMMUNOPHENOTYPE			**0.73**
B-lineage	21 (84 %)	20 (77 %)	
T-lineage	4 (16 %)	6 (23 %)	
PROTOCOL			**0.17**
Non high risk	19 (76 %)	15 (58 %)	
High risk	6 (24 %)	11 (42 %)	

Table I. Patient characteristics of the intervention group and the control group at diagnosis.

Anthropometry and body composition

At diagnosis, height, weight and BMI were not significantly different between the two groups (Table II). BMI significantly increased during the two years of chemotherapy in the intervention group (Δ1BMI = 1.53 SDS) and in the control group (Δ_1BMI = 1.38 SDS); this increase did not significantly differ between both groups (p=0.69). One year after cessation of treatment, BMI had decreased towards normal values of healthy peers in both intervention and control group (Δ_2BMI = -0.95 SDS vs. $\Delta_2$2BMI = -0.47 SDS), but this change was significantly more pronounced in the intervention group (p=0.026) (Figure 2A). On cessation of chemotherapy and one year after cessation of therapy there were no significant differences between the groups in height, weight and BMI (Table II).

	DIAGNOSIS		
MEDIAN SDS	**INTERVENTION GROUP**	**CONTROL GROUP**	**P-VALUE**
Height (cm) SDS	-0.11	0.10	0.66
Weight (kg) SDS	-0.40	-0.09	0.76
Body mass index (kg/m^2) SDS	-0.33	-0.38	0.86
Body fat (%) SDS	0.47	-0.22	0.12
Lean body mass (g) SDS	-0.46	-0.66	0.57

Table II. Standard deviation scores of anthropometry and body composition measurements at diagnosis.

At diagnosis there was no significant difference between the groups in percentage of body fat. During treatment there was no significant difference in the change of body fat percentage between the intervention group and that in the control group ($\Delta_1$fat% = 1.04 SDS vs. $\Delta_1$fat% = 1.56 SDS, p=0.25). One year after cessation of chemotherapy the percentage of body fat had decreased in the intervention group ($\Delta_2$fat% = -1.08 SDS, p<0.001) and in the control group ($\Delta_2$fat% = -0.49 SDS, p=0.001). The decrease was more prominent in the intervention group than in the control group (p=0.013) (Figure 2B).

At diagnosis there was no significant difference in LBM between the intervention group and the control group. We found no differences between the two groups in decline of LBM from start to cessation of treatment (intervention group: Δ_1LBM = -0.61 SDS vs. control group: Δ_1LBM = -0.12 SDS, p=0.16). One year after treatment LBM had increased equally in both groups (intervention group: Δ_2LBM = 0.29 SDS vs. control group: Δ_2LBM = 0.22 SDS, p=0.66) (Figure 2C).

Bone mineral (apparent) density

At diagnosis there were no significant differences between the intervention group and the control group in BMD_{TB} (-0.10 SDS vs. −0.18 SDS, p=0.87), BMD_{LS} (-0.42 SDS vs. -0.96 SDS, p=0.22) and $BMAD_{LS}$ (0.14 SDS vs. −0.48 SDS, p=0.09). Between start and cessation of treatment BMD_{TB} decreased significantly in both groups (intervention group: Δ_1BMD_{TB} = -0.75 SDS, p=0.03 and control group: Δ_1BMD_{TB} = -0.96 SDS, p=0.003). The decrease of BMD_{TB} did not differ between both groups (p=0.65). One year after discontinuation of treatment BMD_{TB} had recovered in both groups (intervention group: Δ_2BMD_{TB} = 0.42 SDS, p=0.004 and control group: Δ_2BMD_{TB} = 0.35 SDS, p=0.002). This recovery of BMD_{TB} was not different between both groups (p=0.70) (Figure 3A).

BMD_{LS} did not change in either group during treatment (intervention group: Δ_1BMD_{LS} = -0.15 SDS, p=0.69 vs. control group: Δ_1BMD_{LS} = -0.04 SDS, p=0.90) nor one year after cessation of chemotherapy (intervention group: Δ_2BMD_{LS} = 0.10 SDS, p=0.54 vs. control group: Δ_2BMD_{LS} = 0.14 SDS, p=0.30) (Figure 3B). In addition, after correction for bone size, we found no difference between the intervention and control group in the development of the BMD of the lumbar spine during chemotherapy (Δ_1BMAD_{LS} = -0.66 SDS vs. Δ_1BMAD_{LS} = -0.36 SDS, p=0.47) or during the year after cessation of therapy (Δ_2BMAD_{LS} = 0.12 SDS vs. Δ_2BMAD_{LS} = 0.04 SDS, p=0.77) (Figure 3C).

Skeletal complications

During the study period seven children in the intervention group and three controls sustained fractures (29% vs. 12%, p=0.17). Each group contained one patient with 2 fractures.

Motor performance

At diagnosis motor performance of the patients was significantly impaired compared to healthy peers (-1.41 SDS, p<0.001). There was a trend to improvement in motor performance of both groups combined from -1.41 SDS at diagnosis to -1.00 SDS at cessation of treatment (p=0.055). There was no significant difference between the intervention and control group in change of motor performance during the course of chemotherapy treatment (Δ_1= 0.37 SDS vs. Δ_1= 0.68 SDS, p=0.44) (Figure 4).

Passive ankle dorsiflexion

Mean passive ankle dorsiflexion of both groups combined changed significantly from 9.1° (SD 4.6) at diagnosis to 4.2° (SD 5.8) at cessation of treatment (p=0.001). However, there was no significant difference in decrease in passive dorsiflexion mobility during the course of treatment between the intervention and control group (Δ_1= -5.2° vs. Δ_1= -4.6°, p=0.76) (Figure 5). In the intervention group five children had been supplied with night splints to maintain ankle dorsiflexion mobility versus none in the control group (p=0.017).

Questionnaires

On completion of chemotherapy forty-two questionnaires were sent out: four were not returned leaving 38 available for analysis (19 in the intervention group, 19 controls). Ninety-five percent of all parents stated that they had appreciated the regular testing of motor performance and 84% of the parents in the intervention group appreciated the physiotherapy follow-up sessions. Adherence to the exercise program in the intervention group varied considerably: 11% of the parents performed exercises daily with their child, 37% more than once a week, 16% once weekly, and the other 36% less than once a week. Eight children in the intervention group (42%) and seven children in the control group (37%) had been referred to a local physiotherapist (p=0.52).

DISCUSSION

The present study showed no difference in change of BMI, body fat, LBM, BMD, motor performance or passive ankle dorsiflexion between childhood ALL patients who received the exercise program and those who received standard care. Therefore, we conclude that the exercise program was not more beneficial than standard care to prevent motor problems and reduction in bone mass during ALL treatment. However, increased BMI and body fat of ALL patients in the intervention group normalised faster than in the control group during the year after cessation of chemotherapy, which may point to an educational effect of the intervention program.

The results may be explained by several reasons. First, although the study was based on a reasonable concept, it may not be possible to maintain a continuous 2-year exercise program during childhood ALL treatment. In our cohort, disappointing adherence to the exercise program seems to underscore this and may be important for the lack of impact on the primary end points. Another explanation might be that the intervention and control group were not well matched. However, the similarity of the groups in age, gender, body weight and immunophenotype or risk-group stratification does not support this hypothesis. Although the percentage of children treated with a high-risk protocol was not significantly different, it was slightly higher in the control group. Children treated with a high-risk protocol received more MTX, but slightly less vincristine and corticosteroids. Although this may have had a negative effect on the results of the control group, the conclusion that the exercise program is not more beneficial remains the same.

Scarce information is available in the literature on the value of regular exercise to prevent reduction in BMD, fractures, altered body composition, and impairment of motor performance and passive ankle dorsiflexion during childhood ALL. The exercise program aimed to diminish loss of BMD, muscle power and ankle mobility. According to the retrospectively filled out questionnaires the adherence to

the exercise program was low. In order to promote adherence we ensured that parents, and if possible children, were educated regarding the side-effects of chemotherapy, difficulties that could arise and the need for exercises. A large number of exercises were provided to make variation possible and the exercises had been constructed to fit into children's normal daily activities. However, reasons mentioned for not exercising were that periodically exercising daily did not seem necessary, or that the child was physically unable to exercise daily. The follow-up visits to the hospital-based paediatric physiotherapist always coincided with other appointments, but were missed regularly. Adherence to long-term programs tends to be lower than to short-term interventions[29] and exercising throughout the treatment duration of 2 years may have been too long to remain motivated. Marchese et al. reported a positive effect of an exercise program on active ankle dorsiflexion and knee extension strength. This may be explained by the fact that the intervention in their study lasted for four months only[23]. Whether this effect was maintained for the remainder of the treatment period is unknown. Progression of symptoms may have affected adherence negatively[30]. Another important consideration is that also 37% of the children in the control group had received physiotherapy, albeit locally. It may well be that children who develop motor problems are referred in a timely fashion and that standard care is already adequate. Finally, because parents met regularly in the outpatient department, some exchange of information about the exercise program between parents of children in the intervention group and of the controls cannot be ruled out.

Several animal studies showed that mechanical loads are a crucial factor in the stimulation of bone acquisition[15, 16]. In addition, studies in postmenopausal women[31] and in healthy children[17-21] have shown that exercise (including short-burst high-intensity activities) is an effective way to increase BMD. Therefore, we hypothesized that an exercise program with short-burst high-intensity activities might impede the reduction in BMD in childhood ALL. However, the reduction of BMD was not prevented in our intervention group. Furthermore, we found no difference in number of patients with fractures between both groups.

Both BMI and body fat are in inverse relation to physical activity in healthy children[32]. We found no influence of the exercise program on BMI or body fat during the treatment period of two years, but the increased body fat was lost faster after cessation of chemotherapy in the intervention group. An educational effect of the intervention may have resulted in a lifestyle change.

Motor performance of children with ALL was already decreased at diagnosis and was still decreased at cessation of chemotherapy. It is known that although motor performance improves, it is still lower than in healthy peers two years after cessation of treatment[2]. Ankle mobility in the current study decreased during the first year of therapy and remained stable in the second year, but we do not know whether improvement occurred after cessation of chemotherapy. It is therefore recommended to monitor mobility and motor performance not only during treatment but also after therapy is completed.

In conclusion, effects of the exercise program on body composition, BMD, motor performance and passive ankle dorsiflexion were not beneficial over standard care. Low adherence to the intervention program or adequate standard care may have influenced the results. Increased BMI and body fat of ALL patients in the intervention group normalised faster after cessation of chemotherapy, which may be due to the educational effect of the intervention program. Further studies are necessary to analyse physical activity intervention programs of shorter duration in order to improve adherence.

A. BONE MINERAL DENSITY OF THE TOTAL BODY

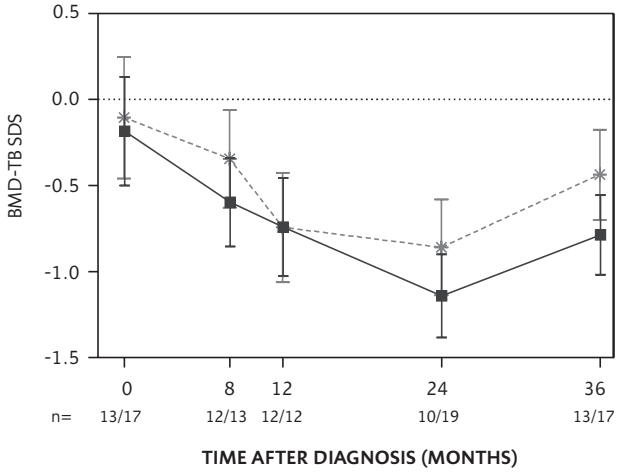
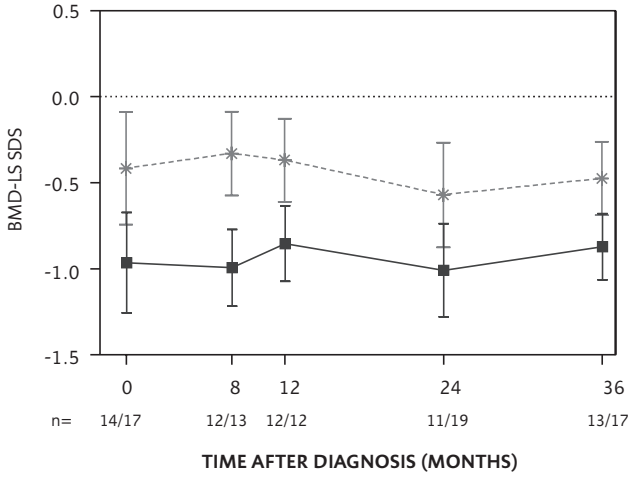


Figure 3A. Development of bone mineral density of the total body of intervention and control group.

*Values are expressed as mean +/- standard error of the mean. Intervention group: *---, control group: ■—. Abbreviations: SDS = standard deviation score, BMD-TB = bone mineral density of the total body.*

B. BONE MINERAL DENSITY OF THE LUMBAR SPINE



C. BONE MINERAL APPARENT DENSITY OF THE LUMBAR SPINE

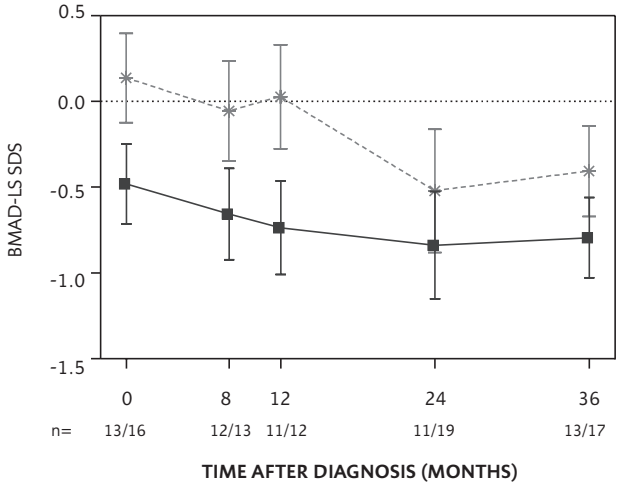


Figure 3B and 3C. Development of bone mineral density of the lumbar spine and of bone mineral apparent density of the lumbar spine of intervention and control group.

*Values are expressed as mean +/- standard error of the mean. Intervention group: *---, control group: ■—. Abbreviations: SDS = standard deviation score, BMD-TB = bone mineral density of the total body, BMD-LS = bone mineral density of the lumbar spine, BMAD = bone mineral apparent density.*

MOTOR PERFORMANCE

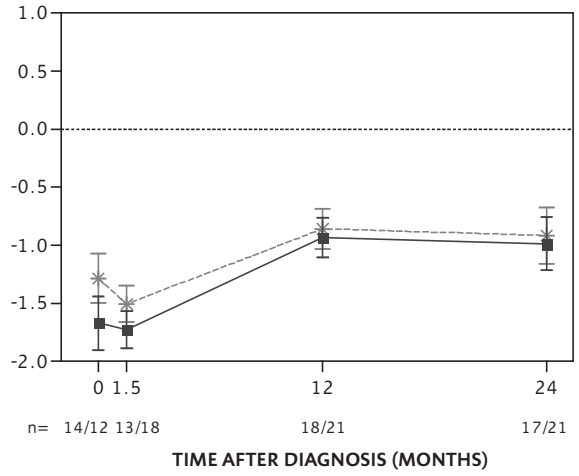


Figure 4. Development of motor performance of intervention and control group. *Values are expressed as mean +/- standard error of the mean. Intervention group: *---, control group: ■—. Abbreviations: SDS = standard deviation score.*

ANKLE DORSIFLEXION

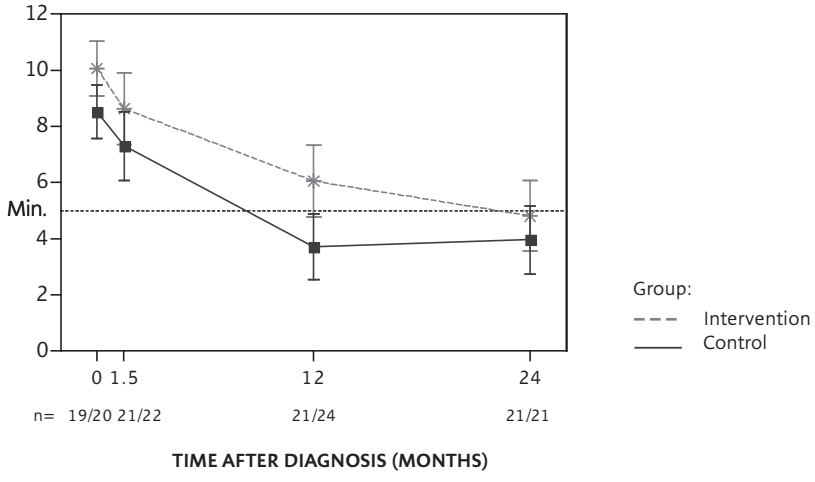
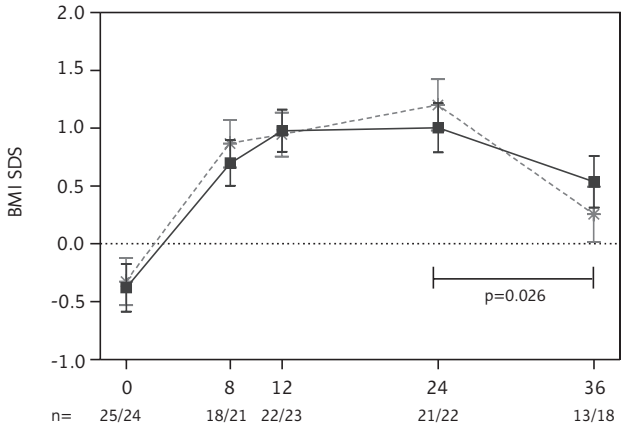
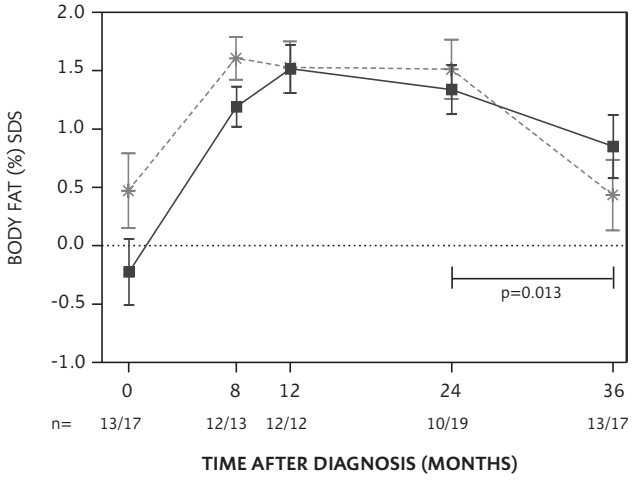


Figure 5. Passive ankle dorsiflexion of intervention group and controls. *Values are expressed as mean +/- standard error of the mean. Intervention group: *---, control group: ■—. Dotted line: minimal limit of normal.*

A. BODY MASS INDEX



B. PERCENTAGE OF BODY FAT



C. LEAN BODY MASS

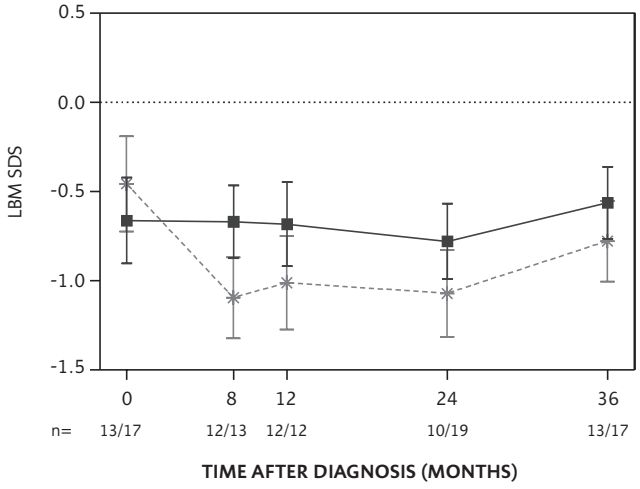


Figure 2. Development of body composition of the intervention and the control group.

