Original Article

Phase 2 study for nonmetastatic extremity high-grade osteosarcoma in pediatric and adolescent and young adult patients with a risk-adapted strategy based on ABCB1/P-glycoprotein expression: An Italian Sarcoma Group trial (ISG/OS-2)

Emanuela Palmerini, MD, PhD[1]; Cristina Meazza, MD[2]; Angela Tamburini, MD[3]; Gianni Bisogno, MD[4]; Virginia Ferraresi, MD[5]; Sebastian D. Asaftei, MD[6]; Giuseppe M. Milano, MD[7]; Luca Coccoli, MD[8]; Carla Manzitti, MD[9]; Roberto Luksch, MD[2]; Massimo Serra, BSc[1]; Marco Gambarotti, MD[10]; Davide M. Donati, MD[11,12]; Katia Scotlandi, PhD[13]; Rossella Bertulli, MD[2]; Claudio Favre, MD[3]; Alessandra Longhi, MD[1]; Massimo E. Abate, MD[14]; Silverio Perrotta, MD[15]; Maurizio Mascarin, MD[16]; Paolo D'Angelo, MD[17]; Marilena Cesari, MD[1]; Eric L. Staals, MD, PhD[11,12]; Emanuela Marchesi, BSc[18]; Elisa Carretta, PhD[1]; Toni Ibrahim, MD, PhD[1]; Paolo G. Casali, MD[2]; Piero Picci, MD[18]; Franca Fagioli, MD[6,19]; and Stefano Ferrari, MD[1]

BACKGROUND: According to retrospective osteosarcoma series, ABCB1/P-glycoprotein (Pgp) overexpression predicts for poor outcomes. A prospective trial to assess a risk-adapted treatment strategy using mifamurtide in Pgp+ patients was performed. **METHODS:** This was a phase 2, multicenter, uncontrolled trial including patients 40 years old or younger with nonmetastatic extremity high-grade osteosarcoma stratified according to Pgp expression. All patients received high-dose methotrexate, doxorubicin, and cisplatin (MAP) preoperatively. In Pgp+ patients, mifamurtide was added postoperatively and combined with MAP for a good histologic response (necrosis ≥ 90%; good responders [GRs]) or with high-dose ifosfamide (HDIFO) at 3 $g/m^2/d$ on days 1 to 5 for a histologic response < 90% (poor responders [PRs]). Pgp– patients received MAP postoperatively. After an amendment, the cumulative dose of methotrexate was increased from 60 to 120 g/m^2 (from 5 to 10 courses). The primary end point was event-free survival (EFS). A postamendment analysis was performed. **RESULTS:** In all, 279 patients were recruited, and 194 were included in the postamendment analysis: 70 (36%) were Pgp–, and 124 (64%) were Pgp+. The median follow-up was 51 months. For Pgp+ patients, 5-year EFS after definitive surgery (null hypothesis, 40%) was 69.8% (90% confidence interval [CI], 62.2%-76.2%): 59.8% in PRs and 83.7% in GRs. For Pgp– patients, the 5-year EFS rate was 66.4% (90% CI, 55.6%-75.1%). **CONCLUSIONS:** This study showed that adjuvant mifamurtide, combined with HDIFO for a poor response to induction chemotherapy, could improve EFS in Pgp+ patients. Overall, the outcomes compared favorably with previous series. Mifamurtide and HDIFO as salvage chemotherapy are worth further study. *Cancer* **2022;128:1958-1966.** © 2022 The Authors. *Cancer* published by Wiley Periodicals LLC on behalf of American Cancer Society This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

KEYWORDS: adolescents and young adults (AYAs), ATP binding cassette subfamily B member 1 (ABCB1), chemotherapy, high-grade bone sarcoma, mifamurtide, osteosarcoma, pediatric bone tumors, P-glycoprotein.

INTRODUCTION

High-grade osteosarcoma is the most frequent primary bone tumor that usually occurs in pediatric and adolescent and young adult (AYA) patients.[1-3] A 5-year survival rate of nearly 70% can be expected after multimodality treatments, including wide surgical resection and chemotherapy regimens based on methotrexate, doxorubicin,

and cisplatin (MAP) and ifosfamide.[4-6] In the last 2 decades, a sort of plateau of survival has been reached, and new treatment strategies and agents are being investigated.

A potential strategy for improving the prognosis of nonmetastatic osteosarcoma is treatment differentiation based on risk stratification. There is evidence that the expression of ATP binding cassette subfamily B member 1 (ABCB1), also known as P-glycoprotein (Pgp), an efflux pump that reduces the intracellular concentration of doxorubicin, is associated with poorer survival in patients with osteosarcoma.[7-10] On the basis of these data, the Italian Sarcoma Group (ISG) planned the current study, in which patients with nonmetastatic osteosarcoma were stratified according to the expression of Pgp. The backbone of the treatment was the drugs active in osteosarcoma: methotrexate, doxorubicin, and cisplatin (ie, MAP).

Mifamurtide was approved by the European Medicines Agency for the treatment of patients with a diagnosis of localized osteosarcoma. At that time, there was discussion about a potential synergistic effect of mifamurtide and ifosfamide.

Therefore, mifamurtide was administered postoperatively to high-risk patients with Pgp expression (Pgp+). Moreover, Pgp+ patients who responded poorly to induction chemotherapy also received high-dose ifosfamide (HDIFO).

Mifamurtide is a fully synthetic lipophilic derivative of the muramyl dipeptide that is encapsulated into liposomes. It binds to extracellular toll-like receptor 4 and activates monocytes and macrophages, and it promotes antitumor activity.[11]

The drug was authorized on the basis of the phase 3 INT-0133 trial, which showed a significant benefit of mifamurtide for overall survival (OS; hazard ratio, 0.71; 95% confidence interval [CI], 0.52-0.96; $P = .03$), with the 6-year survival rate improving from 70% to 78%.[12] However, the therapeutic benefit of mifamurtide for patients with nonmetastatic osteosarcoma remains controversial.[13] Moreover, it is unclear which patients could potentially benefit from mifamurtide.

