Risk factors for symptomatic osteonecrosis in childhood ALL: A retrospective study of a Slovenian pediatric ALL population between 1970 and 2004

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Abstract. Treatment induced non-traumatic osteonecrosis (ON) has been reported increasingly in children treated for acute lymphoblastic leukemia (ALL). Several risk factors for ON have been identified in childhood cancer patients; however, their diagnostic and prognostic power is limited and the etiology of the disease remains unclear. Therefore, a continuous effort is focused on the identification of additional ON risk factors. We performed a retrospective study of 313 childhood ALL patients to test the association between the ON occurrence in children receiving ALL therapy and common polymorphisms in potential target genes: Thiopurine S-methyltransferase (*TPMT*; 460G>A, 719A>G), 5,10-methylenetetrahydrofolate reductase (*MTHFR*; 677C>T, 1298A>C), estrogen receptor alpha 1 (*ESR1*; *XbaI*) and collagen type I, α1 (*COL1A1*; Sp1). In the present cohort, higher age and more recently developed treatment protocols were independent risk factors for ON. In children >14.5 years old, *TPMT* genotype modulated the risk of ON. Additionally, in children <12.9 years old *ESR1* genotypes were also implicated in the pathogenesis of ON. Besides greater age and more recent treatment protocols, genetic factors (polymorphisms in *ESR1* and *TPMT* genes) were suggested to be implicated in the pathogenesis of ON and could be potentially used as genetic prognostic markers for ON.

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Abbreviations: ACP-1, acid phosphatase 1; ALL, acute lymphoblastic leukemia; BFM, Berlin-Frankfurt-Münster; BMD, bone mineral density; COL1A1, collagen, type I, α1; ESR1, estrogen receptor alpha 1; GC, glucocorticoide; 6-MP, 6-mercaptopurine; MSC, mesenchimal stem cell; MTHFR, 5,10-methylenetetrahydrofolate reductase; MTX, methotrexate; ON, osteonecrosis; PAI-1, plasminogen activator inhibitor-1; POG, pediatric oncology group; TPMT, thiopurine S-methyltransferase; TYMS, tymidylate synthetase

Key words: acute lymphoblastic leukemia, collagen, type I, α1, estrogen receptor alpha 1, 5,10-methylenetetrahydrofolate reductase, osteonecrosis, thiopurine S-methyltransferase

Introduction

Osteonecrosis (ON), also known as aseptic or avascular necrosis of the bone, is a disorder characterized by segmental death of one or more osseous sites (1). Although non-traumatic ON is rare in young people, it has been reported increasingly in children treated for acute lymphoblastic leukemia (ALL), as ALL survival rates continue to improve (1). The symptoms of ON are highly variable, ranging from mild discomfort to decreased mobility, severe pain and articular collapse (2). Although up to 25% of children treated for ALL exhibit radiographic evidence of osteopenia (3) and one-third of ALL patients develop symptomatic ON (1), the majority of patients are asymptomatic, some showing the spontaneous regression or even complete resolution of the disorder (1). Due to heterogenous diagnostic criteria, the reported incidence of ON varies widely in children with cancer; between 0.3 and 9% (2,4). The pathogenesis of ON is complex and includes the enhanced differentiation of mesenchymal stem cells (MSCs) into lipocytes at the expense of osteogenesis, as well as damage to the venous system, vascular stasis and ischemia (1). Several risk factors for ON, such as older age, white race and glucocorticoid therapy (1), have been identified in infant cancer patients; however, their diagnostic power is limited and the etiology of the disease remains unclear. Therefore, there is a continuous effort to identify additional ON risk factors. In addition to glucocorticoids (GCs), other therapeutic agents such as methotrexate (MTX) and 6-mercaptopurine (6-MP) have been indicated in bone morbidity during ALL treatment (5-7). Recently, several polymorphisms have been identified as potential risk factors for ON during ALL treatment in genes involved in various processes, including: Bone metabolism, vitamin D receptor (8), collagen type II, α1 (1) and acid phosphatase 1 (*ACP-1*) (9); thrombosis, 5,10-methylenetetrahydrofolate reductase (*MTHFR*) (9) and plasminogen activator inhibitor-1 (*PAI-1*) (10); and ALL pharmacogenetics, tymidylate synthetase (*TYMS*) (8). The present