*Values are expressed as mean +/- standard error of the mean. Intervention group: *---, control group: ■—. Abbreviations: SDS = standard deviation score, BMI = body mass index, LBM = lean body mass.*

REFERENCES

1. Wright MJ, Halton JM, Martin RF, Barr RD. Long-term gross motor performance following treatment for acute lymphoblastic leukemia. Med Pediatr Oncol 1998;31(2):86-90.
2. Hartman A, van den Bos C, Stijnen T, Pieters R. Decrease in motor performance in children with cancer is independent of cumulative vincristine dose. Cancer 2006;106(6):1395-1401.
3. Marchese V, Chiarello L, Lange B. Strength and functional mobility in children with acute lymphoblastic leukemia. Med Pediatr Oncol 2003;40:230-232.
4. Hartman A, van den Bos C, Stijnen T, Pieters R. Decrease in peripheral muscle strength and ankle dorsiflexion as long-term side effects of treatment for childhood cancer. Pediatr Blood Cancer 2008;50(4):833-7.
5. Salehian B, Kejriwal K. Glucocorticoid-induced muscle atrophy: mechanisms and therapeutic strategies. Endocr Pract 1999;5(5):277-81.
6. Boot AM, van den Heuvel-Eibrink MM, Hahlen K, Krenning EP, de Muinck Keizer-Schrama SM. Bone mineral density in children with acute lymphoblastic leukaemia. Eur J Cancer 1999;35(12):1693-7.
7. Atkinson SA, Halton JM, Bradley C, Wu B, Barr RD. Bone and mineral abnormalities in childhood acute lymphoblastic leukemia: influence of disease, drugs and nutrition. Int J Cancer Suppl 1998;11:35-9.
8. Van der Sluis IM, van den Heuvel-Eibrink MM, Hahlen K, Krenning EP, de Muinck Keizer-Schrama SM. Altered bone mineral density and body composition, and increased fracture risk in childhood acute lymphoblastic leukemia. J Pediatr 2002;141(2):204-10.
9. van der Sluis IM, van den Heuvel-Eibrink MM, Hahlen K, Krenning EP, de Muinck Keizer-Schrama SM. Bone mineral density, body composition, and height in long-term survivors of acute lymphoblastic leukemia in childhood. Med Pediatr Oncol 2000;35(4):415-20.
10. van Beek RD, de Muinck Keizer-Schrama SM, Hakvoort-Cammel FG, van der Sluis IM, Krenning EP, Pieters R, et al. No difference between prednisolone and dexamethasone treatment in bone mineral density and growth in long term survivors of childhood acute lymphoblastic leukemia. Pediatr Blood Cancer 2006;46(1):88-93.
11. Brennan BM, Mughal Z, Roberts SA, Ward K, Shalet SM, Eden TO, et al. Bone mineral density in childhood survivors of acute lymphoblastic leukemia treated without cranial irradiation. J Clin Endocrinol Metab 2005;90(2):689-94.
12. Kaste SC, Rai SN, Fleming K, McCammon EA, Tylavsky FA, Danish RK, et al. Changes in bone mineral density in survivors of childhood acute lymphoblastic leukemia. Pediatr Blood Cancer 2006;46(1):77-87.
13. Warner JT, Evans WD, Webb DK, Bell W, Gregory JW. Relative osteopenia after treatment for acute lymphoblastic leukemia. Pediatr Res 1999;45(4 Pt 1):544-51.
14. Kadan-Lottick N, Marshall JA, Baron AE, Krebs NF, Hambidge KM, Albano E. Normal bone mineral density after treatment for childhood acute lymphoblastic leukemia diagnosed between 1991 and 1998. J Pediatr 2001;138(6):898-904.
15. Rubin CT, Lanyon LE. Regulation of bone formation by applied dynamic loads. J Bone Joint Surg Am 1984;66(3):397-402.
16. Lanyon LE. Control of bone architecture by functional load bearing. J Bone Miner Res 1992;7 Suppl 2:S369-75.
17. Jones G, Dwyer T. Bone mass in prepubertal children: gender differences and the role

of physical activity and sunlight exposure. J Clin Endocrinol Metab 1998;83(12):4274-9.
18. Lehtonen-Veromaa M, Mottonen T, Irjala K, Nuotio I, Leino A, Viikari J. A 1-year prospective study on the relationship between physical activity, markers of bone metabolism, and bone acquisition in peripubertal girls. J Clin Endocrinol Metab 2000;85(10):3726-32.
19. Fuchs RK, Bauer JJ, Snow CM. Jumping improves hip and lumbar spine bone mass in prepubescent children: a randomized controlled trial. J Bone Miner Res 2001;16(1):148-56.
20. Kemper HC, Twisk JW, van Mechelen W, Post GB, Roos JC, Lips P. A fifteen-year longitudinal study in young adults on the relation of physical activity and fitness with the development of the bone mass: The Amsterdam Growth And Health Longitudinal Study. Bone 2000;27(6):847-53.
21. Nilsson M, Ohlsson C, Mellstrom D, Lorentzon M. Previous Sport Activity During Childhood and Adolescence is Associated with Increased Cortical Bone Size in Young Adult Men. J Bone Miner Res 2008.
22. Wright MJ, Hanna SE, Halton JM, Barr RD. Maintenance of ankle range of motion in children treated for acute lymphoblastic leukaemia. Ped Physiother 2003;15:146-152.
23. Marchese VG, Chiarello LA, Lange BJ. Effects of physical therapy intervention for children with acute lymphoblastic leukemia. Pediatr Blood Cancer 2004;42(2):127-33.
24. Veerman AJ, Hahlen K, Kamps WA, Van Leeuwen EF, De Vaan GA, Solbu G, et al. High cure rate with a moderately intensive treatment regimen in non-high-risk childhood acute lymphoblastic leukemia. Results of protocol ALL VI from the Dutch Childhood Leukemia Study Group. J Clin Oncol 1996;14(3):911-8.
25. van der Sluis IM, de Ridder MA, Boot AM, Krenning EP, de Muinck Keizer-Schrama SM. Reference data for bone density and body composition measured with dual energy x ray absorptiometry in white children and young adults. Arch Dis Child 2002;87(4):341-7; discussion 341-7.
26. Fredriks AM, van Buuren S, Burgmeijer RJ, Meulmeester JF, Beuker RJ, Brugman E, et al. Continuing positive secular growth change in The Netherlands 1955-1997. Pediatr Res 2000;47(3):316-23.
27. Henderson S, Sugden D. Movement Assessment Battery for Children. Sidcup, England: The Psychological Corporation; 1992.
28. Bayley N. Manual for the Bayley Scales of Infant Development, 2nd ed. San Antonio: The Psychological Corporation; 1993.
29. Sackett D, Snow J. The magnitude of compliance and noncompliance. In: Haynes R, Taylor D, Sackett D, editors. Compliance in health care. Baltimore: Johns Hopkins Press; 1979. p. 11-22.
30. Prasad SA, Cerny FJ. Factors that influence adherence to exercise and their effectiveness: application to cystic fibrosis. Pediatr Pulmonol 2002;34(1):66-72.
31. Wolff I, van Croonenborg JJ, Kemper HC, Kostense PJ, Twisk JW. The effect of exercise training programs on bone mass: a meta-analysis of published controlled trials in pre- and postmenopausal women. Osteoporos Int 1999;9(1):1-12.
32. White J, Flohr JA, Winter SS, Vener J, Feinauer LR, Ransdell LB. Potential benefits of physical activity for children with acute lymphoblastic leukaemia. Pediatr Rehabil 2005;8(1):53-8.

NATIONAL OSTEONECROSIS STUDY: A PROSPECTIVE STUDY ON INCIDENCE, RISK FACTORS AND LONG-TERM OUTCOME OF OSTEONECROSIS IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

Mariël L. te Winkel, Rob Pieters, Wim C.J. Hop, Hester A. de Groot-Kruseman, Maarten H. Lequin, Inge M. van der Sluis, Jos P.M. Bökkerink, Jan A. Leeuw, Marrie C.A. Bruin, R. Maarten Egeler, Anjo J.P. Veerman, Marry M. van den Heuvel-Eibrink

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ABSTRACT

Purpose

We studied cumulative incidence, risk factors, therapeutic strategies and outcome of symptomatic osteonecrosis in pediatric ALL patients.

Methods

Prospectively, the cumulative incidence of osteonecrosis was assessed in 694 patients treated with the dexamethasone-based DCOG-ALL9 protocol. Osteonecrosis was defined by development of symptoms (NCI grade 2-4) during treatment/within the year after treatment discontinuation, confirmed by MRI. We evaluated risk factors for osteonecrosis using logistic multivariate regression. To describe outcome, we reviewed clinical and radiologic information after antileukemic treatment, ≥1 year after osteonecrosis diagnosis.

Results

The cumulative incidence of osteonecrosis at 3 years was 6.1%. After adjustment for treatment center, logistic multivariate regression identified age (OR=1.47,P<0.01) and female gender (OR=2.21,P=0.04) as independent risk factors. Median age at diagnosis of ALL in patients with and without osteonecrosis was 13.5 versus 4.7 years. In 21 of 38 osteonecrosis patients (55%) chemotherapy was adjusted. Seven patients (18%) underwent surgery: five joint-preserving procedures, two total-hip arthroplasties. Clinical follow-up of 35 patients was evaluated, median follow-up was 4.9 years. In 14 patients (40%) symptoms completely resolved, 14 (40%) had symptoms interfering with function but not with activities of daily living (ADL) (grade 2), seven (20%) had symptoms interfering with ADL (grade 3). In 24 patients radiologic follow-up was available; in six (25%) lesions improved/disappeared, in 13 (54%) lesions remained stable, five (21%) had progressive lesions.

Conclusion

6% of pediatric ALL patients developed symptomatic osteonecrosis during/shortly after treatment. Older age and female gender were risk factors. After a median follow-up of five years, 60% of the patients had persistent symptoms.

INTRODUCTION

As survival rates for pediatric acute lymphoblastic leukemia (ALL) have improved, awareness of side effects becomes increasingly important[1]. Osteonecrosis is one of these side effects that can occur during or shortly after pediatric ALL treatment[2]. Osteonecrosis causes severe pain, limited range of motion of joints and eventually joint destruction. The general pathologic mechanism of osteonecrosis is a compromised bone vascularization leading to death of bone, resulting in demineralization and trabecular thinning and subsequently mechanical failure. The exact pathogenesis of osteonecrosis in childhood ALL patients is not fully understood[3] and the risk factors for osteonecrosis need to be elucidated prospectively. Although corticosteroids have been identified as the main cause of osteonecrosis in childhood ALL patients, also other drugs may contribute to the development of osteonecrosis.

Published prevalences of osteonecrosis in pediatric ALL patients vary widely, depending on the definition of osteonecrosis, the used treatment protocol and the selection of ALL patients. The true prevalence is probably underestimated[2], as most published studies were retrospective[4-8]. We performed a prospective study to determine the prevalence and risk factors for symptomatic osteonecrosis. In addition, we evaluated the therapeutic strategies used for osteonecrosis and described the long-term outcome of osteonecrosis.

PATIENTS AND METHODS

Study population

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[9]. Patients were stratified into non-high risk (NHR) and high risk (HR) groups. HR criteria were white blood cell count ≥$50x10^9$/L, T-cell immunophenotype, mediastinal mass, central nervous system involvement, testes infiltration, t(9;22) and 11q23/*MLL* gene rearrangements. The 2-years' treatment schedules included dexamethasone during induction for 6 weeks and repetitive pulses during maintenance therapy (cumulative doses: 1,244mg/m^2 (HR) and 1,370mg/m^2 (NHR)). Total cumulative dose of methotrexate was 13,650mg/m^2 in the HR and 8,100mg/m^2 in the NHR arm. No patient received central nervous system irradiation. All pediatric oncology centers participating in the DCOG-ALL9 protocol participated in the current osteonecrosis study, except for one. Exclusion criteria for the osteonecrosis study were Down syndrome, pre-existent diseases affecting the locomotor system and administration of major parts of treatment abroad. Patients were prospectively evaluated from diagnosis of ALL until one year after cessation of treatment and data were obtained by Case Report Forms which were centrally collected by the DCOG. For patients who did not complete the protocol (due to relapse, hematopoietic stem-cell transplantation or death) data prior to going off-study were included. The Medical Ethical Committee approved the study and written informed consent according to the Helsinki agreement was obtained from all parents and patients ≥12 years.

Baseline characteristics

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, weight and BMI were compared with reference values of healthy controls matched for age and gender and expressed as standard deviation scores[10-11]. Puberty was documented according to the Tanner staging system and the maximum of developmental scales was used[12]; patients were classified as prepubertal (stage 1), pubertal (stage 2-4), and mature (stage 5).

Definition of osteonecrosis

Symptomatic osteonecrosis was defined as persistent pain in arms or legs, not due to vincristine administration, in combination with typical findings on magnetic resonance imaging (MRI)[13]. Osteonecrosis was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI), version 3.0. Patients who developed symptomatic osteonecrosis (NCI grade 2-4) during treatment or within the first year after cessation of treatment, were considered to have osteonecrosis.

To describe the outcome of osteonecrosis, we retrospectively evaluated the clinical outcome and radiologic examinations (MRI/X-ray) after stop of ALL treatment and ≥1 year after diagnosis of osteonecrosis. Information regarding clinical follow-up was extracted from the medical records. For determining reversibility of radiologic lesions, the last radiologic examinations were used.

Statistical analysis

To estimate the cumulative incidence of osteonecrosis, we performed a Kaplan-Meier survival analysis. To compare patient characteristics between patients with and without osteonecrosis, we used the Mann-Whitney U-test for continuous variables and the chi-square test for categorical variables. To evaluate which variables significantly contributed to the risk to develop osteonecrosis, we performed logistic multivariate regression. The following putative risk factors were investigated: age at diagnosis, gender, risk group of ALL treatment, and BMI at diagnosis. Analyses were adjusted for treatment center. A backward-elimination approach was used to determine the major risk factors. As the effect of age on the risk of developing osteonecrosis might differ for boys and girls, we investigated this by an appropriate interaction term. In addition, quadratic and cubic terms for continuous covariates were added to test for linear, quadratic or cubic relationships.

To evaluate whether the occurrence of osteonecrosis is related to the event-free survival (EFS), Cox-regression was used with osteonecrosis as time-dependent variable. The EFS was defined as the time from diagnosis to induction failure, relapse, death in remission, or second malignancy. Patients who underwent hematopoietic stem-cell transplantation or who were changed to an alternative treatment protocol due to toxicity were withdrawn from further analyses thereafter. Patients who did not achieve complete remission because of resistant disease, were included in the analyses and considered as treatment failures on day 0. For patients alive at January 1st, 2009 (censored observations) EFS was calculated until this date.

Statistical analyses were performed with SPSS version 17.0 (SPSS Inc.,Chicago,IL,USA) and $P<0.05$ (two-sided) was considered statistically significant.

RESULTS

Patients

Of the 859 DCOG-ALL9-treated patients, 751 (466 male) were treated in the centers participating in the current study, of whom 694 (426 male) were eligible (Figure 1). Of the 57 excluded patients, 35 were excluded due to pre-existing conditions (23 Down syndrome and 12 diseases affecting the locomotor system), 20 patients from abroad received only induction therapy in the Netherlands and were not followed for toxicity registration afterwards, and two patients were excluded for other reasons (Figure 1).

Occurrence of osteonecrosis

Thirty-eight out of the 694 patients were diagnosed with symptomatic osteonecrosis during or within one year after stop of antileukemic treatment. One additional patient developed osteonecrosis, however this occurred within half a year after surgery for a proximal femoral fracture. Likely, the blood supply to the bone was damaged due to the fixation material, leading to necrosis of the bone. As the pathophysiology of this kind of local osteonecrosis is probably different than that of chemotherapy-related osteonecrosis, we excluded this patient for further analyses. Figure 2 depicts a Kaplan-Meier curve showing the estimated cumulative incidence of symptomatic osteonecrosis at 3 years of 6.1%.

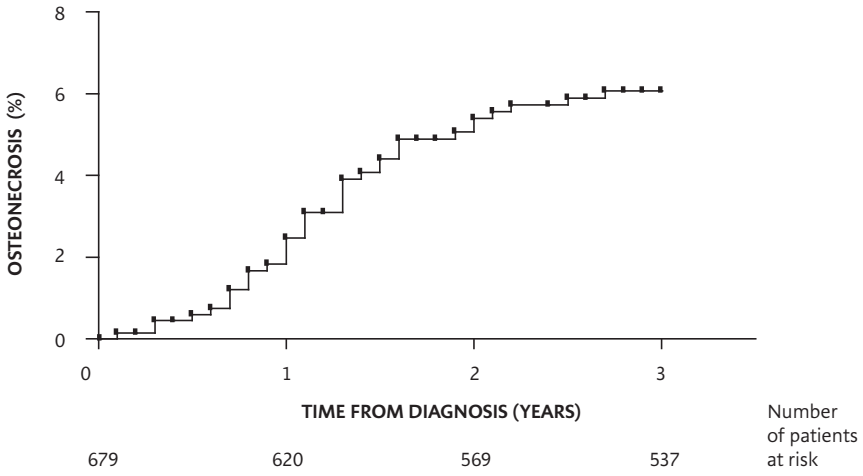

Figure 2. Cumulative incidence of symptomatic osteonecrosis in pediatric acute lymphoblastic leukemia patients during treatment and the year thereafter, for patients who achieved complete remission (n=679).

Of the 693 included patients, 574 completed the whole 3-year study period. Of the patients who ended the study earlier, 14 did not reach complete remission with the DCOG-ALL9 protocol, 12 underwent a stem-cell transplantation as a component of the HR treatment arm, 3 patients changed treatment protocol (2 due to non-skeletal toxicity and 1 with a second malignancy), 67 patients relapsed (44 during therapy and 23 during the first year after cessation of therapy), and 23 patients died in remission during therapy. Duration of follow-up for patients who completed the entire study period and for the different groups of patients who did not, are mentioned in Figure 1.

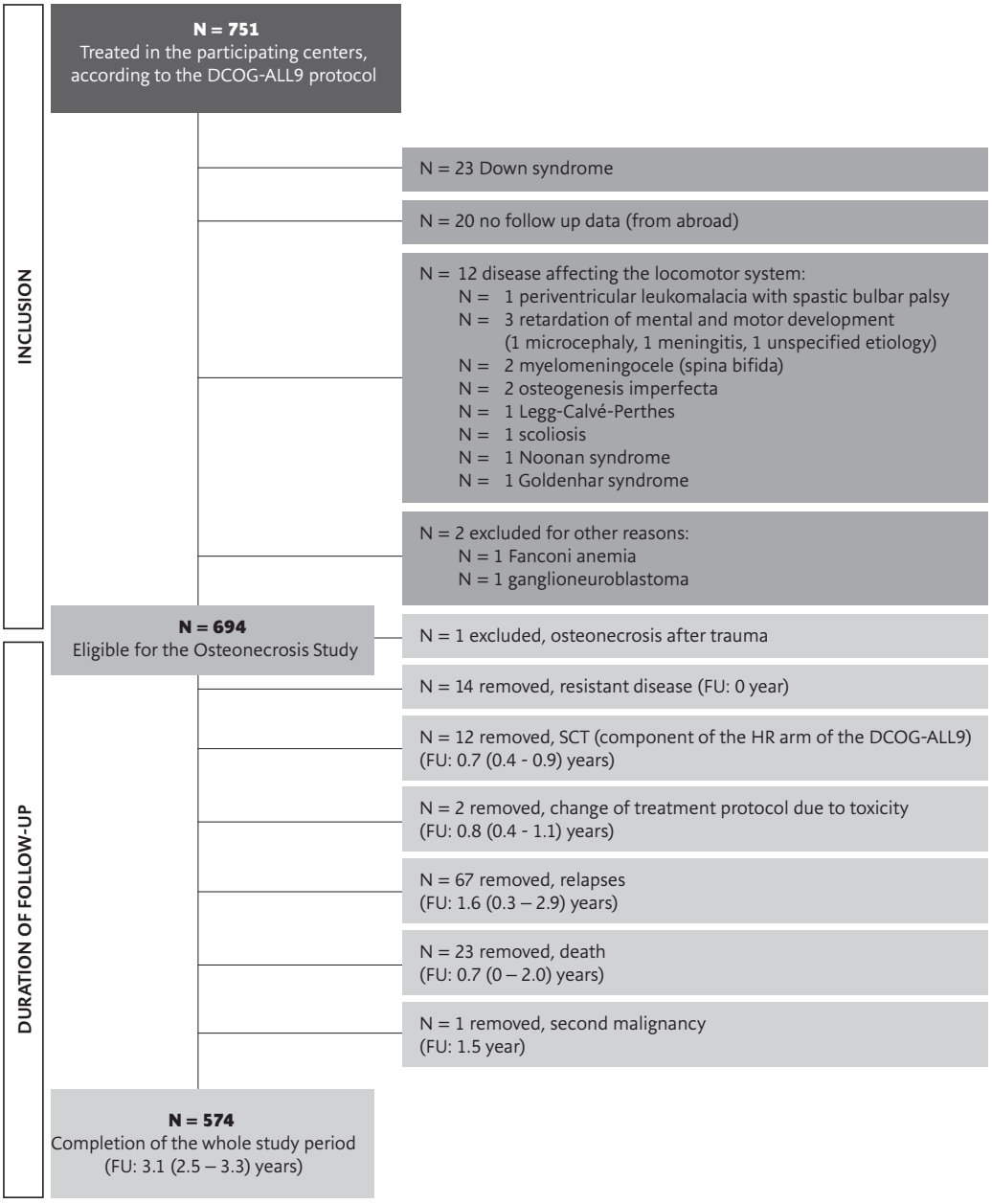

Figure 1. Flowchart that shows the inclusion of patients in the study and duration of follow-up.

Abbreviations: N = number, DCOG = Dutch Childhood Oncology Group, ALL = acute lymphoblastic leukemia, FU = mean duration of follow-up (range), SCT = stem cell transplantation, HR = high risk.

In 35 patients osteonecrosis became apparent during treatment (induction phase (n=1), intensification phase (n=1), and maintenance phase (n=33)) and in 3 patients symptoms started during the first year after stop of therapy. As illustrated in Table 1, the incidence of osteonecrosis was not different in the NHR and HR arms. The mean time-interval between diagnosis of ALL and presentation of osteonecrosis was 1.2 (range:0.1-2.7) years.

The primary location of the symptoms of osteonecrosis was in all 38 patients in the weight-bearing joints of the lower limb; the hip in 11 patients, the knee in 25 patients, and the ankle in 2 patients. The majority of patients (n=34) reported multifocal symptomatic involvement of osteonecrosis (Supplemental Table).