In the current study, the objective was the evaluation of event-free survival (EFS) and OS in pediatric and AYA patients with nonmetastatic osteosarcoma of the extremities treated according to a risk-adapted chemotherapy regimen based on the expression of ABCB1/Pgp.

MATERIALS AND METHODS

Study Design and Participants

ISG/OS-2 was an Italian, phase 2, multicenter, uncontrolled trial evaluating the efficacy and toxicity of risk-adapted chemotherapy regimens; it was conducted from June 2011 to March 2018. Written informed consent was obtained from the patients or, in the case of pediatric subjects, from their guardians. The local ethics committees approved the protocol.

Patients' inclusion and exclusion criteria are summarized in Supporting Table 1 and included the following: a diagnosis of primary, central, high-grade osteosarcoma of the extremity (ie, grade 3 or 4 according to Broders[14]); an age ≤ 40 years; no prior surgery or chemotherapy for osteosarcoma; and no organ dysfunction (normal hepatic, renal, bone marrow, and cardiac function). The main exclusion criteria were metastatic disease at diagnosis (ie, lung nodules more than 1 mm were considered metastatic), previous treatment for osteosarcoma, and medical contraindications (Supporting Table 1).

The assessment of Pgp was centralized at the IRCCS Istituto Ortopedico Rizzoli. Expression of Pgp was evaluated by immunohistochemistry on 4- to 6-μm-thick sections obtained from paraffin-embedded tumor biopsies. Immunohistochemistry was performed via an avidin-biotin peroxidase complex method (Vectastain ABC kit; Vector Laboratories, Inc, Burlingame, California). Pgp immunodetection was performed with 3 monoclonal antibodies that react with different, mutually exclusive epitopes of this protein: JSB-1 (Monosan Sanbio, Uden, the Netherlands), MRK16 (MyBioSource Aurogene Srl, Roma, Italy), and C494 (Invitrogen, Ltd, Paisley, United Kingdom), as previously described.[7] For each specimen, both negative and positive controls for immunostaining were performed. Negative controls were performed by replacement of the primary antibody with normal horse serum. As positive controls, sections of human normal kidney were used because of their physiological overexpression of Pgp in proximal tubuli. In addition, a positive control for the antigenicity of the tumor specimen was performed by incubating 1 tumor section with the V9 anti-vimentin monoclonal antibody (Dako Agilent, Santa Clara, California). A semiquantitative scoring system was used (Supporting Fig. 1).

Interventions

The chemotherapy protocol is outlined in Figure 1. Neoadjuvant chemotherapy consisted of 2 cycles of MAP for all patients enrolled in the study. After removal of the primary tumor, surgical margins were assessed according
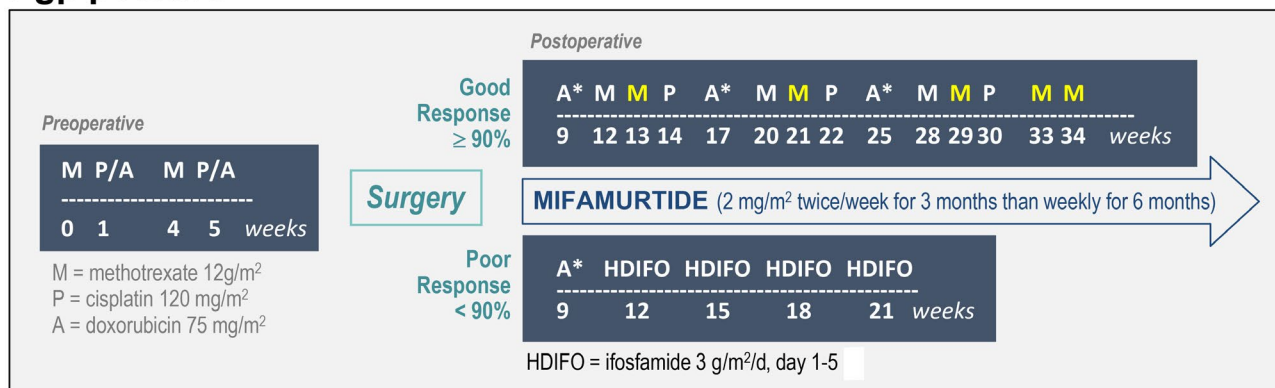
Pgp negative
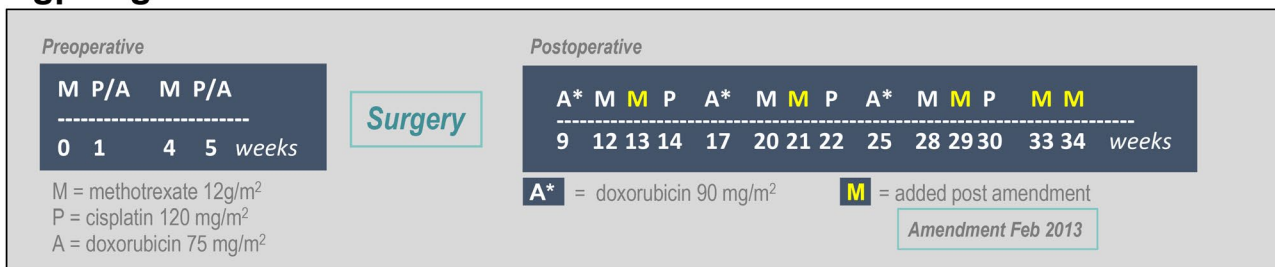
Pgp positive

Figure 1. Chemotherapy and mifamurtide schedule and doses by Pgp expression. Pgp indicates P-glycoprotein.

to Enneking et al,[15] and chemotherapy-induced necrosis was expressed as a percentage. When the percentage of tumor necrosis was 90% or higher, patients were classified as good responders; when the percentage of tumor necrosis was less than 90%, patients were defined as poor responders.