exploratory retrospective study was conducted to assess whether common polymorphisms in the target genes thiopurine S-methyltransferase (*TPMT*), *MTHFR*, estrogen receptor alpha 1 (*ESR1*) and collagen type I, α1 (*COL1A1*) are associated with ON in children receiving ALL therapy and could be used as prognostic genetic markers for ON. Target genes were selected to investigate all three aspects of ON risk: Thrombotic (*MTHFR, ESR1*); bone metabolism (*ESR1, COL1A1* and *MTHFR*); and pharmacogentic (*TPMT, MTHFR*). TPMT and MTHFR are key enzymes in the metabolism of 6-MP and MTX (11), respectively, and are crucial to the majority of ALL treatment protocols (12). They are administered in all phases of the therapy and are the primary drugs used in the maintenance phase, during which ON typically emerges (12). In addition, *MTHFR* variants (9,13), as well as hyperestrogenemia (11), have been implicated in ON due to thrombophilia in adults. Finally, *ESR1* and *COL1A1* were associated with bone mineral density (BMD) and other osteoporotic phenotypes in candidate gene studies (14), in addition to large-scale association (15,16) and genome-wide studies (17,18). Additionally, MTHFR has been suggested to be associated with osteoporotic phenotype in certain studies (17-19).

Materials and methods

Patients. Slovenian pediatric ALL patients diagnosed and treated at the University Children's Hospital, University Medical Centre (Ljubljana, Slovenia) between 1970 and 2004 were identified via the national oncology patients registry. Among the 405 registered patients with childhood ALL, 62 had inadequate medical records, 3 patients received non-Hodgkin's lymphoma Berlin-Frankfurt-Münster (NHL-BFM) treatment, 3 underwent bone marrow transplantation prior to maintenance therapy and 15 patients succumbed or relapsed prior to receiving maintenance therapy. A further 9 patients were later excluded from the study due to unsuccessful DNA extraction. The final study group consisted of 313 patients with childhood ALL. The study was conducted in accordance with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects. Ethical approval was obtained from the Medical Ethics Committee of Slovenia.

Different therapy protocols were applied in the study periods from 1970 to 2004. Pediatric oncology group (POG) protocols (20) were used from 1970 to 1983, and thereafter BFM protocols [BFM-83 (21), -86 (22), -90 (23), -95 (24) and intercontinental (IC) trial-BFM 2002 (25)]. Therapy data, which were systematically recorded by attending physicians, such as incidence of toxic effects, were obtained for 313 childhood ALL patients. From patients' medical records, 12 patients with symptomatic ON were identified, corresponding to grades 3 and 4 adverse events of NCI Common Toxicity Criteria (version 2.0) (26).

Genotyping. Genotyping was performed after the therapy data were extracted from patients' medical files and analyzed by researchers who were blinded to patients' medical data.

DNA was extracted from archival bone marrow smears of patients at the diagnosis of ALL using a semi-automated ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems; Thermo Fisher Scientific, Inc., Foster City, CA, USA).

TPMT (*3B: 460G>A, rs1800460 and *3C: 719A>G, rs1142345), *MTHFR* (677C>T, rs1801133 and 1298A>C, rs1801131), *ESR1* (*Xba*I, rs9340799) and *COL1A1* (*Sp1*, rs1800012) polymorphisms were determined using TaqMan chemistry on ABI Prism® 7000 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). TaqMan chemical reagents were purchased from Applied Biosystems). As 460G>A and 719A>G polymorphisms in *TPMT* are in linkage disequilibrium and inherited together in cis, patients carrying both polymorphisms were considered to be heterozygotes for TPMT*3A allele. All low activity *TPMT* alleles were designated as TPMT*3 and wild-type as TPMT*1. We combined *MTHFR* 677C>T and 1298A>C genotypes and established that 677T rarely occurred in cis with 1298C. In the group of 313 patients, only a single patient (0.3%) exhibited the 677TT/1298AC genotype, which is in accordance with previous results (27). The 677CC/1298AA genotype was designated a wild type and all other as mutated genotypes.