	Patients with ON		Patient without ON		
	Mean	**SD**	**Mean**	**SD**	**P (MWU)**
AGE (YEARS)	**12.5**	**3.5**	**6.0**	**3.8**	**<0.01**
ANTHROPOMETRY					
Weight (sds)	0.28	1.28	0.08	1.07	0.50
Height (sds)	-0.17	1.18	-0.07	1.10	0.55
Body mass index (sds)	-0.38	1.25	-0.17	1.16	0.41
	No.	**%**	**No.**	**%**	**P (χ2)**
GENDER					**0.10**
Male	18	47	398	62	
Female	20	53	243	38	
PUBERTAL STAGE					**<0.01**
Prepubertal	10	33	432	93	
Pubertal	11	37	27	6	
Postpubertal	9	30	8	2	
Not documented	8	-	174	-	
RISK GROUP					**0.99**
Non-high risk	27	71	446	70	
High risk	11	29	195	30	
TREATMENT CENTER					**0.01**
1	18	47	132	21	
2	6	16	72	11	
3	5	13	144	22	
4	4	10	135	21	
5	1	3	78	12	
6	3	8	57	9	
7	1	3	23	4	

Table 1. Baseline characteristics of patients with and without symptomatic osteonecrosis.

Abbreviations: sds = standard deviation score, ON = symptomatic osteonecrosis, SD = standard deviation, MWU = Mann-Whitney U-test, χ2 = chi-square test, No. = number of patients

Risk factors

The clinical baseline characteristics of patients with and without symptomatic osteonecrosis are shown in Table 1. The median age at diagnosis of ALL was 13.5 years for patients who developed osteonecrosis and 4.7 years for those without osteonecrosis (Figure 3). The median age at diagnosis of osteonecrosis was 14.8 (range:6.0-18.1) years.

Stepwise elimination of BMI and risk group in the logistic multivariate regression analysis showed that age and gender were the major risk factors for osteonecrosis after adjustment for treatment center. The regression coefficients of the tested models are shown in Table 2. The model fit did not improve with the interaction term age*gender (P=0.09), indicating that the effect of age on the risk of osteonecrosis was not different for boys and girls. Furthermore, the fit of the model did not improve by adding a quadratic or cubic term to the model (respectively P=0.51 and P=0.59), corresponding with a linear relation between age and risk of osteonecrosis.

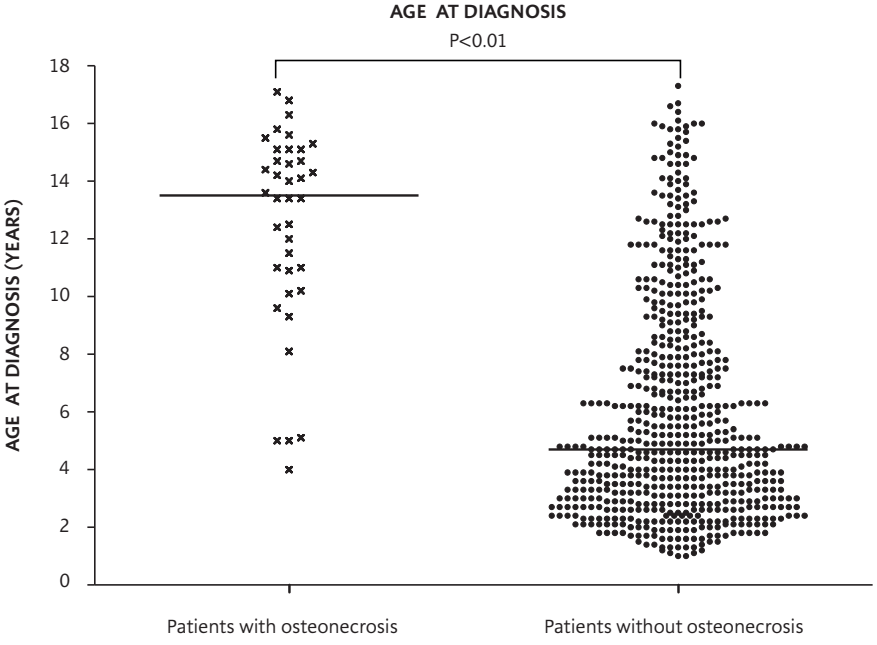

Figure 3. Age at diagnosis in patients with and without symptomatic osteonecrosis. *Abbreviations: ON = symptomatic osteonecrosis. Age at diagnosis was reflected by a "x" for patients with osteonecrosis and a "•" for patients without osteonecrosis. The straight horizontal lines reflect the median age at diagnosis of the two groups.*

MULTIVARIATE LOGISTIC REGRESSION									
	INITIAL MODEL			ELIMINATION OF BMI			ELIMINATION OF BMI AND RISK GROUP (FINAL MODEL)		
RISK FACTOR	**OR**	**95% CI**	***P***	**OR**	**95% CI**	***P***	**OR**	**95% CI**	***P***
Age at diagnosis of ALL (years)	1.47	1.33-1.63	<0.001	1.47	1.33-1.64	<0.001	1.47	1.32-1.63	<0.001
BMI at diagnosis (sds)	0.88	0.64-1.20	0.41	-	-	-	-	-	-
GENDER									
Male	1.00			1.00			1.00		
Female	2.13	0.99-4.62	0.05	2.20	1.02-4.74	0.04	2.23	1.04-4.81	0.04
RISK GROUP									
Non-high risk	1.00			1.00			-		
High risk	0.69	0.30-1.60	0.39	0.67	0.29-1.55	0.35	-	-	-

Table 2. Multivariate logistic regression analysis of symptomatic osteonecrosis in relation to investigated risk factors and adjusted for treatment center. *Abbreviations: OR = odds ratio, CI = confidence interval, BMI = body mass index, sds = standard deviation score.*

Management

The DCOG-ALL9 protocol did not comprise a stringent guideline for the management of osteonecrosis. Results of the analysis on how osteonecrosis was managed in each patient are reported in the Supplemental Table. In 21 patients (55%) chemotherapy was adjusted because of osteonecrosis. All patients received instructions on limiting weight bearing. Part of the patients additionally used orthoses (n=2), crutches (n=11) or wheelchairs (n=11). Seven patients (18%) underwent surgery; five underwent joint-preserving procedures (arthroscopy of the knee with drilling and removal of osteochondral lesions (n=2), bone impacting grafting with decompression of the hip (n=2)), and osteotomy of the hip (n=1)) and two endured total arthroplasty of both hips. Patients who underwent surgery were treated in four different centers. They had a median age of 15.2 (range:6.1-17.6) years when symptoms of osteonecrosis appeared and underwent surgery at a median age of 16.7 (range:10.3-18.4) years. One patient had surgery 2 months before stop of therapy, and the other six patients underwent surgery after treatment discontinuation.

Follow-up

For the description of the outcome of osteonecrosis in our patients, clinical follow-up data of 35 patients could be evaluated as two patients died within one year after diagnosis of osteonecrosis (one due to relapse and one because of sepsis), and one patient was lost to follow-up. The median follow-up time of the remaining 35 patients was 4.9 (range:1.2-8.9) years after diagnosis of osteonecrosis. In 14 patients (40%) symptoms resolved completely, 14 patients (40%) had symptoms interfering with function but not with activities of daily living (ADL) (NCI grade 2), and seven patients (20%) remained to have symptoms interfering with ADL (NCI grade 3). Clinical grading was not different for patients with chemotherapy adaptations and those without. All patients who had surgery remained symptomatic during follow-up (median time after surgery was 2.0 (range:0.8-5.4) years; at last follow-up four patients had NCI stage 2 and

three NCI stage 3. After exclusion of radiological examinations that were performed <1 year after diagnosis of osteonecrosis, 24 patients were available for the analysis of radiological outcome, (median follow-up time 3.8 (range:1.1-8.9) years). All these radiologic examinations were in the post-intervention period. Six patients (25%) had partially or completely reversible lesions, 13 (54%) showed stable lesions, and in five patients (21%) lesions were progressive. Two of the six patients with improved radiologic lesions remained symptomatic, in 10 of the 13 patients with stable radiologic lesions symptoms remained and in all five patients with progressive lesions symptoms remained.

The 5-year EFS for the whole group was 82.3%, and after the occurrence of osteonecrosis the 5-year EFS was 72.4%. On univariate analysis Cox-regression showed a decreased EFS after the occurrence of osteonecrosis (Hazard ratio=2.07,P=0.029). However, after adjustment for age, gender and risk group of ALL, multivariate Cox-regression showed no significant effect of osteonecrosis on subsequent EFS (P=0.75).

DISCUSSION

This prospective study shows a cumulative incidence of symptomatic osteonecrosis of 6.1% in pediatric ALL patients who were treated with a dexamethasone-based protocol. In literature, the reported incidences of osteonecrosis vary widely, depending on the used definition of osteonecrosis, the selection criteria of the study population and the antileukemic treatment (Table 3)[4-8,14-21]. The majority of published studies were performed retrospectively, probably resulting in an underestimation of the true incidence of symptomatic osteonecrosis. Prospective studies that have been performed, mostly report on the combination of symptomatic and asymptomatic osteonecrosis. We chose to include symptomatic patients only, as it remains debatable whether screening for osteonecrosis by MRI is clinically relevant, as many asymptomatic patients eventually do not experience symptoms[18]. The incidence of symptomatic osteonecrosis found in our study is within the range of 1%-9% as reported by other large, but retrospective studies[4-7]. Recently, an MRI-screening study for osteonecrosis in childhood ALL patients reported a higher incidence of grade 2-4 osteonecrosis of 17.6%[15]. The knowledge of lesions on initial MRI may have lead to an increased awareness of symptoms of both the clinician and the patient, possibly overestimating the real number of symptomatic osteonecrosis.

Corticosteroids play a pivotal role in the treatment of ALL, but may cause skeletal toxicity. Dexamethasone is not incontrovertibly proven to be more toxic to the skeleton than prednisone in childhood cancer patients[22], although this is often suggested. We showed no difference in bone density between prednisolone and dexamethasone-containing regimens in ALL survivors[23]. Straus et al. described that dexamethasone was associated with a higher risk of fractures, but not with a higher risk of osteonecrosis. Mattano et al. reported a higher incidence of osteonecrosis in pediatric ALL patients treated with dexamethasone during induction phase than in those treated with prednisone (respectively 11.6% and 8.7%)[14]. This difference between these types of corticosteroids was observed only in patients ≥13 years, suggesting that older children may be more vulnerable to the effect of dexamethasone. In the current dexamethasone-based study, we confirmed older age at diagnosis of ALL to be an independent risk factor for symptomatic osteonecrosis. The fact that older children are prone to develop osteonecrosis has been suggested to be due to higher circulating sex-hormone levels and closure of the growth plate, resulting in less buffering capacity of increased intra-osseous pressures[4,24]. Nevertheless, the incidence of osteonecrosis seems to

be higher in adolescents than in adults[25]. We previously reported that a therapy-induced hypercoagulable state, resulting from a lower dexamethasone-related increase of antithrombin and protein S and subsequent decline of these anticoagulants after introduction of asparaginase, may contribute to the development of osteonecrosis[26]. Age-related differences in dexamethasone-induced changes in the coagulation system during ALL treatment may reflect the higher incidence of osteonecrosis at older age[27].

In the current prospective study, we showed that gender is an independent risk factor for symptomatic osteonecrosis, with a two times higher risk in females than in males. This female predominance was seen in some previous retrospective reports[4,7], although other retrospective studies did not confirm this[5,18,28]. Reasons for the higher risk of osteonecrosis in girls than in boys may be the earlier progress through puberty with subsequent changes in hormone levels, earlier closure of the growth plate, and different changes in the lipid metabolism.

High BMI theoretically may increase the risk of osteonecrosis by a reduced blood flow in the bone marrow due to hypertrophic fat cells, by fat embolisms or by an increase of the mechanical force on joints. Two small studies described conflicting results on the role of BMI on the development of radiographic osteonecrosis in ALL patients[18,29]. In our large prospectively evaluated cohort of pediatric ALL patients, BMI appeared not to be a risk factor for osteonecrosis.

In the present study, symptomatic osteonecrosis was primarily located in weight-bearing bones and mostly multiple joints were involved, consistent with prior reports[21,30]. More than half of the patients remained to have symptoms over a period of approximately five years. As symptoms resolved completely in 40% of the patients, the necessity of surgical procedures needs careful consideration. Given the concerns about surgical procedures in growing individuals and the lifespan of implants, we recommend, if possible, a non-invasive or at least a joint-conserving approach.

Finally, some limitations of our study should be mentioned. First, there may be an unequal awareness of the medical staff in the participating centers, illustrated by differences in incidence of osteonecrosis. Therefore, we adjusted for treatment center in the multivariate logistic regression analyses. In addition, different distributions of ethnic groups among areas may contribute to differences in occurrence of osteonecrosis between centers. Our study population has a rather homogeneous ethnic origin, although we do not have specific information on ethnicity of each patient. Second, it is possible that more severe cases of osteonecrosis (particularly those with a surgical intervention) were reported more accurately than those that were treated conservatively.

In patients with a high risk of osteonecrosis, one may argue to decrease corticosteroid doses. Due to the difficulty of adequately defining patients with a high risk of osteonecrosis and the concerns about the negative consequences for the EFS of ALL, this is not yet a generally accepted approach. Mattano et al. described a trend toward improved EFS among osteonecrosis patients and speculated that this could be due to the high corticosteroids sensitivity in osteonecrosis patients4. In an MRI-screening study, dexamethasone plasma levels turned out to be higher in those with grade 3-4 osteonecrosis[15]. In our Dutch study, we could not confirm a difference in EFS after occurrence of osteonecrosis, although it should be mentioned that the dose of corticosteroids was often reduced due to osteonecrosis. Results of the Children's Cancer Group-1961 study suggest that discontinuous administration instead of continuous

administration of corticosteroids reduces the risk of osteonecrosis, despite a higher cumulative dose[31-32].

In conclusion, this large, prospective study shows that 6.1% of the pediatric ALL patients develop symptomatic osteonecrosis during or shortly after antileukemic treatment. Older age and female gender are the main determinants for the risk of symptomatic osteonecrosis. About 60 percent of the osteonecrosis patients remain to have symptoms over a period of approximately five years, pointing out the importance of awareness of this disabling complication. Due to growing numbers of pediatric ALL survivors, future challenges are to prospectively study long-term consequences of osteonecrosis on joint mobility, ADL and quality of life, and to develop evidence-based guidelines for the management of osteonecrosis during or after pediatric ALL treatment. These guidelines need to address the role of chemotherapy adaptations, avoidance of surgery and the use of bone-modifying agents.

REFERENCE (PROTOCOL)	ASYMPTOMATIC/ SYMPTOMATIC	N=	DESCRIPTION OF THE STUDY POPULATION AND INCIDENCES
Retrospective studies			
Mattano et al. [4] (CCG-1882)	Symptomatic	111/ 1409	**Protocol for HR ALL patients:** - 3-y life-table incidence: 9.3% - <10y: 0.9% vs. ≥10y: 14.2% (Sig) - 10-20y: F: 17.4% vs. M: 11.7% (Sig) 2x21-day DEX: 23.2% vs. 1x21-day DEX: 16.4% (NS) whites: 16.7% vs. blacks: 3.3% vs. other: 6.7% (Sig) Highest: - F 10-15y: 19.2% - M 16-20y: 20.7%
Retrospective studies			
Wei et al. [21] (LR: various protocols, HR: CCG- 1882)	Symptomatic	8/ 202	Incidence: - overall: 4.0% - LR: 2/ 144 (1.4%) vs. HR: 6/ 58 (10.3%)
Strauss et al. [5] (DFCI 87-01, DFCI 91-01)	Symptomatic	13/ 176	5-y cum inc: - overall: 7% - <9y: 4% vs. 9-18y: 21% (Sig) - DEX: 9% vs. PRED: 6% (NS) - Sex, risk group, presenting WBC (NS)
Arico et al. [7] (AIEOP-ALL 95)	Symptomatic	15/ 1421	5-y cum inc: 1.6% - F: 2.5% vs. M: 0.7% (Sig) - 0-5y: 0.3%, 6-9y: 0.7%, 10-17y: 7.4% (Sig) - SR 2.4%, intermediate: 1.0%, HR: 5.8% (Sig) Highest: - F + 10-17y + HR
Burger et al. [6] (ALL-BFM 95)	Symptomatic	31/ 1951	5-y cum inc: - overall: 1.8% - <10y: 0.2% vs. ≥10y: 8.9% (Sig) - SR: 0.2% vs. MR: 2.7% (Sig), MR vs. HR: 3.5% (NS) - M: 1.4% vs. F: 2.4% (NS)
Elmantaser et al. [8] (UKALL97, UKALL97/01, UKALL2003)	Symptomatic	18/ 186	Incidence: - overall: 9.7% - age >9y risk factor for ON (Sig) - DEX: 11% vs. PRED: 3.5% (Sig) - M: 10%, F: 9% (NS)

Table 3. Overview of publications on the incidence of osteonecrosis in pediatric ALL (published from the year 2000)

Abbreviations: N = number, ALL = acute lymphoblastic leukemia, y = years, Sig = significant, NS = not significant, F = female, M = male, DEX = dexamethasone, PRED = prednisone, LR = low risk, SR = standard risk, MR = medium risk, HR = high risk, cum inc = cumulative incidence, ON = osteonecrosis, NHL = Non-Hodgkin lymphoma, WBC = white blood-cell count, BMI = body mass index, MTX = methotrexate, na = not available.

PAGE 102-103: Supplemental table. Detailed description of the patients who developed symptomatic osteonecrosis.

*Abbreviations: M = male, F = female, y = years, na = not available, Dx = diagnosis, ON = symptomatic osteonecrosis, NHR = non-high risk, HR = high risk, DEX = dexamethasone, PRED = prednisone, CS = corticosteroids, VCR = vincristin, 6MP = 6-mercaptopurine, MTX = methotrexate, maint. = maintenance, CR = complete remission. # Pubertal stage according to Tanner staging system: 1 = prepubertal, 2-4 = pubertal, 5=mature. * The change from dexamethasone to prednisone was also because of other side effects of dexamethasone (psychological/ psychiatric problems). ** Stop corticosteroids and vincristin because of constipation. *** Early stop of maintenance because of neutropenia and infections. † No follow-up due to death shortly after diagnosis of ON. ‡ No clinical follow-up data available. ‡‡ No radiologic examination were performed ≥1 year after diagnosis of ON.*

REFERENCE (PROTOCOL)	ASYMPTOMATIC/ SYMPTOMATIC	N=	DESCRIPTION OF THE STUDY POPULATION AND INCIDENCES
Prospective studies			
Ribeiro et al. [18]	Asymptomatic + symptomatic (MRI-screening	ALL: 15/ 107 6 asympt 9 sympt	ALL + NHL patients combined: Risk factors for ON: - >10 y (Sig) - WBC, BMI, sex, cum doses of MTX and corticosteroids, treatment with DEX (NS)
Mitchell et al. [16] (ALL97, ALL97/99)	Symptomatic (NCI grade 3-4)	15/ 1603	DEX vs. PRED relative risk (95%CI): 0.67 (0.24-1.88) (NS) Risk factors: older age, F-gender, second phase of trial (ALL97/99)
Kawedia et al. [15] (St Jude Total XV)	Asymptomatic + symptomatic (MRI-screening)	259/ 364 190 asympt 69 sympt	Cum inc during therapy: - asympt + sympt: 71.8% (asympt: 53.9%, sympt: 17.6%)
Abstracts only (limited data available)			
Mattano et al. [14] (interim analysis COG AALL0232)	Asymptomatic + symptomatic	1 asympt + symp	Protocol for HR ALL patients: 2y-cum inc: - overall: 10.4% - <10y: 2.6% vs.≥10y: 15.2% (Sig) - DEX: 11.6% vs. PRED: 8.7% (Sig) (≥13y: DEX: 18.9% vs. PRED: 9.9%) (Sig), ≥10y: DEX: 17.2% vs. PRED: 12.6% (Sig), <10y: DEX vs. PRED (NS)) - Capizzi MTX: 10.4% vs. HD-MTX: 9.8% (NS)
Vora et al. [19] (interim analysis UK ALL2003	Symptomatic	na	Incidence: - overall: 4% - <10y:1% vs. 10-15y:13% vs. >16y:16% (Sig) - M:3.5% vs. F:4% (NS) - Escalating treatment intensity from A to C: A=1%, B=8%, C=6% (NS)
Moricke et al. [17] (ALL-BFM 2000)	Symptomatic	111/ 3048	Incidence: - overall: 3.6% - <10y: F 0.8%, M 0.7% (NS) - 10y: F 18.4%, M 7.6% (Sig) - Induction: 60 mg/m2/day PRED: 3.2% vs. 10 mg/m^2/day DEX: 3.0% (NS)
Vrooman et al. [20] (DFCI 00-01)	na	Na	5-y cum inc: - 1-10y: DEX: 2.6% vs. PRED: 4.3% (NS) - 10-18y: DEX:23% vs. PRED: 4.7% (Sig