Postoperatively, Pgp– patients continued the 3-drug treatment (MAP) for a total duration of 27 weeks. Pgp+ patients received mifamurtide (2 mg/m^2 twice per week for 3 months and then weekly for 6 months up to a total of 48 administrations as per the approved drug data sheet) in the postoperative phase. For this group, in the case of a good histologic response (tumor necrosis ≥ 90%), postoperative chemotherapy consisted of MAP and mifamurtide for a total duration of 48 weeks. For Pgp+ patients with a poor histologic response (tumor necrosis < 90%), the postoperative treatment was completely modified: after 1 cycle of doxorubicin at 90 mg/m^2, the patients received 4 consecutive cycles of HDIFO (3 g/m^2/d, days 1-5, continuous infusion) and mifamurtide for a total duration of 48 weeks. In February 2013, based on the results of a previous prospective observational study,[16] an amendment increased the total number of methotrexate

cycles from 5 to 10 (all additional cycles delivered postoperatively) for a total of 34 weeks.

The primary tumor was evaluated on plain radiographs, computed tomography (CT) scans, and/or magnetic resonance imaging (MRI) scans.

Baseline studies included CT or MRI of the primary tumor with contrast media, complete blood count and blood chemistry tests, serum electrolytes, serum alkaline phosphatase and lactate dehydrogenase levels, bilirubin and aminotransferase levels, creatinine clearance in 24-hour urine, and echocardiography with the ejection fraction.

Screening for metastases was performed with chest CT scanning and ^{18}F-fludeoxyglucose–positron emission tomography/CT or Tc99 bone scintigraphy.

Eligible patients underwent primary chemotherapy. Before surgery, CT and/or MRI of the primary tumor and CT of the chest were repeated.

At the end of chemotherapy treatment, an x-ray of the operated limb and a chest CT scan were performed, and then clinical and radiological follow-up was started as follows: a chest CT scan every 3 months for 1 year, every 4 months during the second and third years, and

subsequently every 6 months until the fifth year and yearly until 10 years.

If for any reason the expression of Pgp could not be evaluated, the patient was treated according to the ISG/OS/Oss study.

Data collection was performed through an electronic case report form by the ISG (www.isg-area-riservata.org/dh/).

Toxicity data were analyzed and graded according to the Common Toxicity Criteria for Adverse Events (version 4.0).[17]

Outcomes

The primary outcome was the EFS calculated until recurrence (local or distant), death from all causes, the appearance of secondary tumors, or the last follow-up examination. The time-to-event outcome was estimated after definitive surgery for the Pgp+ patients (event-free survival after definitive surgery [EFS-DS]) and from the time to the first day of chemotherapy for Pgp– patients (EFS). The secondary outcome was the OS calculated from the time to the first day of chemotherapy to the date of death or to the date of the last follow-up examination.

Sample Size Considerations

On the basis of previous ISG protocols, an annual enrollment of 45 patients per year was expected, with an expected study duration of 5 years of enrollment and 2 years of observation from last patient enrollment, for a total of 225 patients.

On the basis of data from previous studies, the percentage of patients with Pgp overexpression was estimated to be between 40% and 50% (18-23 patients per year).[7]

The population overexpressing Pgp represents a poor-prognosis population.[7] Under the assumption of a 5-year EFS probability of 40%, a sample of 96 patients with Pgp overexpression had 95% power to detect a 20% increase in 5-year EFS with a 1-sided α error of 5%.[18] Because of the amendment, which increased the number of cycles of methotrexate (from 5 to 10 for a total of 34 weeks), the study duration was increased by 18 months to ensure the planned sample size for the cohort of patients overexpressing Pgp and treated with the postamendment regimen.

Statistical Analysis

The relationship between Pgp expression and histologic response was assessed with the Fisher exact test and relative risks with 95% CIs. Time-to-event outcomes (EFS and OS) along with 90% CIs were estimated with the Kaplan-Meier method. The median follow-up was calculated with the reverse Kaplan-Meier estimator. The postamendment risk-adapted chemotherapy regimen was considered effective in the Pgp-overexpressed population if the lower bound of the 90% CI of the 5-year EFS excluded the null hypothesis of 40%.

Toxicities were summarized in each cohort defined by Pgp expression as frequencies. Moreover, methotrexate-related toxicity was compared between children (younger than 18 years) and adults (18-40 years old) by a logistic regression model using the generalized estimating equations approach and the exchangeable correlation matrix. For all the analyses, a P value < .05 was considered statistically significant. Statistical analyses were performed with SAS 9.4 (SAS Institute, Inc, Cary, North Carolina).

RESULTS

Two hundred seventy-nine patients were treated from June 2011 to March 2018. Pgp was not evaluable for 15 patients, and 70 patients were recruited before the amendment. Overall, 194 patients were included in the postamendment analysis: 64% were Pgp+, and 36% were Pgp– (Fig. 2).

Clinical characteristics are reported in Table 1. The median age was 14 years (range, 4-38 years); 146 patients (75%) were pediatric (younger than 18 years). All cases had a diagnosis of high-grade osteosarcoma: 44 were grade 3 (23%), and 150 (77%) were grade 4. Alkaline phosphatase at diagnosis was available in 177 cases: it was above the normal range (high) in 54 (28%) and within the normal range (low) in 123 (63%).