Statistical analysis. To assess differences in age, gender, treatment protocol, *TPMT, MTHFR, ESR1* and *COL1A1* genotypes between ON and non-ON group the Fisher exact test was used.

For multivariate analysis, we applied the classification tree method to determine the interaction between multiple predictive variables and to evaluate the risk of ON associated with specific subgroups. ON was treated as the dependent variable, while all other variables (age, gender, treatment protocol and genotypes) were treated as independent variables. All variables were considered when constructing the classification tree. The classification and regression tree growing method was used and the minimum number of cases in parent and child node was set to 5 and 2, respectively. Due to the small number of ON cases the results of the classification tree were confirmed using Fisher exact test. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using the SPSS software, version 16.0 for Windows (SPSS, Inc., Chicago, IL, USA).

Results

Characteristics of the patients. The study group consisted of 313 childhood ALL patients, 12 (3.8%) of whom were found to have had symptomatic ON (NCI grades 3 and 4). In the group of 313 childhood ALL patients, the mean age at ALL diagnosis was 5.9±4.2 years (range, 0.2-17.0 years). There were 148 (47.3%) female and 165 (52.7%) male patients. Furthermore, 94 (30.0%), 37 (11.8%), 55 (17.6%), 59 (18.8%), 55 (17.6%) and 13 (4.2%) of patients were treated with POG, BFM-83, -86, -90, -95 and IC-BFM 2002 protocols, respectively (Table I).

The genotyping for *TPMT* and *MTHFR* polymorphisms was successful for all 313 patients, while *ESR1* and *COL1A1* genotypes were not determined for 23 and 52 patients, respectively, due insufficient extracted DNA from bone marrow smears. With respect to *TPMT* genotype, there were no homozygous mutated (*3/*3) patients, 21 (6.7%) patients were heterozygotes (*1/*3) and the remainder were wild-type (*1/*1). A total of 32 (10.2%) patients were *MTHFR* wild type, while 281 (89.8%) had at least one mutated allele in one of the two *MTHFR* locus. Among the 261 patients that were successfully genotyped for *COL1A1*, 201 (77.0%) had GG, 51 (19.5%) GT

Table I. Analysis of independent risk factors for osteonecrosis in acute lymphoblastic leukemia patients with and without osteonecrosis.

Parameter	Osteonecrosis (N=12)	No osteonecrosis (N=301)	P
Age, years	11.3±5.9	5.6±4.0	0.007
Gender, n (%)			0.774
Male	7 (58.3)	158 (52.5)	
Female	5 (41.7)	143 (47.5)	
Treatment protocol, n (%)			0.018
POG	2 (16.7)	92 (30.6)	
BFM-83	0 (0.0)	37 (12.3)	
BFM-86	0 (0.0)	55 (18.3)	
BFM-90	5 (41.7)	54 (17.9)	
BFM-95	3 (25.0)	52 (17.3)	
IC-BFM 2002	2 (16.7)	11 (3.7)	
TPMT genotype, n (%)			0.188
*1/*1	10 (83.3)	282 (93.7)	
*1/*3	2 (16.7)	19 (6.3)	
MTHFR genotype, n (%)			0.620
Wild type[a]	0 (0.0)	32 (10.8)	
Mutated[b]	12 (100)	269 (89.4)	
ESR1 genotype, n (%)			0.465
GG	4 (33.3)	53 (19.1)	
GA	4 (33.3)	121 (43.5)	
AA	4 (33.3)	104 (37.4)	
COL1A1 genotype, n (%)			0.797
GG	10 (90.9)	191 (76.4)	
GT	1 (9.1)	50 (20.0)	
TT	0 (0.0)	9 (3.6)	

[a]677 CC/1298 AA. [b]All other genotype combinations with at least one mutated allele. Independent samples t-test were used for the age and Fisher's exact test was used for all the other parameters. POG, pediatric oncology group; BFM, Berlin-Frankfurt-Münster; IC, intercontinental; TPMT, thiopurine S-methyltransferase; MTHFR, 5,10-methylenetetrahydrofolate reductase; ESR1, estrogen receptor alpha 1; COL1A1, collagen, type I, α1.

and 9 (3.4%) TT genotype. The distribution of *ESR1* genotypes in the 290 successfully genotyped patients was as follows: GG, 57 (19.7%); AG, 125 (43.1%); and AA, 108 (37.2%). All examined polymorphisms were in Hardy-Weinberg equilibrium.