PROSPECTIVE									
PATIENT CHARACTERISTICS AT DX OF ON					COMPLETION OF PROTOCOL	TIME ALL-ON (Y)	LOCALISATION OF SYMPTOMS (CONFIRMED **WITH MRI)**	MANAGEMENT OF ON	
No	Sex	Age (y)	Pubertal stage	Risk group				Conservative treatment	
1	M	6.1	1	NHR	Yes	2.1	Hips, knees, ankles	Crutches	
2	M	6.6	1	NHR	Yes	1.6	Knees	Weight-bearing restrictions	
3	M	14.5	5	NHR	No: relapse	0.3	Knees	Weight-bearing restrictions	
4	F	17.6	5	NHR	Yes	2.5	Hips, knee	Crutches	
5	M	16.1	2-4	NHR	Yes	0.8	Hips , knees, shoulder	Wheelchair	
6	F	11.2	2-4	HR	Yes	1.9	Hip, knees	Crutches	
7	F	11.6	1	HR	Yes	1.5	Hip, ankles	Wheelchair	
8	F	15.3	2-4	NHR	No: death	1.0	Knees	Wheelchair	
9	M	15.7	5	NHR	No: relapse	1.1	Hips, knees	Crutches	
10	M	10.9	1	HR	Yes	0.7	Hips, knees, ankles	Wheelchair	
11	M	11.7	1	HR	Yes	0.7	Knees, ankles	Wheelchair	
12	F	12.6	2-4	HR	Yes	1.1	Hips, knees, shoulders	Wheelchair	
13	F	12.9	2-4	HR	Yes	0.9	Hips	Wheelchair	
14	F	14.0	2-4	HR	Yes	1.6	Hips, knees, ankles, shoulder	Crutches	
15	M	13.8	2-4	HR	Yes	1.3	Hips	Crutches	
16	F	16.4	5	HR	Yes	1.3	Hip	Weight-bearing restrictions	
17	M	17.7	5	HR	Yes	2.2	Hips and knees	Crutches	
18	F	18.1	5	HR	Yes	1.0	Hip, knees	Crutches	
19	M	6.4	1	NHR	Yes	1.3	Hips, knees	Weight-bearing restrictions	
20	F	9.2	1	NHR	Yes	1.1	Hips, knees, ankles, wrist	Weight-bearing restrictions	
21	F	10.2	na	NHR	Yes	0.6	Knees	Weight-bearing restrictions	
22	M	12.2	1	NHR	Yes	1.3	Knee	Weight-bearing restrictions	
23	F	12.5	2-4	NHR	Yes	1.5	Hips and knees	Wheelchair	
24	M	14.1	2-4	NHR	Yes	0.7	Hip	Weight-bearing restrictions	
25	F	14.8	5	NHR	Yes	1.4	Hips	Wheelchair	
26	F	16.3	5	NHR	Yes	2.7	Knees	Weight-bearing restrictions	
27	F	14.8	2-4	NHR	Yes	0.8	Hips, knees	Wheelchair	
28	M	15.2	2-4	NHR	Yes	1.1	Knee	Orthosis	
29	M	16.4	2-4	NHR	Yes	2.0	Knees	Crutches	
30	F	16.3	5	NHR	Yes	1.6	hips, knees, ankles	Crutches	
31	M	15.5	5	NHR	Yes	0.8	hips, knees, shoulders	Wheelchair	
32	F	16.4	5	NHR	Yes	1.3	hips, knees, ankles	Weight-bearing restrictions	
33	M	16.6	5	NHR	Yes	1.0	knees	Weight-bearing restrictions	
34	F	15.8	5	NHR	Yes	0.1	knees	Weight-bearing restrictions	
35	M	16.8	Na	NHR	Yes	0.5	hips, knees, shoulders, elbow	Weight-bearing restrictions	
36	F	17.1	5	NHR	Yes	0.3	hips	Crutches	
37	F	15.4	na	NHR	Yes	2.0	knee, ankle	Weight-bearing restrictions	
38	M	6.0	1	NHR	Yes	1.0	feet	Orthosis	

RETROSPECTIVE						
MANAGEMENT OF ON			FU TIME OUTCOME (Y)		STATUS PRAESENS AT LAST FU	
	Treatment adaptations during maintenance due to ON	Surgical intervention	Clin	Rad	ON	**ALL**
	No	Osteotomy (hip)	4.9	5.0	NCI grade 3	Relapse
	No	No	5.6	5.6	asymptomatic	CR
	Reduced DEX dose	No	†	†	†	Death
	No (ON after completion of treatment)	Bone impaction grafting with decompression (femoral head)	2.8	‡‡	NCI grade 2	CR
	No	Bone impacting grafting with decompression (femoral head)	2.5	5.2	NCI grade 3	CR
	Stop CS, stop VCR	No	4.2	4.2	asymptomatic	CR
	Reduced DEX dose	No	3.4	4.4	NCI grade 2	CR
	No	No	†	†	†	Death
	PRED instead of DEX*	No	4.7	4.3	NCI grade 3	Relapse
	Stop CS, stop VCR, continuous 6MP&MTX	No	6.0	6.0	NCI grade 3	CR
	Stop CS, continuous 6MP&MTX	No	3.5	‡‡	asymptomatic	CR
	Reduced DEX dose, continuous 6MP&MTX	No	6.1	5.7	NCI grade 2	CR
	Reduced DEX dose, continuous 6MP	No	6.8	‡‡	asymptomatic	CR
	PRED instead of DEX	Two-sided total hip arthroplasty; arthroscopy (knee) with removal of fragments	4.3	4.2	NCI grade 3	Relapse
	No	No	1.8	‡‡	asymptomatic	CR
	No	No	8.1	‡‡	asymptomatic	CR
	No (ON after completion of treatment)	No	8.9	8.9	NCI grade 3	CR
	Stop CS, continuous 6MP&MTX	No	2.4	2.3	NCI grade 2	CR
	Early stop maint. (request of parents)	No	1.2	‡‡	asymptomatic	CR
	Stop CS, stop VCR, continuous 6MP&MTX	No	8.2	8.2	NCI grade 2	CR
	Stop CS, continuous 6MP&MTX	No	1.9	1.8	asymptomatic	Relapse
	No**	No	2.5	2.5	NCI grade 2	CR
	Stop CS, continuous MTX	No	6.2	‡‡	NCI grade 3	CR
	No	No	5.9	1.5	asymptomatic	CR
	Stop CS, stop VCR, continuous 6MP&MTX	Two-sided total hip arthroplasty	5.0	1.1	NCI grade 2	Relapse
	No (ON after completion of treatment)***	No	5.4	‡‡	NCI grade 2	CR
	No	No	5.2	2.2	asymptomatic	Relapse
	Stop CS, stop VCR	Arthroscopy + arthrotomy (knee) with removal of fragments	3.3	3.3	NCI grade 2	CR
	Stop CS, stop VCR	No	1.8	3.0	asymptomatic	CR
	No	No	5.7	‡‡	asymptomatic	CR
	Stop CS, continuous 6MP&MTX	Arthroscopic drilling (knee)	6.5	2.0	NCI grade 2	CR
	No	No	2.8	1.1	NCI grade 2	CR
	Reduced DEX dose	No	6.4	‡‡	asymptomatic	Relapse
	No	No	7.3	8.0	NCI grade 2	CR
	Stop CS, continuous 6MP&MTX, early stop maint.	No	2.8	3.5	NCI grade 2	Relapse
	No	No	7.2	‡‡	NCI grade 2	CR
	No	No	‡	1.6	‡	CR
	Stop CS	No	2.7	5.0	asymptomatic	CR

REFERENCES

1. Kamps WA, van der Pal-de Bruin KM, Veerman AJ, et al: Long-term results of Dutch Childhood Oncology Group studies for children with acute lymphoblastic leukemia from 1984 to 2004. Leukemia 24:309-19, 2010
2. Barr RD, Sala A: Osteonecrosis in children and adolescents with cancer. Pediatr Blood Cancer 50:483-5; discussion 486, 2008
3. Mankin HJ: Nontraumatic necrosis of bone (osteonecrosis). N Engl J Med 326:1473-9, 1992
4. Mattano LA, Jr., Sather HN, Trigg ME, et al: Osteonecrosis as a complication of treating acute lymphoblastic leukemia in children: a report from the Children's Cancer Group. J Clin Oncol 18:3262-72, 2000
5. Strauss AJ, Su JT, Dalton VM, et al: Bony morbidity in children treated for acute lymphoblastic leukemia. J Clin Oncol 19:3066-72, 2001
6. Burger B, Beier R, Zimmermann M, et al: Osteonecrosis: a treatment related toxicity in childhood acute lymphoblastic leukemia (ALL)--experiences from trial ALL-BFM 95. Pediatr Blood Cancer 44:220-5, 2005
7. Arico M, Boccalatte MF, Silvestri D, et al: Osteonecrosis: An emerging complication of intensive chemotherapy for childhood acute lymphoblastic leukemia. Haematologica 88:747-53, 2003
8. Elmantaser M, Stewart G, Young D, et al: Skeletal morbidity in children receiving chemotherapy for acute lymphoblastic leukaemia. Arch Dis Child 95:805-9, 2010
9. Veerman AJ, Kamps WA, van den Berg H, et al: Dexamethasone-based therapy for childhood acute lymphoblastic leukaemia: results of the prospective Dutch Childhood Oncology Group (DCOG) protocol ALL-9 (1997-2004). Lancet Oncol 10:957-66, 2009
10. Fredriks AM, van Buuren S, Burgmeijer RJ, et al: Continuing positive secular growth change in The Netherlands 1955-1997. Pediatr Res 47:316-23, 2000
11. Fredriks AM, van Buuren S, Wit JM, et al: Body index measurements in 1996-7 compared with 1980. Arch Dis Child 82:107-12, 2000
12. Tanner JM, Whitehouse RH: Clinical longitudinal standards for height, weight, height velocity, weight velocity, and stages of puberty. Arch Dis Child 51:170-9, 1976
13. Saini A, Saifuddin A: MRI of osteonecrosis. Clin Radiol 59:1079-93, 2004
14. Mattano LA, Jr., Nachman JB, Devidas M, et al: Increased Incidence of Osteonecrosis (ON) with a Dexamethasone (DEX) Induction for High Risk Acute Lymphoblastic Leukemia (HR-ALL): A Report from the Children's Oncology Group (COG). ASH Annual Meeting Abstracts 112:898-, 2008
15. Kawedia JD, Kaste SC, Pei D, et al: Pharmacokinetic, pharmacodynamic, and pharmacogenetic determinants of osteonecrosis in children with acute lymphoblastic leukemia. Blood 117:2340-7, 2011
16. Mitchell CD, Richards SM, Kinsey SE, et al: Benefit of dexamethasone compared with prednisolone for childhood acute lymphoblastic leukaemia: results of the UK Medical Research Council ALL97 randomized trial. Br J Haematol 129:734-45, 2005
17. Moricke A, Zimmermann M, Schrauder A, et al: No Influence on the Incidence of Osteonecroses When Dexamethasone Replaces Prednisone during Induction Treatment for Childhood ALL: Results of Trial ALL-BFM 2000. ASH Annual Meeting Abstracts 112:899-, 2008
18. Ribeiro RC, Fletcher BD, Kennedy W, et al: Magnetic resonance imaging detection of avascular necrosis of the bone in children

receiving intensive prednisone therapy for acute lymphoblastic leukemia or non-Hodgkin lymphoma. Leukemia 15:891-7, 2001

19. Vora A, Wade R, Mitchell C, et al: Incidence and Outcome of Osteonecrosis in Children and Young Adults with Acute Lymphoblastic Leukaemia Treated on a Dexamethasone Containing Protocol: Results of the Medical Research Council UK Trial ALL 2003. ASH Annual Meeting Abstracts 112:910-, 2008
20. Vrooman LM, Neuberg DS, Stevenson KE, et al: Dexamethasone and Individualized Asparaginase Dosing Are Each Associated with Superior Event-Free Survival in Childhood Acute Lymphoblastic Leukemia: Results From DFCI-ALL Consortium Protocol 00-01. ASH Annual Meeting Abstracts 114:321-, 2009
21. Wei SY, Esmail AN, Bunin N, et al: Avascular necrosis in children with acute lymphoblastic leukemia. J Pediatr Orthop 20:331-5, 2000
22. Sala A, Mattano LA, Jr., Barr RD: Osteonecrosis in children and adolescents with cancer - an adverse effect of systemic therapy. Eur J Cancer 43:683-9, 2007
23. van Beek RD, de Muinck Keizer-Schrama SM, Hakvoort-Cammel FG, et al: No difference between prednisolone and dexamethasone treatment in bone mineral density and growth in long term survivors of childhood acute lymphoblastic leukemia. Pediatr Blood Cancer 46:88-93, 2006
24. Ruderman RJ, Poehling GG, Gray R, et al: Orthopedic complications of renal transplantation in children. Transplant Proc 11:104-6, 1979
25. Patel B, Richards SM, Rowe JM, et al: High incidence of avascular necrosis in adolescents with acute lymphoblastic leukaemia: a UKALL XII analysis. Leukemia 22:308-12, 2008
26. te Winkel ML, Appel IM, Pieters R, et al: Impaired dexamethasone-related increase of anticoagulants is associated with the development of osteonecrosis in childhood acute lymphoblastic leukemia. Haematologica 93:1570-4, 2008
27. Appel IM, Hop WC, van Kessel-Bakvis C, et al: L-Asparaginase and the effect of age on coagulation and fibrinolysis in childhood acute lymphoblastic leukemia. Thromb Haemost 100:330-7, 2008
28. Hogler W, Wehl G, van Staa T, et al: Incidence of skeletal complications during treatment of childhood acute lymphoblastic leukemia: comparison of fracture risk with the General Practice Research Database. Pediatr Blood Cancer 48:21-7, 2007
29. Niinimaki RA, Harila-Saari AH, Jartti AE, et al: High body mass index increases the risk for osteonecrosis in children with acute lymphoblastic leukemia. J Clin Oncol 25:1498-504, 2007
30. Murphy RG, Greenberg ML: Osteonecrosis in pediatric patients with acute lymphoblastic leukemia. Cancer 65:1717-21, 1990
31. Seibel NL, Steinherz PG, Sather HN, et al: Early postinduction intensification therapy improves survival for children and adolescents with high-risk acute lymphoblastic leukemia: a report from the Children's Oncology Group. Blood 111:2548-55, 2008
32. McNeer JL, Nachman JB: The optimal use of steroids in paediatric acute lymphoblastic leukaemia: no easy answers. Br J Haematol 149:638-52, 2010

IMPAIRED DEXAMETHASONE-RELATED INCREASE OF ANTICOAGULANTS IS ASSOCIATED WITH THE DEVELOPMENT OF OSTEONECROSIS IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

Mariel L. te Winkel, Inge M. Appel, Rob Pieters and Marry M. van den Heuvel-Eibrink

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ABSTRACT

Backgrounds and Objectives

Alterations of coagulation accompanied by impairment of microcirculation may be involved in the pathogenesis of osteonecrosis (ON) in childhood acute lymphoblastic leukemia (ALL). ALL induction treatment includes dexamethasone and asparaginase, both influencing coagulation.

Design and Methods

Retrospectively, we evaluated coagulation parameters of 161 ALL patients: 24 who developed ON (ON-positive, median age: 13.8 (4.0–17.2) years) and 137 who did not (ON-negative, median age: 4.9 (1.0-16.7) years). Thrombin generation, fibrinolysis, procoagulant and anticoagulant factors were determined at diagnosis and during induction treatment.

Results

Coagulation parameters of ON-positive and ON-negative patients were similar at diagnosis. After 4 weeks induction treatment including dexamethasone, the anticoagulants antithrombin and protein S were significantly less increased in the ON-positive than in the ON-negative patients. Subsequently, after administration of 4 doses asparaginase and tapering dexamethasone, these coagulation parameters equally decreased in both ON-positive and ON-negative patients. As a result, the nadirs of antithrombin and protein S were significantly lower in ON-positive than in ON-negative patients, even reaching levels below lower normal limits in the ON-positive group. No dexamethasone or asparaginase induced differences of other coagulation parameters were found between both groups.

Interpretation and Conclusion

We conclude that a reduced dexamethasone related increase of antithrombin and protein S and subsequent decline of these anticoagulants below normal levels after introduction of asparaginase, may result in a hypercoagulable state. This therapy induced hypercoagulable state may be a contributing factor to develop symptomatic ON in childhood ALL.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common malignancy in childhood. Because of the high cure-rate of ALL, the complications of the disease and side-effects during and after therapy become increasingly important. One of these serious complications is osteonecrosis (ON), which is caused by impairment of microcirculation serving a segment of bone that has a poor collateral circulation and insufficient venous drainage[1-3].

It is generally accepted that various factors play a role in the etiology of ON in childhood ALL, one of these factors may be hypercoagulability[4-6]. Dexamethasone and asparaginase, important drugs in the induction treatment of childhood ALL, can both influence the coagulation system[7-14]. Corticosteroids will increase most coagulation protein concentrations, whereas asparaginase can diminish the synthesis of coagulation factors and inhibitors. Micro-thrombi resulting from an imbalance between procoagulant and anticoagulant processes in patients with Legg-Calvé-Perthes disease have shown to play an important etiologic role in the development of ON[15-19]. Recently, it was shown that neither factor V Leiden mutations nor lipoprotein A metabolism influenced the risk of ON in childhood ALL[4, 5]. Nevertheless, only scarce information is available on the role of coagulation dysregulation in the pathogenesis of ON in childhood ALL patients.

Therefore, the main objective of this study is to investigate whether induction therapy related alterations in coagulation, are associated with the development of ON in childhood ALL patients. For this reason procoagulant factors (fibrinogen, factor II, V, VII, IX, X), anticoagulant factors (antithrombin (AT), protein C and protein S), parameters of thrombin generation (prothrombin fragment 1+2 (F1+2), thrombin antithrombin (TAT) complex) and of fibrinolysis (α2-antiplasmin (a2AP), plasminogen, plasmin-α2AP (PAP) complex, D-dimers) were studied.

DESIGN AND METHODS

Patients

Differences in coagulation parameters between pediatric ALL patients with and without ON were studied in a retrospective analysis. Between 1997 and 2004, 174 patients received induction therapy according to the Dutch Childhood Oncology Group (DCOG)-ALL9 treatment protocol at the Erasmus Medical Center-Sophia's Children's Hospital, Rotterdam. This dexamethasone-based protocol aimed to reproduce the results of the DCOG-ALL6 treatment protocol in a larger cohort of Dutch ALL patients and included induction therapy as depicted in figure 1[20]. Cumulative doses of the chemotherapeutic agents used in the non-high risk versus the high risk DCOG-ALL9 protocol are reported previously[21]. In the DCOG-ALL9 protocol newly diagnosed ALL patients older than 365 days and younger than 19 years were included, with exception of mature B cell ALL patients. Coagulation parameters, which were routinely measured during induction therapy to monitor asparaginase toxicity, were available of 161 patients (of which 24 developed ON).

Clinical data (age, gender, immunophenotype of leukemia) and data on thrombotic events during intensification and maintenance treatment of the DCOG-ALL9 treatment schedule were collected from the medical records. The Medical Ethics Committee of Erasmus Medical Center, Rotterdam, the Netherlands, approved of the study and written informed consent was obtained from all participants and/or their parents.

Methods

Patients were considered ON-positive if they developed symptomatic ON during or within one year after cessation of treatment. Symptomatic ON was defined as persistent pain in the arms or legs not associated with recent vincristine administration, in combination with typical findings on magnetic resonance imaging (MRI)[22-24].

Peripheral blood (PB) samples were taken at diagnosis and at 5 time points (day 29, 33, 36, 40 and 43) during induction therapy of ALL (Figure 1). On day 29, 33, 36 and 40 PB samples were taken immediately before each asparaginase infusion. Procoagulant factors (fibrinogen and factor II, V, VII, IX, X), anticoagulant factors (AT, protein C and protein S), parameters of thrombin generation (F1+2 and TAT complex) and fibrinolysis (a2AP, plasminogen, PAP complex and D-dimers) were determined, using sample tubes containing a fixed amount of citrate as anticoagulant. Samples were chilled immediately on ice and centrifuged 30 minutes at 20,000 rpm at 4°C. The supernatant was withdrawn and stored until the time of analysis at -80°C.

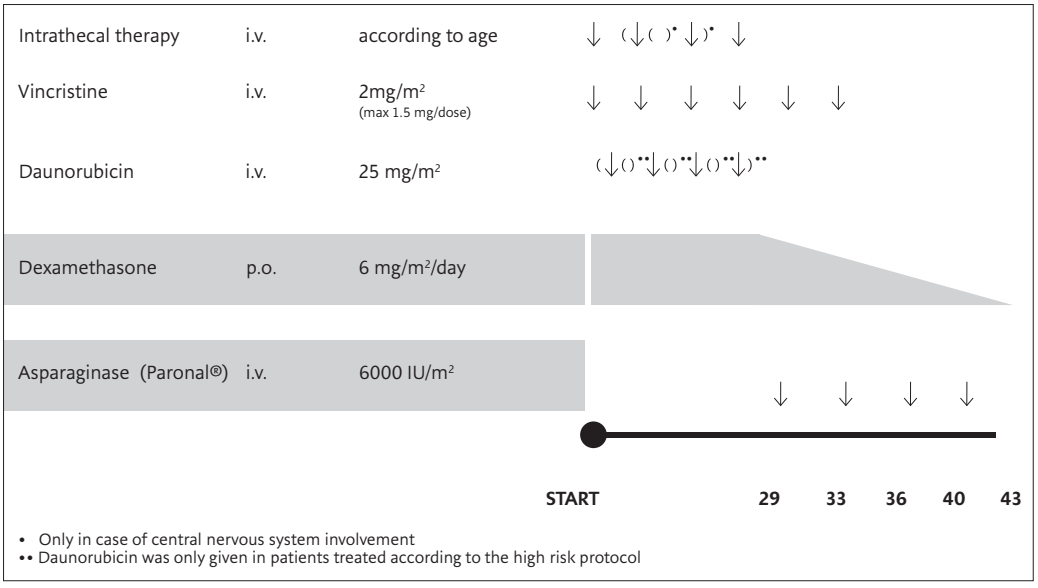

Figure 1. DCOG (Dutch Childhood Oncology Group)-ALL9 induction treatment schedule. *Abbreviations: i.v., intra venous; p.o., per os. Asparaginase (Paronal®, Christiaens B.V., Breda, the Netherlands).*

Coagulation assays

Fibrinogen was determined using the Claus method[25] (Dade® Thrombin Reagent; Dade Behring GmbH, Marburg, Germany). Factor II, V, VII, IX, X were assessed using factor deficient plasma and standard one stage factor assays (Coagulation Factor II, VII and X, Coagulation Factor V Deficient Plasma and Coagulation Factor IX; Dade Behring GmbH, Marburg, Germany).