All patients underwent surgery: resection in 184 patients (95%), amputation in 9 cases (5%), and rotationplasty in 1 patient (0.5%). Margins were adequate (wide or radical) in 190 cases (98%), marginal in 3 cases (1.5%), and contaminated in 1 case (0.5%).

The histologic response was good in 90 patients (46%) and poor in 104 patients (54%). Pgp expression did not have a statistically significant prognostic value for the histologic response to induction chemotherapy: 48% were good responders among patients with positive Pgp, and 44% were good responders among patients with negative Pgp (relative risk, 1.1; 95% CI, 0.8-1.4; $P = .76$).

Outcomes
EFS

The median follow-up time for patients with Pgp expression was 51 months (interquartile range, 36-65 months). The EFS estimates are reported in Table 2 and Figure 3A,C,E. The 5-year EFS-DS rate among Pgp+
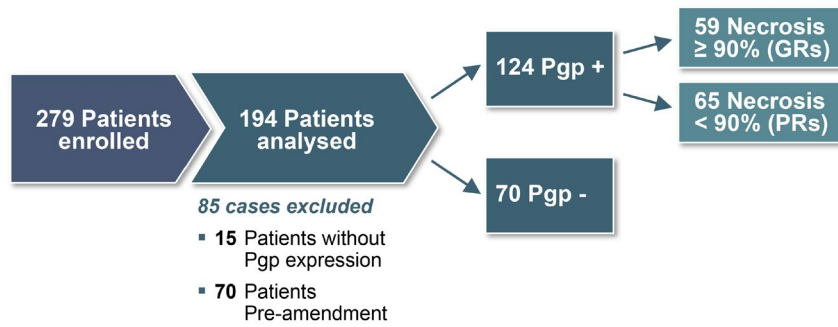
Figure 2. Study profile. GR indicates good responder; Pgp, P-glycoprotein; PR, poor responder.

TABLE 1. Demography at Presentation and Local Treatment for Patients Treated According to the ISG/OS-2 Trial

	ISG/OS-2 Population		
	All (n = 194)	Pgp+ (n = 124)	Pgp– (n = 70)
Age, median (range), y	14 (4-38)	13 (5-33)	15.5 (4-38)
Age group, No. (%)			
<18 y	146 (75)	100 (81)	46 (66)
18-40 y	48 (25)	24 (19)	24 (34)
Gender, No. (%)			
Male	122 (63)	75 (60)	47 (67)
Female	72 (37)	49 (40)	23 (33)
Serum ALP, No. (%)			
High	54 (28)	31 (25)	23 (33)
Normal	123 (63)	85 (69)	38 (54)
Unknown	17 (19)	8 (6)	9 (13)
LDH, No. (%)			
High	47 (24)	28 (23)	19 (27)
Normal	132 (68)	85 (68)	47 (67)
Unknown	15 (8)	11 (9)	4 (6)
Grade, No. (%)			
3	44 (23)	19 (15)	25 (36)
4	150 (77)	105 (85)	45 (64)
Surgery, No. (%)			
Resection	184 (95)	118 (95)	66 (94)
Amputation	9 (5)	6 (5)	3 (4)
Rotationplasty	1 (0)	0	1 (1)
Margin, No. (%)			
Adequate	190 (98)	123 (99)	67 (96)
Inadequate	4 (2)	1 (1)	3 (4)

Abbreviations: ALP, alkaline phosphatase; ISG, Italian Sarcoma Group; LDH, lactate dehydrogenase; Pgp, P-glycoprotein.

patients was 69.8% (90% CI, 62.2%-76.2%). Two patients experienced progression before surgery and were not included in the EFS analysis stratified by necrosis. The 5-year EFS-DS rate among good responders were 83.7% (90% CI, 73.3%-90.2%), whereas among poor responders, it was 59.8% (90% CI, 48.6%-69.3%). The 5-year EFS rate for patients without overexpression of Pgp was 66.4% (90% CI, 55.6%-75.1%).

TABLE 2. EFS-DS, EFS, and OS in Patients With Nonmetastatic Extremity Osteosarcoma Treated in the ISG/OS-2 Trial

	5-y EFS-DS, % (90% CI)	5-y OS, % (90% CI)
Pgp+	69.8 (62.2-76.2)	81.2 (73.4-87.0)
Necrosis < 90% (PRs)	59.8 (48.6-69.3)	74.4 (62.7-82.9)
Necrosis ≥ 90% (GRs)	83.7 (73.3-90.2)	88.5 (76.3-94.6)
	5-y EFS (90% CI)	5-y OS (90% CI)
Pgp–	66.4 (55.6-75.1)	79.4 (67.8-87.2)

Abbreviations: CI, confidence interval; EFS, event-free survival; EFS-DS, event-free survival after definitive surgery; ISG, Italian Sarcoma Group; GR, good responder; OS, overall survival; Pgp, P-glycoprotein; PR, poor responder.

OS

During the follow-up, 31 patients (16%) died. The OS estimates are reported in Table 2 and Figure 3B,D,F. Among patients who overexpressed Pgp, the 5-year OS rate was 81.2% (90% CI, 73.4%-87.0%). As for necrosis after surgery, the 5-year OS rate for good responders was 88.5% (90% CI, 76.3%-94.6%), whereas for poor responders, it was 74.4% (90% CI, 62.7%-82.9%). The 5-year OS rate for patients without overexpression of Pgp was 79.4% (90% CI, 67.8%-87.2%).

Toxicity

One hundred ninety-two patients were evaluable for the safety analysis. Grade 3 or higher toxicities are reported in Table 3, whereas details concerning any grades are reported in the supporting tables (Supporting Tables 2 and 3). Most of the patients (93%) experienced higher grade neutropenia, which was associated with septic death in 1 Pgp+ patient treated with a high dose of ifosfamide.