Independent risk factors for ON. To identify independent risk factors for ON, we compared all variables (age, gender, treatment protocol and studied genotypes) between ON patients and non-ON patients. Higher age at diagnosis and more recent treatment protocols were identified as independent risk factors for ON (Table I and Fig. 1).

Combined risk factors for ON. To investigate the interaction between multiple risk factors, we employed classification tree analysis (Fig. 2). The terminal nodes of the classification tree with the highest incidence of ON represented the patient subgroups with the highest risk for ON.

The subgroup with highest (100%) risk for ON consisted of ALL patients >14.5 years old with the mutated *TPMT* genotype. The ON incidence in this subgroup was compared with its incidence in the remainder of the observed population by means of Fisher exact test, which showed a statistically significant difference (P=0.001) (Fig. 3). The group with the second highest risk (50%) for ON consisted of patients 2.4-12.9 years old, who had GG *ESR1* genotype and were treated with protocols BFM-95 and IC-BFM 2002 (P=0.075). Patients aged 2.2-2.4 years with *ESR1* GG and AG genotypes represented the third risk group with ON incidence of 40% (P=0.013). The incidence of ON in all terminal nodes and its comparison to the remainder of the observed population is shown in Fig. 3. Notably, a combination of age 2.4-12.9 years and *ESR1* AA and AG genotypes had a protective effect, as none of the 185 patients in this subgroup developed symptomatic ON (P<0.001).

Discussion

In the studied population of childhood ALL patients, the incidence of ON between 1970 and 2004 was 3.8%. Greater age and later treatment protocols were independent risk factors for

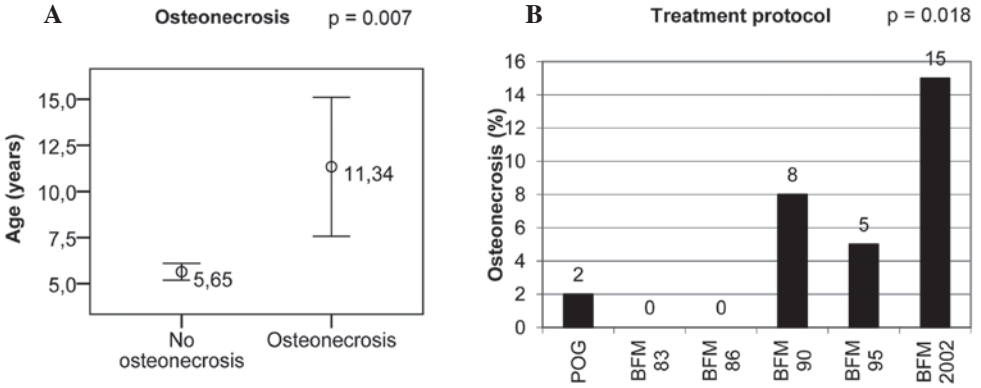

Figure 1. Independent risk factors for osteonecrosis. (A) Age and (B) treatment protocol were independent risk factors for osteonecrosis in childhood acute lymphoblastic leukemia patients; error bars represent 95% confidence interval of the mean. Independent samples t-test were used for the age and Fisher's exact test was used for the treatment protocols. POG, pediatric oncology group; BFM, Berlin-Frankfurt-Münster.