The anticoagulant factors AT and protein C activity were measured using Berichrom Antithrombin III (A) and Berichrom Protein C (Dade Behring GmbH, Marburg, Germany). Total and free protein S

were determined using enzyme-linked immunosorbent assays (ELISA) (Asserachrom® Total Protein S and Asserachrom® Free Protein S; Diagnostica Stago, Asnieres, France). Plasminogen and α2-AP were measured using Berichrom α2-Antiplasmin and Berichrom Plasminogen (Dade Behring GmbH, Marburg, Germany). PAP was measured using PAP micro ELISA (Dade Behring GmbH, Marburg, Germany)[26]. D-dimer levels were measured with an immunoturbidimetric assay (Auto Dimer®; Trinity Biotech plc, Bray, Ireland). The markers of endogenous thrombin generation F1+2 and TAT were measured using ELISA (Enzygnost F1+2 and Enzygnost TAT; Dade Behring GmbH, Marburg, Germany)[27-29].

Data Analysis

The Mann-Whitney U-test and the $\chi 2$-test / Fisher exact test were used to compare patient characteristics between the ON-positive and ON-negative groups. Coagulation parameters of the ON-positive and ON-negative patients were compared by means of the non-parametric Mann-Whitney U-test. These statistical analyses were carried out with SPSS for Windows version 11.0.1 (SPSS Inc., Chicago, IL, USA).

Repeated measurements analysis (SAS PROC MIXED; SAS Institute Inc., Cary, North Carolina, USA) was used to confirm the differences in AT, protein C and protein S between both groups[30]. To analyze differences between the ON-positive and the ON-negative groups in trends over time of the anticoagulants AT, protein C and protein S, the model defined by the variables "time", "ON-group" and the interaction variable "time * ON-group" was applied (Figure 2). We used an unstructured repeated covariance type. Differences between ON-positive and ON-negative patients at each moment were estimated using a model without intercept defined by the interaction variable "ON-group * time". The same model was used to evaluate the significance of the slopes of the curves of the anticoagulants over time. P-values less than 0.05 (two-sided) were considered statistically significant in all analyses.

RESULTS

Patient Characteristics

Clinical characteristics of the ON-positive and ON-negative patients are summarized in table 1. No differences were found between ON-positive and ON-negative patients in gender, immunophenotype of leukemia and risk group stratification. As expected, the median age at diagnosis of ALL was significantly higher in ON-positive as compared to ON-negative patients. All but one of the ON-positive patients had clinical signs of ON before stop of treatment.

At a later stage during treatment, i.e. intensification and maintenance treatment of the DCOG-ALL9 schedule, 3 of the 24 ON-positive patients (12.5%) endured a thrombotic event, as compared to 2 of the 137 (1.5%) of the ON-negative patients ($\chi 2$-test, P=0.02). The thrombotic events included a transverse sinus thrombosis (n=1), a pulmonary embolism (n=1) and a thrombus of the brachiocephalic vein possibly related to the presence of an implantable venous access port (Port-A-Cath) (n=1) in the ON-positive patients. In the group of ON-negative patients 1 transverse sinus thrombosis and 1 catheter related thrombosis in the upper venous system of the arm occurred.

CLINICAL DATA	ON-POSITIVE	ON-NEGATIVE	P-VALUE
Patients at diagnosis (N=)	24	137	-
Gender (male / female)	12 / 12	81 / 56	0.40
Age (years) at diagnosis (median (range))	13.8 (4.0 – 17.2)	4.9 (1.0 – 16.7)	<0.01
Age (years) ON (median (range))	16.1 (6.0 – 18.7)	N.A.	-
Immunophenotype precursor B T	 20 4	 116 21	0.77
Risk group stratification Non high risk High risk	 18 6	 93 44	0.63

Table 1. Clinical characteristics of patients with and without osteonecrosis. *Abbreviations: ON, osteonecrosis; ON+, patients with ON; ON-, patients without ON; N.A., not applicable. P-value: Mann Whitney U test/ Chi-square test/ Fisher exact test.*

Coagulation Parameters at Diagnosis

Values of the coagulation parameters at diagnosis are summarized in table 2. At diagnosis no significant differences in any of the coagulation parameters between ON-negative patients and ON-positive patients were observed. Values of the anticoagulant factors AT, protein C activity and total protein S were all within normal ranges for both groups of ALL patients (Figure 2).

COAGULATION VARIABLE	TOTAL (N=)	ON-POSITIVE (N=)	ON-NEGATIVE (N=)	ON-POSITIVE MEDIAN (RANGE)	ON-NEGATIVE MEDIAN (RANGE)	P-VALUE
PT (sec)	128	21	107	14.0 (10.0-21.5)	13.1(10.0-29.0)	0.71
APTT (sec)	159	23	136	34 (19-48)	34 (20-56)	0.77
Fibrinogen (g/L)	161	24	137	3.2 (1.7-4.7)	3.0 (0.8-7.8)	0.69
AT (IU/mL)	97	13	84	0.93 (0.51-1.20)	0.94 (0.47-1.44)	0.41
ProtCact (IU/mL)	68	9	59	0.68 (0.53-1.09)	0.72 (0.37-1.29)	0.66
ProtSfree (IU/mL)	29	3	26	0.51 (0.29-0.64)	0.58 (0.21-1.07)	0.45
ProtStotal (IU/mL)	40	7	33	0.66 (0.56-1.08)	0.74 (0.33-1.49)	0.46
Plasminogen (IU/mL)	39	6	33	0.86 (0.76-1.29)	1.03 (0.43-1.49)	0.52
a2AP (IU/mL)	39	6	33	1.04 (0.92-1.20)	1.10 (0.42-1.35)	0.21
D-dim (mg/L)	72	13	59	0.33 (0.10-3.21)	0.40 (0.03-4.77)	0.11
Factor II (IU/mL)	39	6	33	0.52 (0.49-1.33)	0.70 (0.24-1.12)	0.53
Factor V (IU/mL)	41	6	35	1.15 (0.38-1.61)	0.86 (0.31-1.89)	0.41
Factor VII (IU/mL)	39	6	33	0.74 (0.30-0.80)	0.65 (0.33-0.92)	0.45
Factor IX (IU/mL)	39	6	33	1.23 (0.91-1.87)	1.16 (0.61-2.08)	0.47
Factor X (IU/mL)	39	6	33	0.77 (0.57-0.94)	1.05 (0.14-1.62)	0.07
TAT (ug/L)	39	6	33	9.3 (4.0-56.7)	15.1 (3.6-60.0)	0.18
F1+2 (pmol/L)	39	6	33	549 (104-1500)	576 (145-1500)	0.78
PAP (ug/L)	39	6	33	838 (288-1247)	634 (317-2318)	0.63

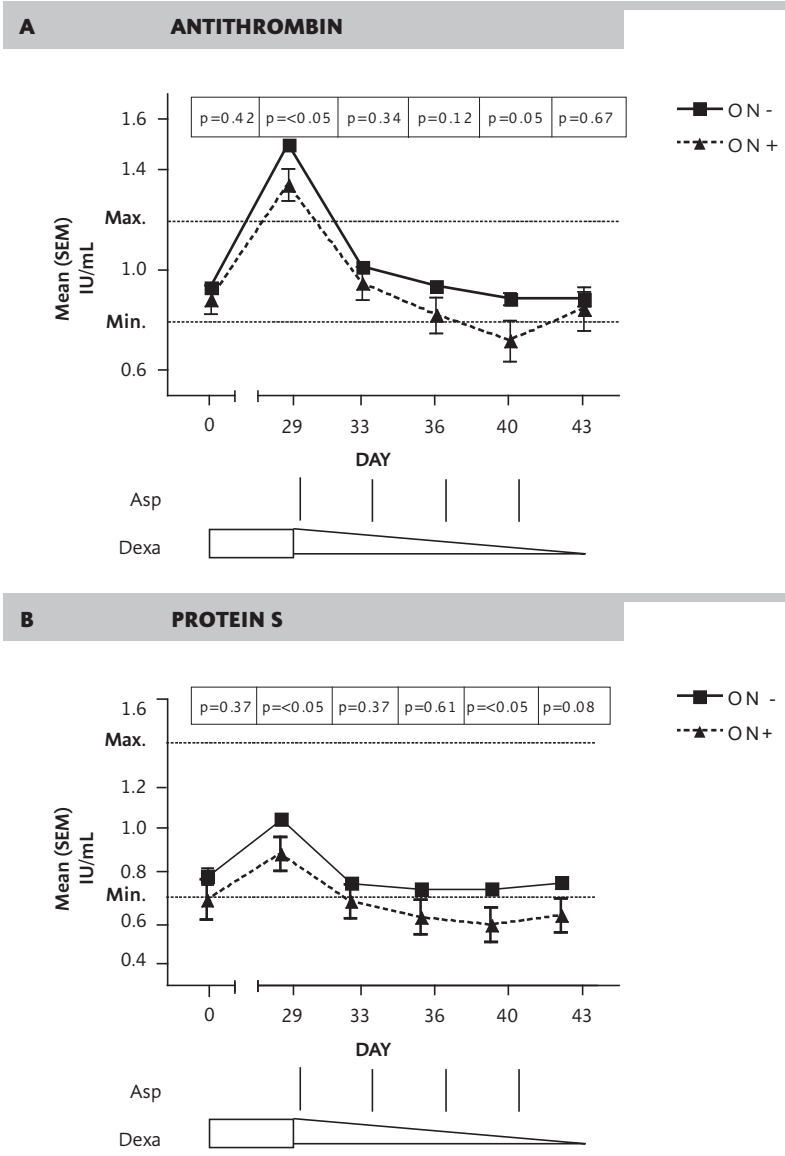

Figure 2. Antithrombin (2A.) and protein S (2B.) at diagnosis and during induction therapy in pediatric patients who did and did not develop osteonecrosis. *Abbreviations: ON+, patients with osteonecrosis; ON-, patients without osteonecrosis; Asp, asparaginase; Dexa, dexamethasone; SEM, standard error of the mean. Normal reference ranges are marked with dotted lines; AT: 0.8-1.2 IU/mL and protein S: 0.7-1.4 IU/mL. P-values indicate differences between ON-positive and ON-negative patients at each moment (mixed model analysis of repeated measures).*

Table 2. Coagulation parameters at diagnosis of pediatric patients with acute lymphoblastic leukemia who did and did not develop osteonecrosis. *Abbreviation: ON, osteonecrosis. P-values: Mann Whitney U test.*

Coagulation Parameters during Induction Treatment

Results of all coagulation parameters measured during DCOG-ALL9 induction therapy after 4 weeks treatment with dexamethasone (day 29) are shown in table 3. Mean values of the anticoagulants AT and total protein S after 4 weeks of induction treatment with dexamethasone at day 29 and during tapering of dexamethasone and administration of 4 doses asparaginase (day 33-43) are depicted in figure 2. Values of AT and total protein S, but not of protein C were significantly lower in ON-positive patients as compared to ON-negative patients after 4 weeks of dexamethasone administration (P<0.05 and P<0.05). Mixed model analysis of repeated measures showed a significant decrease in ON-positive patients of AT (P<0.001) and total protein S (P<0.001) during day 29-43 and also in ON-negative patients there was a significant decrease of AT (P<0.001) and protein S (P<0.001) from day 29-43 (Figure 2). This decrease of AT and total protein S during tapering of dexamethasone and administration of four doses asparaginase was equal in both ON-positive and negative patients, which resulted in a decline below the lower limit of the normal range of AT and protein S in ON-positive patients but not in ON-negative patients.

Factor X was different between ON-positive and ON-negative patients at day 29, but the absolute levels of factor X stayed within the normal range in both groups at all time points. Dexamethasone or asparaginase induced differences in any of the other coagulation parameters between ON-negative and ON-positive patients were not found at any other time point during induction treatment.

COAGULATION PARAMETER	ON-POSITIVE	ON-NEGATIVE	P-VALUE
Fibrinogen (g/L)	0.9 (0.1-2.1)	1.3 (0.1-3.9)	0.11
Plasminogen (IU/mL)	1.04 (0.67-1.61)	1.21 (0.80-2.34)	0.08
a2AP (IU/mL)	1.27 (0.97-1.65)	1.41 (0.95-2.82)	0.06
D-dimers (mg/L)	0.16 (0.07-0.47)	0.11 (0.02-0.64)	0.20
Factor II (IU/mL)	1.21 (0.47-1.39)	1.31 (0.12-2.07)	0.13
Factor V (IU/mL)	1.39 (0.71-1.84)	1.35 (0.81-2.40)	0.67
Factor VII (IU/mL)	0.97 (0.33-1.53)	1.12 (0.54-1.98)	0.16
Factor IX (IU/mL)	1.62 (1.03-2.01)	1.79 (0.41-2.66)	0.41
Factor X (IU/mL)	1.22 (0.67-1.66)	1.56 (0.62-2.52)	<0.05
TAT (ug/L)	6.0 (2.6-22,0)	4.5 (1.6-414.0)	0.57
F1+2 (nmol/L)	1.52 (0.99-5.36)	1.08 (0.49-20.54)	0.07
PAP (ug/L)	306 (3-1403)	251 (51-1023)	0.78
AT (IU/mL)	1.29 (0.96-1.68)	1.47 (1.03-2.02)	<0.05
ProtCact (IU/mL)	1.56 (0.94-2.96)	1.84 (0.84-3.29)	0.09
ProtSfree (IU/mL)	0.89 (0.63-1.50)	1.00 (0.11-1.54)	0.17
ProtStotal (IU/mL)	0.81 (0.64-1.53)	1.03 (0.28-1.53)	<0.05

Table 3. Coagulation parameters measured after 4 weeks induction therapy with dexamethasone in pediatric patients with acute lymphoblastic leukemia who did and did not develop osteonecrosis. *Abbreviation: ON, osteonecrosis. P-values: Mann Whitney U test.*

DISCUSSION

Osteonecrosis is a disabling complication which may occur during treatment of childhood ALL. Although previous studies have shown that older pediatric patients have a higher risk of ON, the pathophysiology is not entirely understood. In general, it has been suggested that a deviation of the coagulation system is one of the predisposing factors for ON, but until now only a few studies have been performed on coagulation dysregulation in the pathogenesis of ON in childhood patients with ALL[15-19]. In 1989 Hanada et al. suggested that L-asparaginase induced coagulopathy in a child with ALL caused osteonecrosis[6]. Kechli et al. investigated the prevalence of hereditary prothrombotic risk factors like *factor V Leiden,* the *prothrombin* 20210G>A polymorphism and the *methylene tetrahydrofolate reductase (MTHFR)* 677C>T variant in a group of 24 children who developed ON during treatment for various forms of cancer including 16 cases of ALL[4]. They did not identify an increased prevalence of these hypercoagulable state mutations. In a previous study we showed that the number of kringle IV repeats in the *Apo(a)* gene (LPA) and lipoprotein (a) levels did not contribute to an increased risk of symptomatic ON in childhood ALL patients[5]. The present study is the first study investigating the influence of coagulation disturbance during induction treatment, as measured by procoagulant factors, anticoagulant factors, parameters of thrombin generation and of fibrinolysis, on the development of ON in a single center cohort of children with ALL, treated according to one treatment protocol.

Our results showed no differences in coagulation parameters at diagnosis between the ALL patients who developed ON and those who did not. This suggests that there are no important pre-existent patient-specific or leukemia-related coagulation aberrations that play a role in the pathogenesis of ON in pediatric ALL.

We suggest that asparaginase plays an important role in the development of ON in ALL patients, illustrated by the significant decrease of antithrombin and protein S in all patients after the introduction of this drug. However, our results implicate that the preceding administration of dexamethasone might play an even more important and discriminative role for developing ON. ON-positive ALL patients showed a less impressive and significant different rise of antithrombin and protein S after administration of dexamethasone, which resulted in a decrease of AT and protein S levels below the lower limit of normal in the ON-positive patients only after asparaginase administration. This may indicate a therapy-induced hypercoagulable state in this subgroup of ALL patients, contributing to the development of ON. The tendency towards hypercoagulability of ON-positive patients may be emphasized by the fact that ON-positive patients experienced more thrombotic events during the remaining part of the treatment, which included a two-weeks period of dexamethasone every 7 weeks during maintenance therapy.

Previous studies showed that ON is a serious complication of childhood ALL treatment, especially in the older patients[31-36]. The explanation for this higher incidence of ON in children above 10 years of age has not been unravelled as yet. The majority of the ON patients in our study were above the age of 10 years (79%). Although in the total group of patients high levels of the anticoagulants AT and protein S after 4 weeks of dexamethasone treatment were found, subgroup analyses showed a significant difference in activation of these anticoagulants in children older than 10 years of age as compared to the younger ALL patients[37]. Athale et al. found older age and high risk disease to be factors predisposing to thrombo-embolism in children with ALL[38]. They reported that the dose of steroids combined with asparaginase

during the intensification phase of therapy, probably also contributes to the development of thrombo-embolism. As our group of ON-positive patients was significantly older as compared to our group of ON-negative ALL patients, we suggest that age related differences in dexamethasone-induced changes in the coagulation system may contribute to this higher incidence of ON at older age. Considering the limitations of our retrospective study design, future prospective studies are necessary to validate the contribution of these age related coagulation aberrations in the development of ON.

In conclusion, the present study indicates that impairment of dexamethasone related increase of AT and protein S and subsequent decline of these anticoagulants below normal levels after introduction of asparaginase, may result in a hypercoagulable state. This impaired activation after dexamethasone in childhood ALL patients may be an important contributing factor to the development of ON, especially in teenagers.