One hundred twenty patients (63%) experienced at least 1 episode of febrile neutropenia. Grade 3 or 4 anemia in 113 patients (59%) and thrombocytopenia in 136
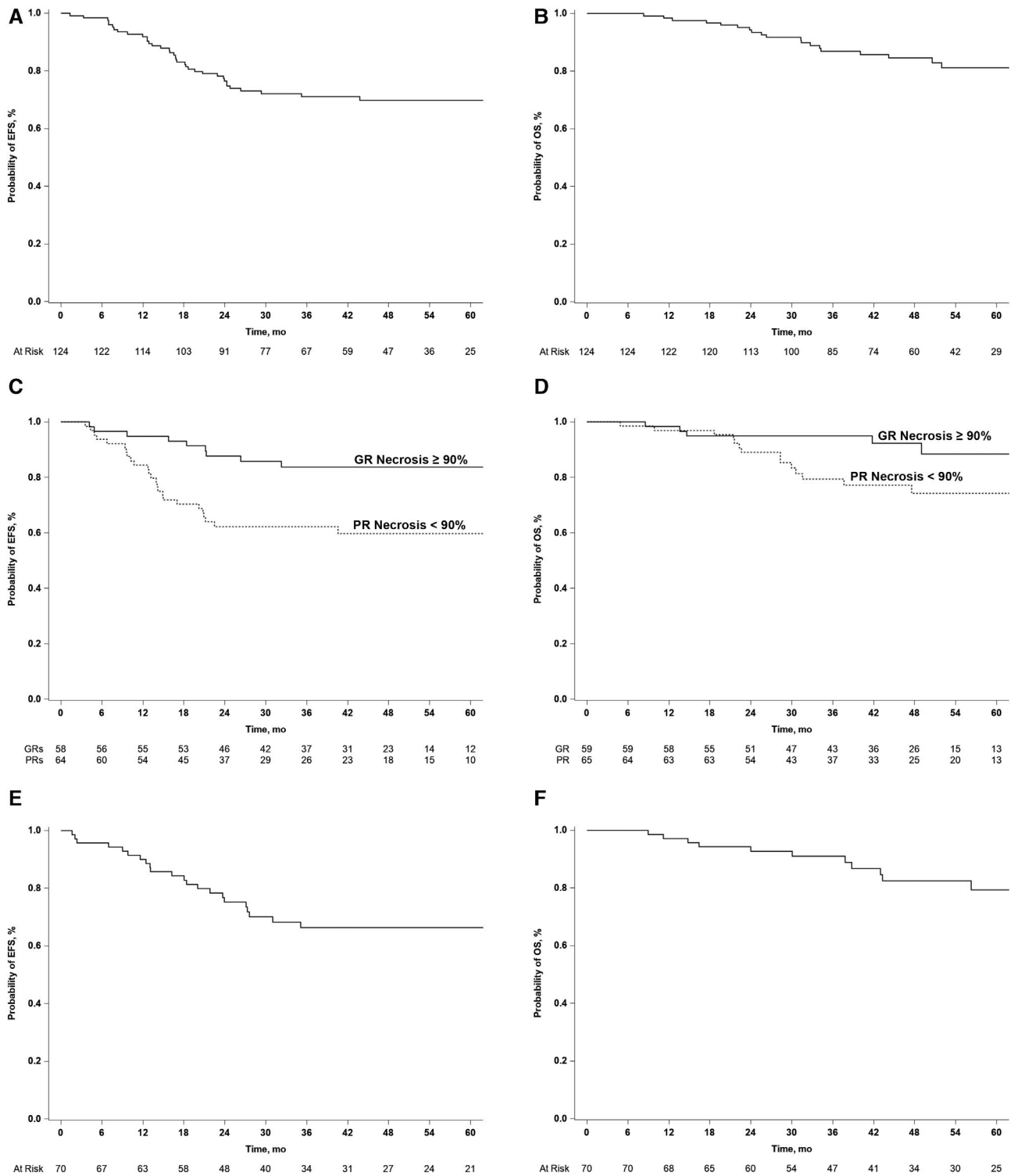
Figure 3. (A) EFS among Pgp+ patients. (B) OS among Pgp+ patients. (C) EFS among Pgp+ patients according to necrosis. (D) OS among Pgp+ patients according to necrosis. (E) EFS among Pgp– patients. (F) OS among Pgp– patients. EFS indicates event-free survival; GR, good responder; OS, overall survival; Pgp, P-glycoprotein; PR, poor responder.

TABLE 3. High-Grade (≥3) Bone Marrow and Organ Toxicities During ISG/OS-2 Treatment in Patients With Nonmetastatic Extremity Osteosarcoma

	Pgp+ (n = 122), No. (%)	Pgp– (n = 70), No. (%)	Overall (n = 192), No. (%)
Leucopenia	107 (88)	63 (90)	170 (89)
Neutropenia	113 (93)	66 (94)	179 (93)
Thrombocytopenia	88 (72)	48 (69)	136 (71)
Anemia	70 (57)	43 (61)	113 (59)
Creatinine	1 (1)	1 (1)	2 (1)
AST	72 (59)	32 (46)	104 (54)
ALT	96 (79)	51 (73)	147 (77)
Central neuropathy	1 (1)	1 (1)	2 (1)
Peripheral neuropathy	1 (1)	2 (3)	3 (2)
Mucositis	18 (15)	15 (21)	33 (17)
Neutropenic fever	73 (60)	47 (67)	120 (63)
Cardiotoxicity	3 (2)	4 (6)	7 (4)
Multiorgan failure	1 (1)	0 (0)	1 (0)

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ISG, Italian Sarcoma Group; Pgp, P-glycoprotein.

patients (71%) were reported. Overall, 113 (59%) and 75 (39%) of the cases required red blood cell and platelet transfusions, respectively (Supporting Table 4).