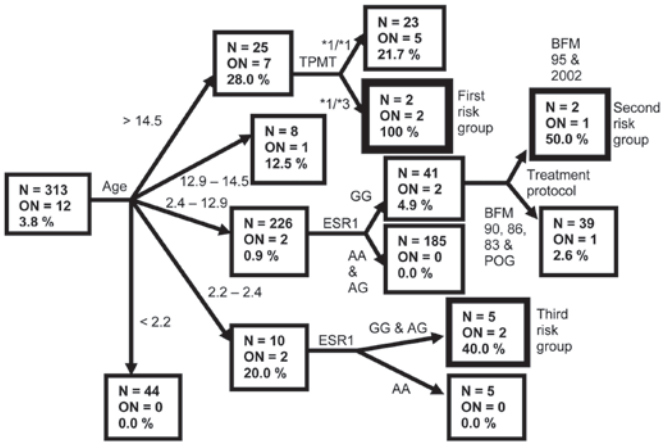

Figure 2. Classification tree for the risk of ON after treatment for childhood acute lymphoblastic leukemia (ALL). Percents represent the incidence of ON in the given subgroup. Analysis of 313 childhood ALL patients from cancer registry of Slovenia. The classification and regression tree growing method was used for the statistical analysis. N, number of patients in the subgroup; ON, number of osteonecrotic patients in the subgroup; ESR1, estrogen receptor alpha 1; TPMT, thiopurine S-methyltransferase; BFM, Berlin-Frankfurt-Muenster protocol.

ON. Furthermore, in patients >14.5 years, a mutated *TPMT* genotype was associated with increased ON risk, while for children younger than 12.9 years *ESR1* GG and AG genotypes were the main risk factor for ON.

In the present cohort age was a strong independent risk factor for ON, which is in accordance with numerous studies (2,4,8,28,29). It has been established that adolescents, particularly those >15 years, are at highest risk for ALL therapy-induced ON. This may reflect the greater vulnerability of rapidly growing bone in adolescent period (1).

Another independent risk factor in the present study was treatment protocol. More recent treatment protocols (BFM-90, -95 and IC-BFM 2002) had higher incidence of ON compared with older protocols. This could be explained by the differences in drug administration schemes in different protocols. All chemotherapeutic agents that are used in the treatment of ALL were found to reduce the number of osteoblast-like cells *in vitro* (5), and combinations of different agents had more toxic effects compared with individual agents (30). GCs were found to exhibit the strongest risk of ON in the clinical setting during ALL therapy (2), although MTX and alkylating agents were also indicated to be associated with ON risk in certain studies (2,3,6). Therefore, we reviewed all used protocols to detect differences in administration schedule and doses of GC and MTX. The main difference between recent (BFM-90, -95 and IC-BFM 2002) and older (POG, BFM-83 and -86) protocols were higher cumulative doses of GC (particularly dexamethasone) and MTX in recent protocols. This is in accordance with the results of two large childhood cancer survivor studies, in which GC therapy was strong risk factor for ON (2) and patients receiving dexamethasone were more likely to develop ON compared with patients who received prednisone alone (2,28). Another important difference in the IC-BFM 2002 was the introduction of intermittent maintenance therapy (IMT) consisting of weekly MTX (20 mg/m^2) and daily 6-MP (50 mg/m^2). IMT was administered in all patient risk groups between the blocks of reinduction therapy (25). Introduction of 2 IMT blocks for standard risk and 3 blocks for intermediate risk group into the reinduction therapy resulted in higher cumulative doses of dexamethasone compared to standard reinduction therapy. In accordance with this, the incidence of ON was highest in the IC-BFM 2002 group (15%), markedly exceeding ON incidence in BFM-90 (8%) and BFM-95 (5%) protocols. Notably, all ON patients treated with the IC-BFM 2002 protocol were assigned to the intermediate risk group.

Deactivating polymorphisms in the *TPMT* gene were a risk factor for ON in patients >14.5 years. TPMT is a S-Adenosyl methionine (SAM)-dependent methyltransferse that catalyses the deactivation of thiopurine drugs, such as 6-MP and 6-thioguanine (31). If the TPMT activity is low (such as in the presence of polymorphisms in the *TPMT* gene), this will result in high concentrations of cytotoxic thioguanine nucleotides (TGN) due to decreased conversion of thiopurine drugs to inactive methylated metabolites (31). The toxic effects of 6-MP on bone and bone cells have been demonstrated *in vitro* (5) and *in vivo* (7). 6-MP reduced cell numbers in osteoblast-like cell line MG63 at therapeutically relevant concentrations (5), and in a retrospective study of childhood ALL survivors number of weeks of 6-MP exposure were strongly correlated with hip BMD reduction (7). High concentrations of TGN exert toxic effects on majority of cells and this effect is most pronounced in