REFERENCES

1. Glimcher MJ, Kenzora JE. The biology of osteonecrosis of the human femoral head and its clinical implications: II. The pathological changes in the femoral head as an organ and in the hip joint. Clin Orthop Relat Res 1979(139):283-312.
2. Liu SL, Ho TC. The role of venous hypertension in the pathogenesis of Legg-Perthes disease. A clinical and experimental study. J Bone Joint Surg Am 1991;73(2):194-200.
3. Jones JP, Jr. Intravascular coagulation and osteonecrosis. Clin Orthop Relat Res 1992 (277):41-53.
4. Kechli AM, Wilimas JA, Pui CH, Park VM, Tonkel S, Deitcher SR. Factor V Leiden and other hypercoagulable state mutations are not associated with osteonecrosis during or after treatment for pediatric malignancy. J Pediatr 1999;134(3):310-4.
5. van Beek RD, Bezemer DD, Meijerink JP, de Muinck Keizer-Schrama SM, Haas OA, Te Winkel L, et al. 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. Leukemia 2006;20(5):879-80.
6. Hanada T, Horigome Y, Inudoh M, Takita H. Osteonecrosis of vertebrae in a child with acute lymphocytic leukaemia during L-asparaginase therapy. Eur J Pediatr 1989;149(3):162-3.
7. Athale UH, Chan AK. Thromboembolic complications in pediatric hematologic malignancies. Semin Thromb Hemost 2007; 33(4):416-26.
8. Appel IM, Hop WC, Pieters R. Changes in hypercoagulability by asparaginase: a randomized study between two asparaginases. Blood Coagul Fibrinolysis 2006;17(2):139-46.
9. Athale UH, Chan AK. Thrombosis in children with acute lymphoblastic leukemia. Part II. Pathogenesis of thrombosis in children with acute lymphoblastic leukemia: effects of the disease and therapy. Thromb Res 2003;111 (4-5):199-212.
10. Caruso V, Iacoviello L, Di Castelnuovo A, Storti S, Mariani G, de Gaetano G, et al. Thrombotic complications in childhood acute lymphoblastic leukemia: a meta-analysis of 17 prospective studies comprising 1752 pediatric patients. Blood 2006;108(7):2216-22.
11. Mauz-Korholz C, Junker R, Gobel U, Nowak-Gottl U. Prothrombotic risk factors in children with acute lymphoblastic leukemia treated with delayed E. coli asparaginase (COALL-92 and 97 protocols). Thromb Haemost 2000;83 (6):840-3.
12. Mitchell LG, Halton JM, Vegh PA, Barr RD, Venneri T, Pai KM, et al. Effect of disease and chemotherapy on hemostasis in children with acute lymphoid leukemia. Am J Pediatr Hematol Oncol 1994;16(2):120-6.
13. Nowak-Gottl U, Ahlke E, Fleischhack G, Schwabe D, Schobess R, Schumann C, et al. Thromboembolic events in children with acute lymphoblastic leukemia (BFM protocols): prednisone versus dexamethasone administration. Blood 2003;101(7):2529-33.
14. Ruud E, Holmstrom H, de Lange C, Natvig S, Albertsen BK, Wesenberg F. Thrombotic effects of asparaginase in two acute lymphoblastic leukemia protocols (NOPHO ALL-1992 versus NOPHO ALL-2000): a single-institution study. Pediatr Hematol Oncol 2006;23(3):207-16.
15. Van Veldhuizen PJ, Neff J, Murphey MD, Bodensteiner D, Skikne BS. Decreased fibrinolytic potential in patients with idiopathic avascular necrosis and transient osteoporosis

of the hip. Am J Hematol 1993;44(4):243-8.
16. Glueck CJ, Freiberg R, Tracy T, Stroop D, Wang P. Thrombophilia and hypofibrinolysis: pathophysiologies of osteonecrosis. Clin Orthop Relat Res 1997(334):43-56.
17. Szepesi K, Posan E, Harsfalvi J, Ajzner E, Szucs G, Gaspar L, et al. The most severe forms of Perthes' disease associated with the homozygous Factor V Leiden mutation. J Bone Joint Surg Br 2004;86(3):426-9.
18. Balasa VV, Gruppo RA, Glueck CJ, Wang P, Roy DR, Wall EJ, et al. Legg-Calve-Perthes disease and thrombophilia. J Bone Joint Surg Am 2004;86-A(12):2642-7.
19. Glueck CJ, Fontaine RN, Gruppo R, Stroop D, Sieve-Smith L, Tracy T, et al. The plasminogen activator inhibitor-1 gene, hypofibrinolysis, and osteonecrosis. Clin Orthop Relat Res 1999(366):133-46.
20. Veerman AJ, Hahlen K, Kamps WA, Van Leeuwen EF, De Vaan GA, Solbu G, et al. High cure rate with a moderately intensive treatment regimen in non-high-risk childhood acute lymphoblastic leukemia. Results of protocol ALL VI from the Dutch Childhood Leukemia Study Group. J Clin Oncol 1996;14(3):911-8.
21. van der Sluis IM, van den Heuvel-Eibrink MM, Hahlen K, Krenning EP, de Muinck Keizer-Schrama SM. Altered bone mineral density and body composition, and increased fracture risk in childhood acute lymphoblastic leukemia. J Pediatr 2002;141(2):204-10.
22. Robinson HJ, Jr., Hartleben PD, Lund G, Schreiman J. Evaluation of magnetic resonance imaging in the diagnosis of osteonecrosis of the femoral head. Accuracy compared with radiographs, core biopsy, and intraosseous pressure measurements. J Bone Joint Surg Am 1989;71(5):650-63.
23. Saini A, Saifuddin A. MRI of osteonecrosis. Clin Radiol 2004;59(12):1079-93.
24. Mitchell DG, Rao VM, Dalinka MK, Spritzer CE, Alavi A, Steinberg ME, et al. Femoral head avascular necrosis: correlation of MR imaging, radiographic staging, radionuclide imaging, and clinical findings. Radiology 1987;162(3): 709-15.
25. Clauss A. [Rapid physiological coagulation method in determination of fibrinogen.]. Acta Haematol 1957;17(4):237-46.
26. Holvoet P, de Boer A, Verstreken M, Collen D. An enzyme-linked immunosorbent assay (ELISA) for the measurement of plasmin-alpha 2-antiplasmin complex in human plasma--application to the detection of in vivo activation of the fibrinolytic system. Thromb Haemost 1986;56(2):124-7.
27. Pelzer H, Schwarz A, Stuber W. Determination of human prothrombin activation fragment 1 + 2 in plasma with an antibody against a synthetic peptide. Thromb Haemost 1991;65(2):153-9.
28. Pelzer H, Schwarz A, Heimburger N. Determination of human thrombin-antithrombin III complex in plasma with an enzyme-linked immunosorbent assay. Thromb Haemost 1988;59(1):101-6.
29. Ries M, Klinge J, Rauch R. Age-related reference values for activation markers of the coagulation and fibrinolytic systems in children. Thromb Res 1997;85(4):341-4.
30. Littell RC, Henry PR, Ammerman CB. Statistical analysis of repeated measures data using SAS procedures. J Anim Sci 1998;76(4):1216-31.
31. Burger B, Beier R, Zimmermann M, Beck JD, Reiter A, Schrappe M. Osteonecrosis: a treatment related toxicity in childhood acute lymphoblastic leukemia (ALL)--experiences from trial ALL-BFM 95. Pediatr Blood Cancer

2005;44(3):220-5.
32. Niinimaki RA, Harila-Saari AH, Jartti AE, Seuri RM, Riikonen PV, Paakko EL, et al. High body mass index increases the risk for osteonecrosis in children with acute lymphoblastic leukemia. J Clin Oncol 2007;25(12):1498-504.
33. Mattano LA, Jr., Sather HN, Trigg ME, Nachman JB. Osteonecrosis as a complication of treating acute lymphoblastic leukemia in children: a report from the Children's Cancer Group. J Clin Oncol 2000;18(18):3262-72.
34. Ribeiro RC, Fletcher BD, Kennedy W, Harrison PL, Neel MD, Kaste SC, et al. Magnetic resonance imaging detection of avascular necrosis of the bone in children receiving intensive prednisone therapy for acute lymphoblastic leukemia or non-Hodgkin lymphoma. Leukemia 2001;15(6):891-7.
35. Arico M, Boccalatte MF, Silvestri D, Barisone E, Messina C, Chiesa R, et al. Osteonecrosis: An emerging complication of intensive chemotherapy for childhood acute lymphoblastic leukemia. Haematologica 2003; 88(7):747-53.
36. Nachman JB. Adolescents with acute lymphoblastic leukemia: a new "age". Rev Clin Exp Hematol 2003;7(3):261-9.
37. Appel IM, van Kessel-Bakvis C, Stigter R, Pieters R. Influence of two different regimens of concomitant treatment with asparaginase and dexamethasone on hemostasis in childhood acute lymphoblastic leukemia. Leukemia 2007;21(11):2377-80.
38. Athale UH, Siciliano SA, Crowther M, Barr RD, Chan AK. Thromboembolism in children with acute lymphoblastic leukaemia treated on Dana-Farber Cancer Institute protocols: effect of age and risk stratification of disease. Br J Haematol 2005;129(6):803-10.

MANAGEMENT AND TREATMENT OF OSTEONECROSIS IN CHILDREN AND ADOLESCENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA: A NARRATIVE REVIEW

M.L. te Winkel, R. Pieters, E.D. Wind, J.H.J.M. Bessems, M.M. van den Heuvel-Eibrink

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GENERAL DISCUSSION AND PERSPECTIVES

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- Dilemma's regarding vertebral fractures in children with acute lymphoblastic leukemia (ALL). Translational Pediatrics 2013; 2(1):40-42

GENERAL DISCUSSION AND PERSPECTIVES

In this thesis, we aimed to reveal the incidence of osteogenic side effects during and after treatment of pediatric acute lymphoblastic leukemia (ALL). We describe risk factors influencing impairment of bone mineral density and osteonecrosis and evaluate potential approaches to diminish these skeletal side effects. In this chapter, we discuss the main findings in the context of current literature, and their implications for future research.

Part I Bone mineral density (BMD) and fracture risk

We performed the first multi-center prospective study that has systematically determined fracture rates and BMD (chapter 2)[1]. A three-years cumulative incidence of fractures of 17.8%, during and just after pediatric ALL treatment was found. This is markedly increased compared to healthy, similarly aged, children, in whom fracture rates are 1-2%[2,3]. Thereby, these results confirm increased fracture rates, previously reported in two smaller retrospective study cohorts[4,5]. Therefore, intervention studies in order to find strategies to reduce the risk for fractures during pediatric ALL treatment are needed.

We showed that BMD of the lumbar spine in pediatric ALL patients was significantly lower than in healthy peers. Although, after discontinuation of treatment, BMD of the lumbar spine recovered, one year after cessation of treatment, BMD was still significantly lower than that of healthy children. Patients who developed fractures had a lower BMD than those without fractures, but treatment-related bone loss was similar in patients with and without fractures. This suggests that low BMD values at diagnosis rather than the treatment-related decline of BMD determines the risk for fractures in pediatric ALL patients. This is in agreement with findings in healthy adolescents and young adults in which BMD is a good predictor for future fractures[6]. Therefore, when considering screening for osteoporosis in clinical practice, we feel that BMD should be determined at diagnosis. However, the value of BMD screening remains debatable as preventative and therapeutic options are yet scarce. In the view of a cost-benefit analysis, screening should probably be restricted to those ALL patients who are most vulnerable to osteoporosis. In order to identify factors predicting the vulnerability to osteoporosis, we studied patient specific, pharmacogenetic and environmental risk factors.

The variation in baseline BMD, may stress the importance of genetic variation as a determinant of bone density in children with ALL. Twin and family studies have shown that bone mass is heavily determined by hereditary factors. So far, knowledge on the role of genetic variation in bone density was mainly obtained from large-scale studies in international consortia in postmenopausal women, in which by now >80 SNPs have been found to be associated with BMD[7]. Only scarce information from small individual studies was available on whether similar genetic variations are related with BMD development during corticosteroid treatment, for example during and after childhood ALL treatment.

We showed that haplotype 3 of the *vitamin-D receptor (VDR)* 5′-end, constructed of the SNPs *Cdx-2* and *GATA*, was a risk factor for a lower lumbar spine BMD during treatment (Chapter 3)[8]. The *collagen type I alpha 1* gene, the *estrogen receptor 1* gene and the *glucocorticoid receptor* gene were not related to BMD nor to fracture risk. Since children with ALL receive high dosages of the antifolate methotrexate, that increases plasma homocysteine levels that may disturb collagen crosslinking, we hypothesized that

the SNPs in the *methylenetetrahydrofolate reductase (MTHFR)* gene and the *methionine synthase reductase (MTRR)* gene influences BMD and fracture risk in pediatric ALL patients (Chapter 4)[9]. Carriage of the *MTHFR* 677 T-allele and the *MTRR* 66 G-allele indeed predicted a lower BMD of the total body. Both the *VDR* gene and the folate metabolism-related SNPs determined the low absolute value of BMD at diagnosis and during ALL treatment, rather than the treatment-induced decline of BMD.

For the future, a prediction model for the vulnerability of fractures during and after pediatric ALL treatment, in which both the aforementioned genetic factors and patient characteristics or environmental components are integrated, would be of value. Before such a prediction model can be designed, confirmation of our results in larger, independent cohorts are necessary. In addition, other pharmacogenetic factors may be of value in such a prediction model. Jones et al. showed that the *corticotrophin-releasing hormone receptor-1 (CRHR1)* polymorphisms have impact on the risk of BMD deficits in pediatric ALL patients in a gender-specific manner[10]. Genome-wide association studies (GWAS) have become a promising approach to further unravel novel pharmacogenetic loci that may affect BMD in pediatric ALL patients. To discover genetic variations that influence response to treatment in ALL patients, a sufficiently powered GWAS has to be performed including replication studies. International consortia of collaborating investigators will be required to make this happen. A GWAS in healthy children (GEFOS Kids project) recently identified the *PPP6R3/LRP5* locus which showed a pleiotropic effect on the peak bone mass[11]. Replication in additional independent cohort of children is currently underway. Moreover, a recently published study investigated whether SNPs that were reproducibly identified with GWAS to be associated with bone density in adult also exerted their effects in childhood. They found the *LACTB2, GPATCH1, DHH, WLS, IDUA, LRP5, SPTBN1* and *STARD3NL* were indeed associated with BMD of the lumbar spine[12]. These findings also need further replication in future. Whether these SNPs that seem to be associated with BMD in healthy children also are important for the treatment-related effects on BMD in pediatric ALL patients, remains yet unknown.

We showed that patient-related or disease-specific characteristics such as age, weight and the immunophenotype of the leukemia are relevant variables that predict osteopenia in childhood ALL. Consequently, those characteristics could be integrated into such a model (Chapter 2). Patients with a precursor-B ALL had a lower BMD at diagnosis than those with a T-ALL. This supports the idea that in the early onset phase of ALL, T-cell lymphoblasts and B-precursor lymphoblasts differently interact with the osteoblasts-osteoclasts homeostasis, congruent with the way healthy B-cells and T-cells both, but authentically, regulate basal bone homeostasis as has been shown in in vitro studies and in vivo mice studies[13,14]. Alternatively, the more rapid development of T-cell ALL compared to B-precursor ALL, and therefore shorter time to affect the bone, may play a role. Finally, the difference in BMD at diagnosis between B-precursor ALL and T-cell ALL may in part reflect the respectively intramedullary or extramedullary original site of leukemognesis.

Several animal studies showed that mechanical strain is a crucial factor in the stimulation of bone acquisition[15,16]. In addition, studies in postmenopausal women[17] and in healthy children[18-22] have shown that exercise (including short-burst high-intensity activities) is an effective way to increase BMD. Therefore, we hypothesized that an exercise program with short-burst high-intensity activities impeded

the reduction in BMD in childhood ALL. However, our randomized control trial that was designed to prove this hypothesis, showed no beneficial effects of an intensive exercise program. This might have been due to non-compliance of such long-term required physical activity during the treatment of ALL (Chapter 5)[23]. As there was no effect on BMD, such exercises did not decrease fracture rates. The optimal duration and intensity of such regular exercises to prevent detrimental reduction in BMD are not known. One might think that a shorter duration of the exercise program and a more intensive guidance by physiotherapists in the primary health care setting, might lead to a more successful intervention.

One of the promising new modalities for future research might be the use of whole-body vibration as a therapeutic approach to increase BMD[24]. Probably, it is most efficient to allocate these physical exercise interventions especially for those patients who are identified to be at risk with the earlier mentioned risk profiles, which includes genetic variation, ALL immunophenotype and decreased BMD at baseline. For the near future, the use of bone modifying agents such as bisphosphonates or RANK-ligand targeted monoclonal antibodies (e.g. Denosumab)[25] may be considered in pediatric ALL patients with a high-risk profile for impaired BMD and high fracture risk. Until now, safety and efficacy studies using these agents in children treated for ALL are not available.

With rising numbers of childhood cancer survivors and increasing ages of survivors, future research projects on osteoporosis in pediatric ALL patients need to consider whether adequate values of peak bone mass will be reached and whether these are maintained during adulthood. In addition, the effects of genetic and environmental risk factors on long-term BMD development in survivors of pediatric ALL, especially in those who are reaching menopause, should be carefully monitored, and screening guidelines for adult survivors at risk for severe osteopenia and subsequent invalidity by increased fracture rates, need to be designed for global application. For that reason, we will address this issue in a large nation-wide study, which is called the Dutch Childhood Oncology Group (DCOG) - LATER Q2008, which will start in 2013 in approximately 7000 long-term survivors of childhood cancer. In the aforementioned large national study, it will be a challenge to analyze BMD measurements after treatment with a wide variety of multi-agent therapy protocols with or without radiotherapy and calibration of the different Dual Energy X-ray Absorptiometry (DXA) machines is warranted[26,27].

PART II OSTEONECROSIS

We found that six percent of all pediatric patients with ALL developed symptomatic osteonecrosis during or shortly after antileukemic treatment (Chapter 6)[28]. Previous reported incidences of osteonecrosis varied widely, depending on the definition of osteonecrosis, selection criteria of the study population, and type, stratification and timing of antileukemic treatment. Many published studies were performed retrospectively, probably resulting in an underestimation of the true incidence of symptomatic osteonecrosis.

Similar to previous studies, we showed that older, and especially pubertal children with ALL are prone to develop symptomatic osteonecrosis. This may result from higher circulating sex-hormone levels and closure of the growth plate, resulting in less buffering capacity of increased intra osseous pressures[29,30]. We identified a novel risk factor of osteonecrosis i.e. the therapy-induced hypercoagulable state, which can result from the tendency towards a lower dexamethasone-related increase of antithrombin and

protein S and subsequent decline of these anticoagulants after the introduction of asparaginase (chapter 7)[31]. Age-related differences in dexamethasone-induced changes in the coagulation system during ALL treatment may reflect the higher incidence of osteonecrosis at older age[32]. Also genetic factors may influence this hypercoagulable state during pediatric ALL treatment, such as the *plasminogen activator inhibitor-1 (PAI-1)* SNP that was previously described[33].

Corticosteroids play a pivotal role in treatment of ALL, and they are notorious for skeletal toxicity. We did not find evidence that dexamethasone is more toxic to the skeleton than prednisone in childhood cancer patients[34], although this has often been suggested[35]. In our dexamethasone-based DCOG-ALL9 study, we did not find a higher incidence of osteonecrosis than in previously reported prednisone-based protocols. Reduction of the cumulative dose of steroids in order to avoid osteonecrosis may hamper the antileukemic efficacy. Therapy reduction decisions should therefore always be taken with great caution. Interestingly, there is evidence that timing of corticosteroids may be even more relevant than the cumulative dose and that reduction of the risk of treatment-related osteonecrosis is feasible by discontinuous, instead of continuous, administration of corticosteroids[36,37]. The exact pathophysiology for the lower osteonecrosis rates with this intermittent administration of corticosteroids remains unravelled. Efficacy of dexamethasone pulses during maintenance on leukemia outcome, has been debated extensively[38-40]. Whether early corticosteroid interruption during induction might further decrease the incidence of osteonecrosis in view of the reported effect of dexamethasone-triggered asparaginase hypercoagulability on the occurrence of osteonecrosis[31], has not been studied.

As shown by our nationwide prospective study, female gender is another important risk factor for the development of avascular bone necrosis in ALL. This female predominance has been suggested in most but not all previous retrospective reports[4,30,41-43]. The higher risk of osteonecrosis in girls than in boys may be explained by the earlier age of puberty development, with subsequent earlier changes in hormone levels and earlier closure of the growth plate. In addition different changes in lipid metabolism might contribute to the female predominance. A study in rabbits showed that treatment with lipid lowering medication prevented corticosteroid induced osteonecrosis[44]. Studies on the role of lipid levels in the development of osteonecrosis during pediatric ALL treatment show conflicting results[45,46]. The *apolipoprotein (a)* gene (LPA) related genetic variation does not seem to play a role[45].

Management and treatment of osteonecrosis in pediatric ALL patients is a real challenge and current available literature provides insufficient evidence to strongly advise any treatment options to reduce the morbidity of symptomatic osteonecrosis (Chapter 8)[35]. However, prevention of osteonecrosis is feasible as the risk of treatment-related osteonecrosis can be reduced by discontinuous, instead of continuous, corticosteroid administration during the intensification phase of treatment.

In ALL patients with osteonecrosis, despite clear evidence, weight-bearing restrictions and adequate pain management are advised to avoid damage to the joints. Lesion size (measured on MRI), seems to be the best predictor for clinical joint outcome[47]. Lesions occupying more than 30% of the femoral head have a high likelihood of joint deterioration or collapse, and therefore non-surgical treatment options are considered by physicians. As no safety and efficacy studies on these non-surgical treatment options, such as bisphosphonates, are available, these bone remodeling agents should only be administered as part of

a clinical trial. Surgical interventions during or shortly after ALL treatment are discouraged, because of the self-limiting course of osteonecrosis in approximately 40% of pediatric ALL patients[28]. In children, only in case of severe deterioration or collapse of a joint, surgical treatment may be required, where joint preserving methods are preferred to partial/ total joint replacements.

Although an extensive search was done to answer the question how to treat childhood cancer patients with osteonecrosis, the question cannot be answered as studies with good quality are lacking. To develop a clinical practice guideline, future high quality research on efficacy and safety of interventions for osteonecrosis in childhood ALL is necessary. Therefore, we encourage research on clinical and genetic risk factors, and the influence of different antileukemic therapy schedules to determine the risk profile of osteonecrosis in ALL and guide preventative and intervention strategies. Also randomized studies on the prophylactic use of for instance bisphosphonates, to those patients who are most vulnerable to develop osteonecrosis, are useful.

Conclusions

We showed that at least one out of five patients suffer from osteogenic toxicity during or shortly after the treatment of childhood ALL. Therefore, awareness of these osteogenic complications and prevention of long-term sequelae are important. The results of this thesis have led to recommendations on screening and treatment of osteogenic toxicity for patients treated according to the national DCOG-ALL11 protocol (Figure 1)[48]. Future research needs to be directed towards the design of prediction models for osteogenic toxicity during and after pediatric ALL treatment, by combining both genetic factors and environmental components. In this way, preventive and therapeutic strategies for those patients that are most susceptible to osteogenic toxicity can be implemented.