Grade 3 or 4 peripheral neurological toxicities were described in 3 patients (2%), and central neuropathy was described in 2 patients: 1 after methotrexate and 1 after HDIFO. In both cases with central neuropathy, symptoms resolved after chemotherapy interruption, hydration, diuretics, and methylene blue.

The analysis of methotrexate-related toxicity was performed on the total number of administrated cycles. Delayed methotrexate elimination was recorded for 218 of 1152 methotrexate cycles (19%), with no differences found between pediatric and adult patients (18.7% for <18 years vs 19.8% for 18-40 years; $P = .778$). In 30 of 1152 cases of delayed methotrexate elimination (2.6%), a creatinine increase was reported (Supporting Table 5).

Delayed methotrexate elimination was associated with a grade 3 or 4 aspartate aminotransferase increase in 31% of cycles and with a grade 3 or 4 alanine aminotransferase increase in 50% of cycles (Supporting Table 6). Hypertransaminemia was more frequent in children than adults (aspartate aminotransferase, 37.7% for <18 years vs 11.8% for 18-40 years; $P = .034$; alanine aminotransferase, 58.7% for <18 years vs 21.6% for 18-40 years; $P = .0060$).

DISCUSSION

This is the first prospective study of osteosarcoma using a risk-adapted strategy based on a prognostic factor. Pgp has been associated with poor survival for patients with osteosarcoma in several studies.[19-22] This has been attributed to both Pgp-mediated drug efflux through the plasma membrane and increased Pgp-mediated drug trapping within lysosomal drug "safe houses."[23] Some controversy exists about the role of immunohistochemical expression of Pgp in patients with osteosarcoma,[7,9,19] and differences in methodology for the immunohistochemical detection of Pgp might jeopardize the interpretation of results. Pgp assessment was centralized in the current study to avoid this bias.

In comparison with previous osteosarcoma cohorts,[7,9] Pgp overexpression was more frequent (58%) in the current trial, and this resulted in an overaccrual of 29% with respect to the original design. As in other studies, there were no differences in the histologic response to induction chemotherapy between Pgp+ and Pgp– cases.[7]

In this study, all patients received MAP for induction chemotherapy, but patients with Pgp expression received adjuvant mifamurtide. Moreover, for Pgp+ patients with a poor response to the preoperative treatment, mifamurtide was combined with 4 consecutive cycles of HDIFO. The group of Pgp+ cases had superior EFS in comparison with previous reports.[7,8]

In addition, the 5-year EFS rate of the current series, 69.8% (90% CI, 62.2%-76.2%), was superior to the most favorable hypotheses of the historical cohorts (upper 95% CI, 49.9%[7] and 61.1%[8]).

Interestingly, the chemotherapy doses in the trial published in 2006 (ISG/SSG1) were similar to those in the current trial: methotrexate at 12 g/m^2, doxorubicin at 75 mg/m^2 preoperatively, and doxorubicin at 90 mg/m^2 postoperatively.

Furthermore, in the subgroup of Pgp+ patients with a poor histologic response (<90% necrosis) to induction chemotherapy, the 5-year EFS rate was 59.8%, which was better than the 5-year EFS in the studies by Serra et al.[7,8] This finding needs to be confirmed by further investigations.

The major limitation of this clinical trial was the lack of a control group due to inherent difficulties in running a randomized trial in the adjuvant setting of a rare disease. Nonetheless, compared with the 5-year EFS for Pgp+ patients in previous trials by Serra et al,[7,8] superior EFS was demonstrated with the current approach. However, we cannot completely rule out that different cumulative doses of methotrexate among trials might have affected outcomes.

In this study, the histologic response to induction chemotherapy remained a strong prognostic predictor within the Pgp+ group.

The results of this clinical trial showed improved survival in comparison with previous ISG studies[19] for the

group of Pgp+ patients. The impact on EFS of ifosfamide, which was added in the case of Pgp positivity and a poor response to preoperative chemotherapy, remains unknown, nor is it clear whether the interaction with mifamurtide was synergic. The European and American Osteosarcoma Study (EURAMOS) study has shown that ifosfamide at 2.8 g/m^2, combined with etoposide at 100 mg/m^2 per day, on days 1 to 5 does not improve survival, increases toxicity, and might not be considered as a salvage therapy for high-risk patients. Compared with the EFS of patients with localized osteosarcoma in the EURAMOS trial,[24,25] EFS in our study was similar for both poor and good responders who were Pgp+. Furthermore, the incidence of central or peripheral neuropathy was rare and no different among patients undergoing MAP only (Pgp−), MAP and mifamurtide, or MAP, HDIFO, and mifamurtide.

Because the most important survival benefit in this study was obtained for Pgp+ patients with a poor response to induction chemotherapy who were treated with both mifamurtide and HDIFO, a positive interaction between these drugs could be hypothesized, and this combination might be proposed as a salvage treatment for unresponsive patients with osteosarcoma. One study from the Children's Oncology Group (INT-0133), using a randomized 2-by-2 factorial design and including patients younger than 30 years with localized osteosarcoma, already hypothesized this interaction. The group's study assigned patients to 4 regimens, including 2 arms with mifamurtide given from diagnosis. This study reported a 3-year EFS rate of 68% in the group that received a combination of mifamurtide and MAP (with methotrexate up to 20 g/m^2 per cycle) and a rate of 78% for those who received mifamurtide combined with MAP and ifosfamide (9 g/m^2 per cycle). In a follow-up study,[12] mifamurtide was associated with an OS benefit and a positive EFS trend. On the basis of these results, an interaction with ifosfamide was hypothesized, but concerns about whether INT-0133's results met generally accepted standards for practice-changing conclusions were raised.[25]