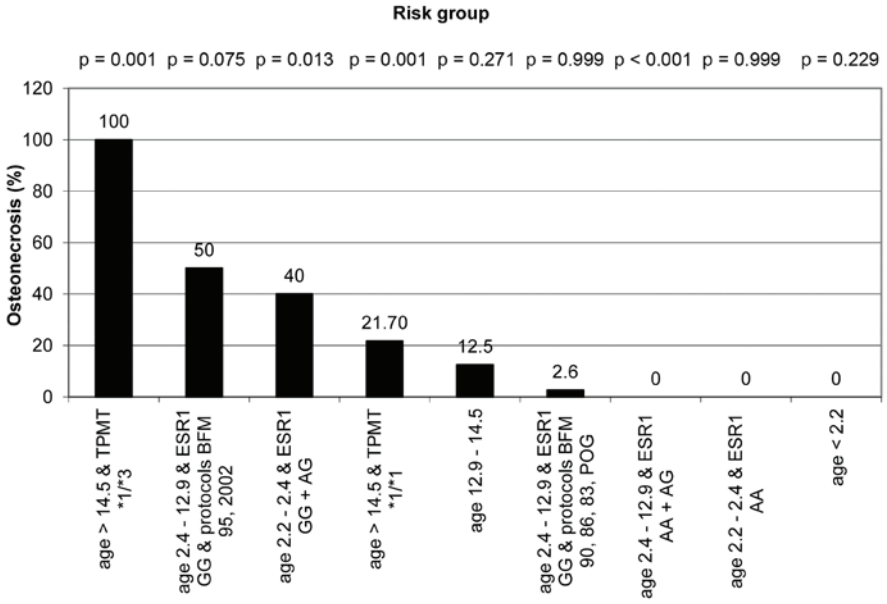

Figure 3. Combined risk factors for osteonecrosis the percentage of patients observed to have osteonecrosis in each risk group (i.e. terminal node of the classification tree); P-values represent comparison to the rest of the observed population. The Fischer's exact test was used for the statistical analysis. TPMT, thiopurine S-methyltransferase; ESR1, estrogen receptor alpha 1; BFM, Berlin-Frankfurt-Münster; POG, pediatric oncology group.

bone marrow cells (32). Since newly formed osteoblasts derive from MSCs residing in bone marrow, we hypothesize that higher levels of TGNs in individuals deficient in TPMT may lead to decreased numbers of MSCs and consequently osteoblasts, leading to the increased ON risk. In this manner, 6-MP could aggravate the effects of GC on MSCs, which include the reduction of MSC number in bone marrow (33) and increased differentiation of MSCs into adipocytes at the expense of osteogenesis (34). Recently it has also been implicated that MSCs may have an important role in the repair process of osteonecrotic lesion (35). MSCs, cultured in the low-oxygen conditions, resembling those in the osteonecrotic lesion, were found to produce high levels of vasculogenic cytokines (35). Since vascular damage is speculated to be one of the primary ON causes, this induction of the vascularization by MSCs may lead to ON improvement (35). Thus, any additional factors decreasing MSC number or differentiation into osteoblasts may worsen the osteonecrotic process, induced by GC. This could explain why only a small fraction of patients receiving GC therapy develop ON and why a relatively small percentage of patients with positive radiograph and MRI osteonecrotic results are symptomatic (35). In the case of 6-MP therapy of the present study, decreased TPMT activity leads to greater bone marrow toxicity and decreased MSC number, which may impair bone formation and repair the forming osteonecrotic lesion. In addition to increased 6-MP toxicity, the impaired TPMT activity may influence bone toxicity through changes in methylation potential. It has been demonstrated *in vitro* that decreased activity of SAM-dependent methyltransferases lead to impaired osteoblast differentiation via DNA-methylation independent mechanism (36). Reduced global methylation decreases the activity of transcription factor Runx2, a key regulator of osteoblast differentiation (36).