Summary: recommendations regarding bone mineral density in pediatric ALL

- Routine DXA measurements are not indicated
- In case of a clinical significant fracture history (vertebral compression fractures, fractures of long bones in the lower extremities, fractures without preceding trauma or 2 fractures), DXA is the preferable method to diagnose osteoporosis
- Guarantee adequate calcium en vitamin D intake
- No antileukemic therapy adjustment are advised (adjustments always require consultation of the chairman of the protocol-committee)
- Therapy:
  - use of bisphosphonates may be considered in case of clinical relevant osteopenia (BMD <2 SDS and clinical significant fractures), but it is not "evidence based practise"

Summary: recommendations regarding osteonecrosis

- Routine radiological screening is not indicated
- In case of symptoms of osteonecrosis, MRI is the standard method for radiological confirmation
- Antileukemic therapy adjustments always requires consultation of the chairman of the protocol-committee
- Therapy:
  - minimize weight-bearing activities
  - adequate pain management
  - interventions with bisphosphonates, lipid lowering agents and anticoagulants are not encouraged as no safety nor efficacy studies are available
- Surgical intervention during treatment is not advised. After treatment, it is only indicated in case of severe deterioration or collapse of the joint, as symptoms of osteonecrosis are reversible in an important part of the patients

Figure 1. Adjusted DCOG-ALL11 guideline for screening and treatment of osteogenic side effects (translated from the original DCOG-ALL11 protocol) *Abbreviations: DXA = Dual energy X-ray Absorptiometry, MRI = Magnetic Resonance Imaging.*

REFERENCES

1. te Winkel ML, Pieters R, Hop WCJ, et al: Bone mineral density at diagnosis determines fracture rate in children treated according to the DCOG-ALL9 protocol. submitted, 2013
2. Landin LA: Fracture patterns in children. Analysis of 8,682 fractures with special reference to incidence, etiology and secular changes in a Swedish urban population 1950-1979. Acta Orthop Scand Suppl 202:1-109, 1983
3. Worlock P, Stower M: Fracture patterns in Nottingham children. J Pediatr Orthop 6:656-60, 1986
4. Strauss AJ, Su JT, Dalton VM, et al: Bony morbidity in children treated for acute lymphoblastic leukemia. J Clin Oncol 19:3066-72, 2001
5. Rayar MS, Nayiager T, Webber CE, et al: Predictors of bony morbidity in children with acute lymphoblastic leukemia. Pediatr Blood Cancer, 2011
6. Boot AM, de Ridder MA, van der Sluis IM, et al: Peak bone mineral density, lean body mass and fractures. Bone 46:336-41, 2010
7. Ralston SH, Uitterlinden AG: Genetics of osteoporosis. Endocr Rev 31:629-62, 2010
8. te Winkel ML, van Beek RD, de Muinck Keizer-Schrama SM, et al: Pharmacogenetic risk factors for altered bone mineral density and body composition in pediatric acute lymphoblastic leukemia. Haematologica 95:752-9, 2010
9. te Winkel ML, de Muinck Keizer-Schrama SM, de Jonge R, et al: Germline variation in the MTHFR and MTRR genes determines the nadir of bone density in pediatric acute lymphoblastic leukemia: a prospective study. Bone 48:571-7, 2011
10. Jones TS, Kaste SC, Liu W, et al: CRHR1 polymorphisms predict bone density in survivors of acute lymphoblastic leukemia. J Clin Oncol 26:3031-7, 2008
11. Medina-Gomez C, Heppe D, Estrada K, et al: The PPP6R3/LRP5 locus influences lean mass in children of different ethnic background and highlights pleiotropic effect on muscle-bone interactions. International conference on children's bone health (ICCBH) abstracts, 2013
12. Zemel B, Kalkwarf H, Li M, et al: Role of bone-associated loci identified in GWAS meta-analyses in the context of longitudinal pediatric BMD in European Americans International conference on children's bone health (ICCBH) abstracts, 2013
13. Li Y, Toraldo G, Li A, et al: B cells and T cells are critical for the preservation of bone homeostasis and attainment of peak bone mass in vivo. Blood 109:3839-48, 2007
14. Pacifici R: T cells: critical bone regulators in health and disease. Bone 47:461-71, 2010
15. Rubin CT, Lanyon LE: Regulation of bone formation by applied dynamic loads. J Bone Joint Surg Am 66:397-402, 1984
16. Lanyon LE: Control of bone architecture by functional load bearing. J Bone Miner Res 7 Suppl 2:S369-75, 1992
17. Wolff I, van Croonenborg JJ, Kemper HC, et al: The effect of exercise training programs on bone mass: a meta-analysis of published controlled trials in pre- and postmenopausal women. Osteoporos Int 9:1-12, 1999
18. Jones G, Dwyer T: Bone mass in prepubertal children: gender differences and the role of physical activity and sunlight exposure. J Clin Endocrinol Metab 83:4274-9, 1998
19. Lehtonen-Veromaa M, Mottonen T, Irjala K, et al: A 1-year prospective study on the relationship between physical activity, markers of bone metabolism, and bone acquisition in

peripubertal girls. J Clin Endocrinol Metab 85:3726-32, 2000
20. Fuchs RK, Bauer JJ, Snow CM: Jumping improves hip and lumbar spine bone mass in prepubescent children: a randomized controlled trial. J Bone Miner Res 16:148-56, 2001
21. Kemper HC, Twisk JW, van Mechelen W, et al: A fifteen-year longitudinal study in young adults on the relation of physical activity and fitness with the development of the bone mass: The Amsterdam Growth And Health Longitudinal Study. Bone 27:847-53, 2000
22. Nilsson M, Ohlsson C, Mellstrom D, et al: Previous sport activity during childhood and adolescence is associated with increased cortical bone size in young adult men. J Bone Miner Res 24:125-33, 2009
23. Hartman A, te Winkel ML, van Beek RD, et al: A randomized trial investigating an exercise program to prevent reduction of bone mineral density and impairment of motor performance during treatment for childhood acute lymphoblastic leukemia. Pediatr Blood Cancer 53:64-71, 2009
24. Slatkovska L, Alibhai SM, Beyene J, et al: Effect of whole-body vibration on BMD: a systematic review and meta-analysis. Osteoporos Int 21:1969-80, 2010
25. Van Poznak CH, Temin S, Yee GC, et al: American Society of Clinical Oncology executive summary of the clinical practice guideline update on the role of bone-modifying agents in metastatic breast cancer. J Clin Oncol 29:1221-7, 2011
26. Dasher LG, Newton CD, Lenchik L: Dual X-ray absorptiometry in today's clinical practice. Radiol Clin North Am 48:541-60, 2010
27. Staal KP, Roos JC, Manoliu RA, et al: Variations in diagnostic performances of dual-energy X-ray absorptiometry in the northwest of The Netherlands. Osteoporos Int 15:335-44, 2004
28. te Winkel ML, Pieters R, Hop WC, et al: Prospective study on incidence, risk factors, and long-term outcome of osteonecrosis in pediatric acute lymphoblastic leukemia. J Clin Oncol 29:4143-50, 2011
29. Ruderman RJ, Poehling GG, Gray R, et al: Orthopedic complications of renal transplantation in children. Transplant Proc 11:104-6, 1979
30. Mattano LA, Jr., Sather HN, Trigg ME, et al: Osteonecrosis as a complication of treating acute lymphoblastic leukemia in children: a report from the Children's Cancer Group. J Clin Oncol 18:3262-72, 2000
31. te Winkel ML, Appel IM, Pieters R, et al: Impaired dexamethasone-related increase of anticoagulants is associated with the development of osteonecrosis in childhood acute lymphoblastic leukemia. Haematologica 93:1570-4, 2008
32. Appel IM, Hop WC, van Kessel-Bakvis C, et al: L-Asparaginase and the effect of age on coagulation and fibrinolysis in childhood acute lymphoblastic leukemia. Thromb Haemost 100:330-7, 2008
33. French D, Hamilton LH, Mattano LA, Jr., et al: A PAI-1 (SERPINE1) polymorphism predicts osteonecrosis in children with acute lymphoblastic leukemia: a report from the Children's Oncology Group. Blood 111:4496-9, 2008
34. Sala A, Mattano LA, Jr., Barr RD: Osteonecrosis in children and adolescents with cancer - an adverse effect of systemic therapy. Eur J Cancer 43:683-9, 2007
35. te Winkel ML, Pieters R, Wind ED, et al: Management and treatment of osteonecrosis in children and adolescents with acute

lymphoblastic leukemia: a narrative review. submitted, 2013
36. Mattano LA, Jr., Devidas M, Nachman JB, et al: Effect of alternate-week versus continuous dexamethasone scheduling on the risk of osteonecrosis in paediatric patients with acute lymphoblastic leukaemia: results from the CCG-1961 randomised cohort trial. Lancet Oncol, 2012
37. van den Heuvel-Eibrink MM, Pieters R: Steroids and risk of osteonecrosis in ALL: take a break. Lancet Oncol, 2012
38. Conter V, Valsecchi MG, Silvestri D, et al: Pulses of vincristine and dexamethasone in addition to intensive chemotherapy for children with intermediate-risk acute lymphoblastic leukaemia: a multicentre randomised trial. Lancet 369:123-31, 2007
39. Eden T, Pieters R, Richards S: Systematic review of the addition of vincristine plus steroid pulses in maintenance treatment for childhood acute lymphoblastic leukaemia - an individual patient data meta-analysis involving 5,659 children. Br J Haematol 149:722-33, 2010
40. De Moerloose B, Suciu S, Bertrand Y, et al: Improved outcome with pulses of vincristine and corticosteroids in continuation therapy of children with average risk acute lymphoblastic leukemia (ALL) and lymphoblastic non-Hodgkin lymphoma (NHL): report of the EORTC randomized phase 3 trial 58951. Blood 116:36-44, 2010
41. Arico M, Boccalatte MF, Silvestri D, et al: Osteonecrosis: An emerging complication of intensive chemotherapy for childhood acute lymphoblastic leukemia. Haematologica 88:747-53, 2003
42. Hogler W, Wehl G, van Staa T, et al: Incidence of skeletal complications during treatment of childhood acute lymphoblastic leukemia: comparison of fracture risk with the General Practice Research Database. Pediatr Blood Cancer 48:21-7, 2007
43. Ribeiro RC, Fletcher BD, Kennedy W, et al: Magnetic resonance imaging detection of avascular necrosis of the bone in children receiving intensive prednisone therapy for acute lymphoblastic leukemia or non-Hodgkin lymphoma. Leukemia 15:891-7, 2001
44. Motomura G, Yamamoto T, Miyanishi K, et al: Combined effects of an anticoagulant and a lipid-lowering agent on the prevention of steroid-induced osteonecrosis in rabbits. Arthritis Rheum 50:3387-91, 2004
45. van Beek RD, Bezemer DD, Meijerink JP, et al: 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. Leukemia 20:879-80, 2006
46. Kawedia JD, Kaste SC, Pei D, et al: Pharmacokinetic, pharmacodynamic, and pharmacogenetic determinants of osteonecrosis in children with acute lymphoblastic leukemia. Blood 117:2340-7, 2011
47. Karimova EJ, Rai SN, Howard SC, et al: Femoral head osteonecrosis in pediatric and young adult patients with leukemia or lymphoma. J Clin Oncol 25:1525-31, 2007
48. DCOG-ALL11 protocol. SNWLK (Stichting Nederlandse Werkgroep Leukemie bij Kinderen), 2012

SUMMARY/ SAMENVATTING

SUMMARY

This thesis provides novel information on the incidence and risk factors of osteogenic side effects during and after childhood acute lymphoblastic leukemia (ALL) treatment. In addition, potential therapeutic approaches for these skeletal side effects are evaluated.

The first part of this thesis is devoted to the studies that were performed on bone mineral density (BMD) of children with ALL. We describe a large multi-center prospective study in children treated for ALL according to the Dutch Childhood Oncology Group (DCOG)-ALL9 protocol to elucidate the incidence of BMD deficits and fractures. A three-years cumulative incidence of fractures of 18% during and just after pediatric ALL treatment was found, which was much higher than the incidence of 2% in healthy peers. The BMD in ALL patients was significantly lower than in healthy peers. Although, after discontinuation of treatment, BMD recovered, one year after cessation of treatment BMD was still significantly lower than that of healthy children. Low BMD values at diagnosis, rather than the treatment-related decline of BMD, determined the risk for fractures during and shortly after pediatric ALL treatment. These low BMD values were influenced by patient-related and disease-specific characteristics such as age, weight and the immunophenotype of the leukemia. As uniformly treated children show a large variation in BMD reduction and fractures, a role for pharmacogenetics in the pathogenesis of these skeletal problems has been suggested. We showed that single nucleotide polymorphisms of the vitamin-D receptor *(VDR)* gene and of the folate metabolism (methylenetetrahydrofolate reductase *(MTHFR)* 677C>T and methionine synthase reductase *(MTRR)* 66A>G) determined the low BMD at diagnosis and during ALL treatment, rather than the treatment-induced decline of BMD. For the future, a prediction model for the vulnerability of fractures during pediatric ALL treatment, in which both genetic factors and environmental components are integrated, would be of value. Finally, we describe the results of a randomized trial investigating an exercise program to prevent reduction of BMD during treatment for childhood ALL. This study was performed because mechanical strain was previously shown to be a crucial factor in the stimulation of bone acquisition. However, our study demonstrated no beneficial effects on BMD and fracture risk. This might have been due to non-compliance of such long-term required physical activity program during the treatment of ALL. One might think that a shorter duration of the exercise program and a more intensive guidance by physiotherapists in the primary health care setting, might lead to a more successful intervention program. Probably, it is most efficient to allocate these physical exercise interventions especially to those patients who are identified to be at risk with the aforementioned risk profiles.

In the second part of this thesis we focus on osteonecrosis. In our prospective multi-center study we found that six percent of all patients developed symptomatic osteonecrosis during or shortly after treatment for pediatric ALL (DCOG-ALL9 protocol). Older age and female gender were independent risk factors for the development of symptomatic osteonecrosis. The fact that older children are more prone to develop osteonecrosis may reflect age-related differences in dexamethasone-induced changes in the coagulation system during ALL treatment, as we showed in one of our studies. Management and treatment of osteonecrosis in pediatric ALL patients is a real challenge and current available literature provides insufficient evidence to strongly advise any treatment options to reduce the morbidity of symptomatic osteonecrosis. However, prevention of osteonecrosis is feasible, as the risk of treatment-

related osteonecrosis is reduced by discontinuous, instead of continuous, corticosteroid administration during the intensification phase of treatment. To develop a clinical practice guideline, future high quality randomized clinical trials on efficacy and safety of interventions for osteonecrosis in childhood ALL are necessary.

In conclusion, we showed that at least one out of five patients suffer from osteogenic toxicity during or shortly after the treatment of childhood ALL. Therefore, awareness of these osteogenic complications and prevention of long-term sequelae are important. The results of this thesis have led to recommendations on screening and treatment of osteogenic toxicity for patients treated according to the national DCOG-ALL11 protocol. Future research needs to be directed towards the design of prediction models for osteogenic toxicity during and after pediatric ALL treatment, by combining both genetic factors and environmental components. In this way, preventative and therapeutic strategies for those patients that are most vulnerable to osteogenic toxicity may be implemented.

SAMENVATTING

Dit proefschrift omvat nieuwe informatie over de incidentie en risicofactoren van osteogene bijwerkingen tijdens en na behandeling van acute lymfatische leukemie (ALL) op de kinderleeftijd. Tevens worden potentiële nieuwe therapeutische benaderingen voor deze bijwerkingen van het skelet geëvalueerd.

Het eerste deel van dit proefschrift is gewijd aan de studies over de bot mineraal dichtheid (BMD) van kinderen met ALL. We beschrijven een groot prospectief multicenter onderzoek onder kinderen die volgens het ALL9 protocol van de Stichting Kinderoncologie Nederland (SKION) werden behandeld voor ALL, om de incidentie van BMD tekorten en fracturen op te helderen. Dit onderzoek toonde een drie-jaars cumulatieve incidentie voor fracturen van 18%, tijdens en vlak na de behandeling van ALL op de kinderleeftijd, welke veel hoger is dan de incidentie van ongeveer 2% in gezonde leeftijdsgenoten. De BMD van kinderen met ALL was significant lager dan dat van gezonde leeftijdsgenoten. Hoewel de BMD herstelde na het staken van de behandeling, was deze een jaar na het beëindigen van de behandeling nog steeds aanzienlijk lager dan dat van gezonde kinderen. Lage waarden van de BMD, en niet de afname van de BMD ten gevolge van de ALL behandeling, bepaalden het risico op fracturen bij kinderen tijdens en kort na de behandeling van ALL. Deze lage BMD waarden worden beïnvloed door patiënt-gerelateerde en ziekte-specifieke kenmerken zoals leeftijd, gewicht en het immunofenotype van de leukemie. Aangezien gelijk behandelde kinderen een grote variatie laten zien in het voorkomen van BMD reductie en fracturen, wordt verondersteld dat farmacogenetica een rol speelt in de pathogenese van deze botproblemen. We toonden aan dat enkel-nucleotide polymorfieën (SNPs) van het vitamine-D-receptor *(VDR)* gen en van het foliumzuurmetabolisme (methyleentetrahydrofolaat reductase *(MTHFR)* 677C> T en methionine synthase reductase *(MTRR)* 66A> G) de lage BMD bij diagnose en tijdens behandeling van ALL bepaalden, en niet zozeer de door de behandeling veroorzaakte reductie van de BMD. Voor de toekomst zou het van waarde zijn om een predictiemodel te ontwikkelen voor de gevoeligheid voor fracturen tijdens de behandeling van ALL op de kinderleeftijd, waarin zowel genetische factoren en omgevingsfactoren zijn geïntegreerd. Tot slot beschrijven we de resultaten van een gerandomiseerde studie, welke de effecten onderzocht van een trainingsprogramma ter preventie van BMD vermindering tijdens de behandeling

van ALL op de kinderleeftijd. Deze studie werd uitgevoerd omdat in de literatuur reeds eerder werd aangetoond dat mechanische belasting een cruciale rol speelt in het stimuleren van bot aanmaak. Onze studie toonde echter geen gunstig effect op de BMD en het fractuurrisico. Dit was mogelijk te wijten aan het niet naleven van een dergelijk langdurig trainingsprogramma tijdens de behandeling van ALL. Mogelijk dat een kortere duur van het trainingsprogramma en een meer intensieve begeleiding door eerstelijns fysiotherapeuten kan leiden tot een meer succesvolle interventie. Waarschijnlijk is het het meest effectief om deze trainingsprogramma´s alleen aan te bieden aan die patiënten, die middels eerder genoemde risicoprofielen, als hoog risico patiënten zullen worden aangeduid.

In het tweede deel van dit proefschrift richten we ons op osteonecrose. In ons prospectieve multicenter onderzoek vonden we dat zes procent van de patiënten symptomatische osteonecrose ontwikkelde tijdens of kort na de behandeling van ALL op de kinderleeftijd (SKION-ALL9 protocol). Oudere leeftijd en vrouwelijk geslacht waren onafhankelijke risicofactoren voor het ontwikkelen van symptomatische osteonecrose. Dat oudere kinderen gevoeliger zijn voor het ontwikkelen van osteonecrose, is mogelijk een weerspiegeling van de, aan de leeftijd gerelateerde, verschillen in dexamethason geïnduceerde veranderingen van het stollingssysteem, zoals blijkt uit een van onze studies. Behandeling van osteonecrose bij pediatrische ALL patiënten is een uitdaging en de huidige beschikbare literatuur levert onvoldoende bewijs om aanbevelingen te doen ten aanzien van chirurgische of niet chirurgische behandelopties om de morbiditeit van symptomatische osteonecrose te reduceren. Preventie van osteonecrose is echter wel mogelijk, aangezien het risico op behandelings- gerelateerde osteonecrose wordt gereduceerd door het intermitterend toedienen van corticosteroïden tijdens de intensiveringsfase van de behandeling, in plaats van continue toediening. Voor verdere ontwikkeling van een definitieve richtlijn, is het noodzakelijk dat gerandomiseerde klinisch trials van goede kwaliteit worden verricht, naar de werkzaamheid en veiligheid van interventies voor osteonecrose tijdens de behandeling van ALL op de kinderleeftijd.

Concluderend, hebben we aangetoond dat ten minste een op de vijf patiënten osteogene toxiciteit ontwikkelt tijdens of kort na de behandeling van ALL op de kinderleeftijd. Daarom zal men bij de behandeling van kinderen met ALL bewust moeten zijn van deze osteogene complicaties en is preventie van deze complicaties van belang. De resultaten van dit proefschrift hebben geleid tot aanbevelingen ten aanzien van screening en behandeling van osteogene toxiciteit bij patiënten die behandeld worden volgens het nationale SKION-ALL11 protocol. Toekomstig onderzoek is bij voorkeur gericht op het ontwerpen van voorspellingsmodellen voor osteogene toxiciteit ten gevolge van de behandeling van ALL, waarin genetische factoren en omgevingsfactoren worden gecombineerd. Zo kunnen preventieve en therapeutische strategieën worden ingezet voor die patiënten die het meest gevoelig zijn voor osteogene toxiciteit.