Some studies have addressed the question whether or not high-dose methotrexate is essential for adequate treatment of osteosarcoma, and it is now well established that outcomes are improved with methotrexate used at high doses (12 g/m^2).[26] Nonetheless, this treatment should be performed at specialized tertiary centers because of the risk of nephrotoxicity, which increases with age.[27] For this reason, methotrexate use has been limited to patients younger than 25 years in other osteosarcoma trials.[28] The current study has confirmed that methotrexate at 12 g/m^2

is safe in pediatric and AYA patients with high-grade osteosarcoma. We did not find differences in the delayed elimination of methotrexate and creatinine increases between pediatric and adult patients. Unexpectedly, this trial showed a clear survival benefit when 10 methotrexate cycles were administered instead of 5 cycles. It might be hypothesized that the cumulative dose of methotrexate is important in the setting of adjuvant chemotherapy for osteosarcoma.

In conclusion, this is a prospective, risk-adapted, uncontrolled trial of the adjuvant treatment of pediatric and AYA patients with high-grade localized osteosarcoma of the extremities stratified according to Pgp expression.

We showed improved EFS in comparison with historical data for patients overexpressing Pgp and treated with risk-adapted postoperative chemotherapy and adjuvant mifamurtide.

Key findings from this study are the importance of the cumulative dose of methotrexate and the potential role of HDIFO combined with mifamurtide as a salvage treatment for cases with an inadequate histologic response to induction chemotherapy.

These results might pave the path for future treatment strategies in select high-risk osteosarcoma groups. No specific indications on the generalized use of mifamurtide should be derived from the current study.

FUNDING SUPPORT

CONFLICT OF INTEREST DISCLOSURES

Emanuela Palmerini has served on advisory boards for Amgen, Daiichi Sankyo, Lilly, Deciphera, Eusa Pharma, and SynOx Therapeutics; has received other research support from Bristol-Myers Squibb, Pfizer, PharmaMar, Daiichi Sankyo, and Incyte; and has received travel support from Lilly, PharmaMar, and Takeda. Virginia Ferraresi has served on advisory boards for Bristol-Myers Squibb, Novartis, MSD, Pierre-Fabre, and PharmaMar. Giuseppe M. Milano has served on advisory boards for GSK and Pfizer. Marco Gambarotti has received institutional funding from Amgen outside the submitted work. Paolo D'Angelo reports being a member of the Associazione Italiana di Ematologia e Oncologia Pediatrica and the International Society of Paediatric Oncology. Eric L. Staals has served on advisory boards for Daiichi Sankyo. Toni Ibrahim reports participation on a board for Amgen-PharmaMar. Piero Picci has received institutional funding from Amgen and PharmaMar outside the submitted work. Stefano Ferrari has received honoraria from Takeda and PharmaMar. The other authors made no disclosures.

AUTHOR CONTRIBUTIONS

Emanuela Palmerini: Acquisition, analysis, and interpretation of research data and drafting of significant parts of the work or critical revisions to

contribute to the interpretation. **Cristina Meazza:** Acquisition, analysis, and interpretation of research data. **Angela Tamburini:** Acquisition, analysis, and interpretation of research data. **Gianni Bisogno:** Acquisition, analysis, and interpretation of research data. **Virginia Ferraresi:** Acquisition, analysis, and interpretation of research data. **Sebastian D. Asaftei:** Acquisition, analysis, and interpretation of research data. **Giuseppe M. Milano:** Acquisition, analysis, and interpretation of research data. **Luca Coccoli:** Acquisition, analysis, and interpretation of research data. **Carla Manzitti:** Acquisition, analysis, and interpretation of research data. **Roberto Luksch:** Acquisition, analysis, and interpretation of research data. **Massimo Serra:** Acquisition, analysis, and interpretation of research data. **Marco Gambarotti:** Acquisition, analysis, and interpretation of research data and drafting of significant parts of the work or critical revisions to contribute to the interpretation. **Davide M. Donati:** Acquisition, analysis, and interpretation of research data. **Katia Scotlandi:** Acquisition, analysis, and interpretation of research data. **Rossella Bertulli:** Acquisition, analysis, and interpretation of research data. **Claudio Favre:** Acquisition, analysis, and interpretation of research data. **Alessandra Longhi:** Acquisition, analysis, and interpretation of research data. **Massimo E. Abate:** Acquisition, analysis, and interpretation of research data. **Silverio Perrotta:** Acquisition, analysis, and interpretation of research data. **Maurizio Mascarin:** Acquisition, analysis, and interpretation of research data. **Paolo D'Angelo:** Acquisition, analysis, and interpretation of research data. **Marilena Cesari:** Acquisition, analysis, and interpretation of research data. **Eric L. Staals:** Acquisition, analysis, and interpretation of research data and drafting of significant parts of the work or critical revisions to contribute to the interpretation. **Emanuela Marchesi:** Acquisition, analysis, and interpretation of research data. **Elisa Carretta:** Acquisition, analysis, and interpretation of research data and drafting of significant parts of the work or critical revisions to contribute to the interpretation. **Paolo G. Casali:** Conception and design of the work; acquisition, analysis, and interpretation of research data; and drafting of significant parts of the work or critical revisions to contribute to the interpretation. **Piero Picci:** Conception and design of the work; acquisition, analysis, and interpretation of research data; and drafting of significant parts of the work or critical revisions to contribute to the interpretation. **Franca Fagioli:** Conception and design of the work and drafting of significant parts of the work or critical revisions to contribute to the interpretation. **Stefano Ferrari:** Conception and design of the work; acquisition, analysis, and interpretation of research data; and drafting of significant parts of the work or critical revisions to contribute to the interpretation. All authors gave final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