In the present study, the *Xba*I polymorphism in the *ESR1* gene represents an ON risk factor only in pre-pubertal children. In children <12.9 years the GG genotype increased ON risk, while AA genotype was protective of ON. ESR1 plays an important role in the regulation of skeletal growth and maintenance of bone mass (37). We investigated a common polymorphism in the *ESR1* gene (*Xba*I), which has already been extensively studied (15,38-40,42). A meta-analysis of association studies involving 5,834 subjects showed a correlation of G allele with higher BMD and decreased risk of fracture (38), while in another large-scale genome-wide study a correlation with fracture that was independent of BMD was observed (15). However, all of the aforementioned studies were performed on adults and not in the context of cancer therapy. In a study by Boot *et al*, the *Xba*I AA genotype was associated with lower lumbar spine BMD in healthy children (39). Furthermore, the effect of ESR1 was more pronounced in pre-pubertal children, which is in accordance with the present results. This may be due to the fact that defects in estrogen receptor have more pronounced clinical consequences in the setting of low estrogen concentrations, such as in pre-pubertal children or post-menopausal women (39). However, the present results indicating *Xba*I GG genotype as risk factor for ON are not in accordance with the results of a previous study on healthy children where the same genotype was associated with higher lumbar spine BMD. Notably, in the aforementioned study (33), *Xba*I was associated with lumbar spine BMD, but not with total body BMD. This may be because the spine is rich in trabecular bone, which has a high bone turnover rate, while the body primarily consists of cortical bone with low turnover (39). Notably, patients in the present study had ON of the hip, where the percent of trabecular bone is lower compared with the spine. There is evidence that estrogens have a more marked effect on cortical than on trabecular bone, possibly due to increased expression of ERa in cortical bone (39). The molecular mechanism by which *ESR1 Xba*I

affects osteoporosis and BMD is unclear; however, there is evidence that it may affect gene transcription. Results of Maruyama *et al* suggest that the presence of *Xba*I G allele can decrease ESR1 activity (40). This is in accordance with the present results that indicate that GG genotype is a risk factor for ON, as decreased levels of estrogen are known to be associated with low BMD and osteoporosis (41). In the context of childhood ALL therapy, polymorphisms in *ESR1* were not correlated with BMD, although they were associated with the recovery of lean body mass after the treatment (42). Finally, all of the discussed previous studies examining the effects of ESR1 on BMD were performed on osteoporotic populations. Although osteoporosis and ON are characterized by low BMD, the pathological mechanism of each diseases is different (1). In osteoporosis, bone resorption and formation are accelerated, while the resorption/formation ratio is increased (43). In ALL therapy-related ON the bone formation is inhibited due to the decreased number of precursor MSCs and their increased differentiation to adipocytes, while at the same time all bone cells are dying at an increased rate due to the decreased level of oxygen and nutrients caused by thrombotic complications (1). This may underlie the discrepancy between the present results regarding osteonecrotic patients and the result of osteoporosis studies in connection to ESR1.

The present exploratory study contained a number of limitations, the first point that needs to be addressed is the number of cases. The study included all pediatric ALL patients from Slovenia from a period of over 30 years. As severe ON is a very rare condition, the number of cases is low despite the lengthy study period. To confirm the present findings, a replication study on another ALL population is required. Secondly, we did not measure BMD in ALL patients as this was not routinely performed during diagnosis and treatment of ALL patients. BMD measurements may offer an additional insight into ON; however, due to the retrospective nature of the study, this was not possible. Thirdly, we employed a hypothesis-based approach and thus did not fully investigate all the possible genetic markers. However, studied genes were selected to cover all three aspects that contribute to the ON development: Bone metabolism (*COL1A1, ESR1, MTHFR*), thrombosis (*MTHFR, ESR1*) and ALL pharmacogenetics (*TPMT*, *MTHFR*). Besides these selected genes, there are numerous other genes that may warrant addition study, such as *ACP-1, PAI-1* and *TYMS*; however, due to the limited quantity of the samples these were impossible to perform. From the same reason, we did not fully cover all genetic variations in the studied genes, but included only those that were clinically and epidemiologically most relevant.

In conclusion, the present retrospective exploratory study of 313 childhood ALL patients suggests that greater age and more recent treatment protocols were independent risk factors for ON. Furthermore, different genetic risk factors were identified in specific age groups. While in patients >14.5 years *TPMT* genotype modulated the risk of ON, in children <12.9 years *ESR1* genotypes were implicated in the pathogenesis of ON. As these genetic markers have potential prognostic value for the prediction of ON in pediatric ALL therapy, these findings require confirmation in a replication study with a larger number of ON patients.

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