APPENDICES

LIST OF ABBREVIATIONS

a2AP	α2-antiplasmin
ADL	activities of daily living
ALL	acute lymphoblastic leukemia
AT	antithrombin
AVN	avascular necrosis of bone
BMD	bone mineral density
BM(A)D_{LS}	bone mineral (apparent) density of the lumbar spine
BMD_{TB}	bone mineral density of the total body
BMI	body mass index
CI	confidence interval
CNS	central nervous system
COLIA1	collagen type I alpha 1 gene
CRHR1	corticotrophin-releasing hormone receptor 1 gene
CT	computed tomography
DCOG	Dutch Childhood Oncology Group
DNA	deoxyribonucleic acid
DXA	dual energy X-ray absorptiometry
EFS	event-free survival
ELISA	enzyme-linked immunosorbent assays
ESR1	estrogen receptor alpha 1 gene
F1+2	prothrombin fragment 1 and 2
GR	glucorcoticoid recepter gene
HD-MTX	high dose methotrexate
HPLC	high-performance liquid chromatography
HR	high risk
HWE	Hardy Weinberg Equilibrium
IRR	incidence-rate ratio
ISCD	Society for Clinical Densitometry

KOCR	kinder oncologisch centrum Rotterdam
LBM	lean body mass
LPA	apolipoprotein (a) gene
MLL	mixed-lineage leukemia gene
movement-ABC	Movement Assessment Battery for Children
MRI	magnetic resonance imaging
MTHFR	methylene-tetrahydrofolate reductase
MTRR	methionine synthase reductase
MTX	methotrexate
MWU	Mann-Whitney U test
NCI	National Cancer Institute
NHR	non-high risk
NRS	non-randomized studies
ON	osteonecrosis
OR	odds ratio
PAI-1	plasminogen activator inhibitor-1
PAP	plasmin- 2-antiplasmin
PB	peripheral blood
PCR	polymerase chain reaction
PTH	parathyroid hormone
RCT	randomised clinical trial
ROI	regions of interest
SD	standard deviation
SDS	standard deviation scores
SEM	standard error of the mean
SKION	stichting kinderoncologie Nederland
SNP	single nucleotide polymorphism
TAT	thrombin antithrombin
VDR	vitamine-D receptor

AFFILIATIONS OF CO-AUTHORS

I.M. Appel	Department of Pediatric Oncology/ Hematology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands.
R.D. van Beek	Department of Pediatric Oncology/ Hematology and Department of Pediatric Endocrinology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands
J.H.J.M. Bessems	Department of Pediatric Orthopedics, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands
J.P.M. Bökkerink	Department of Pediatric Oncology/ Hematology, University Medical Center St Radboud, Nijmegen, the Netherlands
M.C.A. Bruin	Department of Pediatric Oncology, University Medical Center Utrecht, Utrecht, the Netherlands
R. M. Egeler	Department of Pediatrics, Leiden University Medical Center, Leiden, the Netherlands
H.A. de Groot-Kruseman	Dutch Childhood Oncology Group, The Hague, the Netherlands
A. Hartman	Department of Pediatric Physiotherapy, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands
M.M. van den Heuvel-Eibrink	Department of Pediatric Oncology/ Hematology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands
W.C.J. Hop	Department of Biostatistics, Erasmus MC-University Medical Center, Rotterdam, the Netherlands
R. de Jonge	Department of Clinical Chemistry, Erasmus MC-University Medical Center, Rotterdam, the Netherlands
H.C.G. Kemper	EMGO Institute VU University Medical Center, Amsterdam, the Netherlands

W.J.W.Kollen	Department of Pediatrics, Leiden University Medical Center, Leiden, the Netherlands
J.A. Leeuw	Department of Pediatric Oncology, Beatrix Children's Hospital, University of Groningen, Groningen, the Netherlands
M.H. Lequin	Department of Pediatric Radiology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands
S.M.P.F. de Muinck Keizer-Schrama	Department of Pediatric Endocrinology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands
R. Pieters	Department of Pediatric Oncology/ Hematology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands
J.C. Roos	Department of Radiology, VU University Medical Center, Amsterdam, the Netherlands
I.M. van der Sluis	Department of Pediatric Oncology/ Hematology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands
A.G. Uitterlinden	Department of Internal Medicine, Erasmus MC-University Medical Center, Rotterdam, the Netherlands
A.J.P. Veerman	Department of Pediatric Oncology/ Hematology, VU University Medical Center, Amsterdam, the Netherlands.
E.D. Wind	Department of Pediatric Oncology/ Hematology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands

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CURRICULUM VITAE

English

Mariël Lizet te Winkel was born on 29th April 1981 in Winterswijk. She grew up in Aalten, a village in the east of the Netherlands. In 1999 she finished her pre-university education (Atheneum) at the "Christian College Schaersvoorde". She studied medicine in Antwerp for one year, after which she obtained her medical degree at the Erasmus University Rotterdam in 2006 (cum laude). Between September 2006 and September 2011 Lizet performed her PhD at the department of pediatric Oncology of the Erasmus MC-Sophia Children's Hospital (promotor: Prof. Dr. Rob Pieters, and co-promotor Dr. Marry M. van den Heuvel-Eibrink), where the current doctoral thesis was written. Lizet was one of the datamanagers of the Dutch Childhood Oncology Group (DCOG; 2007-2008). She worked as a resident at the department of pediatrics at the Maasstad hospital during one year (2011-2012). In September 2012 she started her training as general practitioner at the Erasmus University Medical Center in Rotterdam. Lizet lives with her husband Ernst-Jan Wind in Rotterdam, in December 2010 their daughter Nore Aimée was born and in November 2013 she expects her second child.

Nederlands

Mariël Lizet te Winkel is geboren op 29 april 1981 te Winterswijk. Ze groeide op in Aalten, een dorp in de Achterhoek nabij de Duitse grens. In 1999 behaalde zij haar Atheneum diploma aan het Christelijk College Schaersvoorde. Hierna studeerde zij geneeskunde, aanvankelijk een jaar in Antwerpen, om vervolgens haar studie in Rotterdam aan de Erasmus Universiteit in 2006 te voltooien (cum laude). Van 2006 tot 2011 was Lizet als promovendus werkzaam op de afdeling Kinderoncologie in het Erasmus MC-Sophia Kinderziekenhuis (promotor: Prof. Dr. Rob Pieters en co-promotor Dr. Marry M. van den Heuvel-Eibrink), alwaar dit proefschrift tot stand is gekomen. Lizet is tevens werkzaam geweest als datamanager van de Stichting Kinderoncologie Nederland (SKION; 2007-2008). Zij werkte gedurende een jaar (2011 tot 2012) als arts-assistent kindergeneeskunde in het Maasstad ziekenhuis te Rotterdam. In september 2012 is zij gestart met haar opleiding tot huisarts aan het huisartseninstituut van het Erasmus MC in Rotterdam. Lizet woont met haar echtgenoot Ernst-Jan Wind in Rotterdam, in december 2010 werd hun dochter Nore Aimée geboren en in november 2013 verwacht zij haar tweede kindje.

LIST OF PUBLICATIONS

- **M.L. te Winkel,** R. Pieters, E.D. Wind, J.H.J.M. Bessems, M.M. van den Heuvel-Eibrink, "Management and treatment of osteonecrosis in children and adolescents with acute lymphoblastic leukemia: a narrative review", submitted 2013.
- **M.L. te Winkel,** R. Pieters, W.C.J. Hop, J.C. Roos, I.M. van der Sluis, J.P.M. Bökkerink, J.A. Leeuw, M.C.A. Bruin, W.J.W. Kollen, A.J.P. Veerman, H.A. de Groot-Kruseman, M.M. van den Heuvel-Eibrink, "Bone mineral density at diagnosis determines fracture rate in children treated according to the DCOG-ALL9 protocol", submitted 2013.
- E. den Boer, S.G. Heil, B.D.van Zelst, R.J. Meesters, B.C. Koch, **M.L. te Winkel,** M.M. van den Heuvel-Eibrink, T.M. Luider, R. de Jonge, "A U-HPLC-ESI-MS/MS-based stable isotope dilution method for the detection and quantitation of methotrexate in plasma", Therapeutic Drug Monitoring 2012; 34(4): 432-439.
- N. Ketharanathan, **M.L. te Winkel,** M.H. Lequin, M.M. van den Heuvel-Eibrink, "An infant with sternal swelling", Ned Tijdschr Geneeskd. 2012; 156(1): A3981.
- **M.L. te Winkel,** R. Pieters, W.C.J. Hop, H.A. de Groot-Kruseman, M.H. Lequin, I.M. van der Sluis, J.P.M. Bökkerink, J.A. Leeuw, M.C.A. Bruin, R.M. Egeler, A.J.P. Veerman, M.M. van den Heuvel-Eibrink, "A prospective study on incidence, risk factors and long-term outcome of osteonecrosis in pediatric acute lymphoblastic leukemia", J Clin Oncol. 2011; 29(31): 4143-50.
- M. van Waas, S.J.C.M.M. Neggers, **M.L. te Winkel,** A. Beishuizen, R. Pieters, M.M. van den Heuvel-Eibrink, "Endocrine late sequelae in ong-term survivors of childhood non-Hodgkin lymphoma", Annals of Oncology 2011; 23(6): 1626-1632.
- **M.L. te Winkel,** S.M.P.F. de Muinck Keizer-Schrama, R. de Jonge, R.D. van Beek, W.C.J. Hop, R. Pieters, M.M. van den Heuvel-Eibrink, "Germline genomic variation in the methylenetetrahydrofolate reductase (MTHFR) and methionine synthase reductase (MTRR) genes determines impairment of bone mineral density in pediatric acute lymphoblastic leukemia", Bone 2011; 48(3): 571-577.
- M.L. te Winkel/ P. de Laat, A.S. Devos, C.E. Catsman-Berrevoets, R. Pieters, M.M. van den Heuvel-Eibrink, "Posterior Reversible Encephalopathy Syndrome (PRES) in Children with Cancer", Annals of Oncology 2011; 22(2): 472-478.
- **M.L. te Winkel,** R. Pieters, M.M. van den Heuvel-Eibrink, " Future perspectives on minimizing bone density reduction in pediatric acute lymphoblastic leukemia", Personalized Medicine 2010; 7(5): 469-471.
- **M.L. te Winkel,** I.M. van der Sluis, M.H. Lequin, R. Pieters, M.M. van den Heuvel-Eibrink, "Letter to the editor in response to "Prospective bone ultrasound patterns during childhood acute lymphoblastic leukemia" by Mussa et al.", Bone 2010; 47(4): 835-6; author reply 837-838.
- L. Zuurbier, I. Homminga, V. Calvert, **M.L. te Winkel,** J.G. Buijs-Gladdines, C. Kooi, W.K. Smits, E. Sonneveld, A.J. Veerman, W.A. Kamps, M. Horstmann, E.F. 3rd Petricoin, R. Pieters, J.P. Meijerink, "NOTCH1 and/or FBXW7 mutations predict for initial good prednisone response but not for improved outcome in pediatric T-cell acute lymphoblastic leukemia patients treated on DCOG or COALL protocols", Leukemia 2010; 24(12): 2014-2022.
- **M.L. te Winkel,** M.H. Lequin, J.R. de Bruyn, C.P. van der Ven, R.R. de Krijger, R. Pieters, M.M.

van den Heuvel-Eibrink, "Self-Limiting Sternal Tumors of Childhood (SELSTOC)", Pediatric Blood and Cancer 2010; 55(1): 81-84.

- **M.L. te Winkel,** R.D. van Beek, S.M.P.F. de Muinck Keizer-Schrama, A.G. Uitterlinden, W.C.J. Hop, R. Pieters, M.M. van den Heuvel-Eibrink, "Pharmacogenetic risk factors for altered bone mineral density and body composition in pediatric acute lymphoblastic leukemia", Haematologica 2010; 95(5): 752-759.
- M.L. te Winkel/ A. Hartman, R.D. van Beek, S.M.P.F. de Muinck Keizer-Schrama, H. Kemper, W.C.J. Hop, M.M. van den Heuvel-Eibrink, R. Pieters, "A randomized trial investigating an exercise programme to prevent reduction of bone mineral density and impairment of motor performance during treatment for childhood acute lymphoblastic leukemia", Pediatric Blood and Cancer 2009; 53(1): 64-71.
- **M.L. te Winkel,** I.M. Appel, R. Pieters, M.M. van den Heuvel-Eibrink, "Impaired dexamethasone related increase of anticoagulants is associated with the development of osteonecrosis in childhood acute lymphoblastic leukemia", Haematologica 2008; 93(10): 1570-1574.
- R.D. van Beek, D.D.L. Bezemer, J.P.P. Meijerink, S.M.P.F. de Muinck Keizer-Schrama, O.A. Haas, **M.L. te Winkel,** R. Pieters and M.M. van den Heuvel-Eibrink, "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 bone (AVN) in pediatric acute lymphoblastic leukemia", Leukemia 2006; 20(5): 879-880.
- M.M. van den Heuvel-Eibrink, R.G.M. Bredius, **M.L. te Winkel,** R. Tamminga, J. Kraker, A.Y.N. Schouten-van Meeteren, M. Bruin and E.T. Korthof, "Childhood paroxysmal nocturnal haemoglobinuria (PNH), a report of 11 cases in the Netherlands", British Journal of Haematology 2005; 128: 571-577.
- **M.L. te Winkel,** "Highlights of the SIOP-congress 2004", "Oncollectie 4-2004".

PHD PORTFOLIO

Name PhD student: Mariël Lizet te Winkel Erasmus MC Department: Pediatric Oncology/Hematology Research School: Molecular Medicine	PhD period: Sept 2006 - Sept 2013 Promotor(s): Prof.Dr. R. Pieters Supervisor: Dr. M.M. v/d Heuvel-Eibrink	
	YEARS	**Workload (Hours/ECTS)**
1. PhD training		
General academic skills		
- Methodologie van patiëntgebonden onderzoek en voorbereiding van subsidieaanvragen	2007	0.2 ECTS
- Biomedical English Writing and Communication	2008	4.0 ECTS
- Presenteren en informatieoverdracht	2009	0.4 ECTS
- Effectief presenteren van wetenschappelijk onderzoek	2009	0.3 ECTS
- Weekly research meetings, department Pediatric Oncology/ Hematology Erasmus MC-Sophia	2006-2011	2.0 ECTS
Statistic courses		
- Classical Methods for Data-Analysis	2007	5.7 ECTS
- Course for the quantitative researcher	2009	1.4 ECTS
- Repeated measurements in clinical studies	2010	1.9 ECTS
In-depth courses (e.g. Research school, Medical Training)		
- Meet the expert on osteonecrosis (L. Mattano and R. Barr, Canada)	2008	0.2 ECTS
- The course molecular diagnostics IV	2009	0.5 ECTS
- SNP Course 2009	2009	1.4 ECTS
- Training course: "Bone - Bedside to Bench and Back"	2013	0.2 ECTS
Oral presentations		
- "Pharmacogenetic Risk Factors for Altered Bone Mineral Density and Body Composition in Pediatric Acute Lymphoblastic Leukemia", 11th International Conference on Long-term Complications of Treatment of Children and Adolescents for Cancer 2010	2010	1.0 ECTS
- "Germline genomic variation in the methylenetetrahydrofolate reductase (MTHFR) and methionine synthase reductase (MTRR) genes determines impairment of bone mineral density in pediatric acute lymphoblastic leukemia", 7th Bi-Annual Childhood Leukemia Symposium (IBFM) 2010	2010	1.0 ECTS
- "Germline genomic variation in the methylenetetrahydrofolate reductase (MTHFR) and methionine synthase reductase (MTRR) genes determines impairment of bone mineral density in pediatric acute lymphoblastic leukemia", 42nd congress of the International Society of Paediatric Oncology (SIOP) 2010	2010	1.0 ECTS
- Oral presentations at Research meetings Pediatrics/ Pediatric Oncology	2006-2011	1.0 ECTS
- "Bottoxiciteit in ALL patiënten", werkgroep kinderoncologie van het Integraal Kankercentrum Nederland (IKNL)	2011	1.0 ECTS
- "Bone mineral density at diagnosis determines fracture rate in children treated according to the DCOG-ALL9 protocol", International Conference on Children's Bone Health (ICCBH) 2013	2013	1.0 ECTS
- Planned: "Bone mineral density at diagnosis determines fracture rate in children treated according to the DCOG-ALL9 protocol", 45th congress of the International Society of Paediatric Oncology (SIOP) 2013	2013	1.0 ECTS

Poster presentations:		
- "Impaired dexamethasone-related increase of anticoagulants is associated with the development of osteonecrosis in childhood acute lymphoblastic leukemia", American Society of Hematology (ASH) 2007	2007	0.5 ECTS
- "Impaired dexamethasone-related increase of anticoagulants is associated with the development of osteonecrosis in childhood acute lymphoblastic leukemia", 13th congress of the European Hematology Association (EHA)	2008	0.5 ECTS
- "Impaired dexamethasone-related increase of anticoagulants is associated with the development of osteonecrosis in childhood acute lymphoblastic leukemia", 40th congress of the International Society of Paediatric Oncology (SIOP) 2008	2008	0.5 ECTS
- "A randomised trial investigating an exercise programme to prevent reduction of bone mineral density and impairment of motor performance during treatment for childhood acute lymphoblastic leukaemia", 40th congress of the International Society of Paediatric Oncology (SIOP) 2008	2008	0.5 ECTS
- "A randomised trial investigating an exercise programme to prevent reduction of bone mineral density and impairment of motor performance during treatment for childhood acute lymphoblastic leukaemia", 50st annual meeting of the American Society of Hematology (ASH) 2008	2008	0.5 ECTS
- "A randomised trial investigating an exercise programme to prevent reduction of bone mineral density and impairment of motor performance during treatment for childhood acute lymphoblastic leukaemia", International Conference on Children's Bone Health (ICCBH) 2009	2009	0.5 ECTS
- "A randomised trial investigating an exercise programme to prevent reduction of bone mineral density and impairment of motor performance during treatment for childhood acute lymphoblastic leukaemia", Nederlandse Vereniging Kindergeneeskunde (NVK) 2009	2009	0.5 ECTS
- "Pharmacogenetic Risk Factors for Altered Bone Mineral Density and Body Composition in Pediatric Acute Lymphoblastic Leukemia", 51st annual meeting of the American Society of Hematology (ASH) 2009	2009	0.5 ECTS
- "Pharmacogenetic Risk Factors for Altered Bone Mineral Density and Body Composition in Pediatric Acute Lymphoblastic Leukemia", International Conference on Children's Bone Health (ICCBH) 2009	2009	0.5 ECTS
- "Germline genomic variation in the methylenetetrahydrofolate reductase (MTHFR) and methionine synthase reductase (MTRR) genes determines impairment of bone mineral density in pediatric acute lymphoblastic leukemia", 11th International Conference on Long-term Complications of Treatment of Children and Adolescents for Cancer 2010	2010	0.5 ECTS
- "A Prospective Study on Incidence, Risk Factors and Long-Term Outcome of Osteonecrosis in 694 Patients with Pediatric Acute Lymphoblastic Leukemia", 53rd annual meeting of the American Society of Hematology (ASH) 2011	2011	0.5 ECTS

- "Prospective Study on Incidence, Risk Factors, and Long-Term Outcome of Osteonecrosis in Pediatric Acute Lymphoblastic Leukemia", 44th congress of the International Society of Paediatric Oncology (SIOP) 2012	2012	0.5 ECTS
- "Bone Mineral Density At Diagnosis Determines Fracture Rate in Children Treated According to the DCOG-ALL9 Protocol", 54th annual meeting of the American Society of Hematology (ASH) 2012	2012	0.5 ECTS
International conferences		
- 45th Annual Meeting of the European Society for Paediatric Endocrinology (ESPE), Rotterdam	2006	0.2 ECTS
- 49th Congres of the American Society of Hematology (ASH), Atlanta	2007	1.0 ECTS
- 48th annual scientific meeting of the British Society for Haematoloy (BSH), Glasgow	2008	1.0 ECTS
- 40th Congress of the International Society of Paediatric Oncology (SIOP), Berlin	2008	1.0 ECTS
- 5th International Conference on Children's Bone Health, Cambridge	2009	1.0 ECTS
- 11th International Conference on on Long-term Complications of Treatment of Children and Adolescents for Cancer 2010, Williamsburg (United States of America)	2010	1.0 ECTS
- 7th Bi-Annual Childhood Leukemia Symposium (IBFM) 2010, Antalya (Turkey)	2010	1.0 ECTS
- 6th International Conference on Children's Bone Health, Rotterdam	2013	1.0 ECTS
Seminars and workshops		
- Research-day Sophia, Rotterdam	2006-2009	0.4 ECTS
- Jaarlijks Kinderoncologie symposium, Rotterdam	2006-2010	1.0 ECTS
- Jaarlijkse Stichting Kinderoncologie Nederland (SKION)-dagen, Utrecht	2007-2009	0.4 ECTS
- Annual Molecular Medicine Day, Rotterdam	2007-2008	0.4 ECTS
- Symposium: "Clinics in Pediatric Endocrinology: a tribute to dr. Sabine de Muinck Keizer-Schrama", Rotterdam	2007	0.2 ECTS
- Erasmus MC science-day, Rotterdam	2008	0.2 ECTS
- Dag voor jonge onderzoekers, Nederlandse vereniging voor kindergeneeskunde (NVK), Veldhoven	2008	0.2 ECTS
- CPO najaarssymposium, "Quality of Life & Quality of Care"	2008	0.1 ECTS
- National "LATER" conferences: "Late Effects of Childhood Cancer", Groningen and Rotterdam	2009-2010	0.2 ECTS
- Congres kindergeneeskunde, Nederlandse vereniging voor kindergeneeskunde (NVK) , Veldhoven	2011	0.2 ECTS

Other		
- KiKa Research Grant: "Genetic and metabolic profiling of folate metabolism to predict methotrexate toxicity in pediatric acute lymphoblastic leukemia", 2010	2010	2.0 ECTS
- Peer reviewing of articles for scientific journals	2009-2011	2.0 ECTS
Award		
- New investigators award of the 6th International Conference on Children's Bone Health 2013	2013	
2. Teaching activities		
Supervising		
- J. de Bruyn, medical student, retrospective research and article writing	2008	1.0 ECTS
- M. Pruissen, medical student, master theses	2008	1.0 ECTS
- P. de Laat, medical student, retrospective research and article writing	2010	1.0 ECTS
- B. Klap, medical student, retrospective research and article writing	2011	1.0 ECTS
- M. van den Hoed, medical student, prospective research and article writing	2012-2013	1.0 ECTS

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