REFERENCES

1. Gitelis S. Bone and Soft Tissue Tumors: Clinical Features, Imaging, Pathology and Treatment. 2nd ed. *J Bone Joint Surg Am.* 2001;83:801-802.
2. Tilsley O. Soft tissue and bone tumours in adults. In: Hanna L, Crosby T, Macbeth F, eds. Practical Clinical Oncology. Cambridge University Press; 2008:335-346.
3. Baumhoer D, Böhling, TO, & Cates, JMM, et al. Osteosarcoma. In: WHO Classification of Tumours - Soft Tissue and Bone Tumours. 5th ed. IARC Press; 2020:403-409.
4. Strauss SJ, Frezza AM, Abecassis N, et al. Bone sarcomas: ESMO-EURACAN-GENTURIS-ERN PaedCan clinical practice guideline for diagnosis, treatment and follow-up. *Ann Oncol.* 2021;32, 1520-1536. https://doi.org/10.1016/j.annonc.2021.08.1995
5. Bielack SS, Kempf-Bielack B, Delling G, et al. Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. *J Clin Oncol.* 2002;20:776-790.
6. Ferrari S, Palmerini E. Adjuvant and neoadjuvant combination chemotherapy for osteogenic sarcoma. *Curr Opin Oncol.* 2007;19:341-346.
7. Serra M, Scotlandi K, Reverter-Branchat G, et al. Value of P-glycoprotein and clinicopathologic factors as the basis for new treatment strategies in high-grade osteosarcoma of the extremities. *J Clin Oncol.* 2003;21:536-542.
8. Serra M, Pasello M, Manara MC, et al. May P-glycoprotein status be used to stratify high-grade osteosarcoma patients? Results from the Italian/Scandinavian Sarcoma Group 1 treatment protocol. *Int J Oncol.* 2006;29:1459-1468.
9. Zhao ZG, Ding F, Liu M, Ma DZ, Zheng CK, Kan WS. Association between P-glycoprotein expression and response to chemotherapy in patients with osteosarcoma: a systematic and meta-analysis. *J Cancer Res Ther.* 2014;10:C206-C209.
10. Pakos EE, Ioannidis JPA. The association of P-glycoprotein with response to chemotherapy and clinical outcome in patients with osteosarcoma: a meta-analysis. *Cancer.* 2003;98:581-589.
11. Kleinerman ES, Jia SF, Griffin J, Seibel NL, Benjamin RS, Jaffe N. Phase II study of liposomal muramyl tripeptide in osteosarcoma: the cytokine cascade and monocyte activation following administration. *J Clin Oncol.* 1992;10:1310-1316.
12. Meyers PA, Schwartz CL, Krailo MD, et al. Osteosarcoma: the addition of muramyl tripeptide to chemotherapy improves overall survival—a report from the Children's Oncology Group. *J Clin Oncol.* 2008;26:633-638.
13. Bielack SS, Hecker-Nolting S, Blattmann C, Kager L. Advances in the management of osteosarcoma. *F1000Res.* 2016;5:2767.
14. Broders AC. Squamous-cell epithelioma of the lip: a study of five hundred and thirty-seven cases. *J Am Med Assoc.* 1920;74:656-664.
15. Enneking WF, Spanier SS, Goodman MA. A system for the surgical staging of musculoskeletal sarcoma. *Clin Orthop Relat Res.* 1980;153:106-120.
16. Ferrari S, Meazza C, Palmerini E, et al. Nonmetastatic osteosarcoma of the extremity. Neoadjuvant chemotherapy with methotrexate, cisplatin, doxorubicin and ifosfamide. An Italian Sarcoma Group study (ISG/OS-Oss). *Tumori.* 2014;100:612-619.
17. Common Terminology Criteria for Adverse Events (CTCAE) v4.0. National Cancer Institute. https://ctep.cancer.gov/protocoldevelopment/electronic_applications/ctc.htm#ctc_40
18. Brookmeyer R, Crowley J. A confidence interval for the median survival time. *Biometrics.* 1982;38:29.
19. Serra M, Picci P, Ferrari S, Bacci G. Prognostic value of P-glycoprotein in high-grade osteosarcoma. *J Clin Oncol.* 2007;25:4858-4860.
20. Liu T, Li Z, Zhang Q, et al. Targeting ABCB1 (MDR1) in multi-drug resistant osteosarcoma cells using the CRISPR-Cas9 system to reverse drug resistance. *Oncotarget.* 2016;7:83502-83513.
21. Smeland S, Bielack SS, Whelan J, et al. Survival and prognosis with osteosarcoma: outcomes in more than 2000 patients in the EURAMOS-1 (European and American Osteosarcoma Study) cohort. *Eur J Cancer.* 2019;109:36-50.
22. Schwartz CL, Gorlick R, Teot L, et al. Multiple drug resistance in osteogenic sarcoma: INT0133 from the Children's Oncology Group. *J Clin Oncol.* 2007;25:2057-2062.
23. Seebacher NA, Richardson DR, Jansson PJ. A mechanism for overcoming P-glycoprotein-mediated drug resistance: novel combination therapy that releases stored doxorubicin from lysosomes via lysosomal permeabilization using Dp44mT or DpC. *Cell Death Dis.* 2016;7:e2510.
24. Marina NM, Smeland S, Bielack SS, et al. Comparison of MAPIE versus MAP in patients with a poor response to preoperative chemotherapy for newly diagnosed high-grade osteosarcoma (EURAMOS-1): an open-label, international, randomised controlled trial. *Lancet Oncol.* 2016;17:1396-1408.
25. Bielack SS, Smeland S, Whelan JS, et al. Methotrexate, doxorubicin, and cisplatin (MAP) plus maintenance pegylated interferon alfa-2b versus MAP alone in patients with resectable high-grade osteosarcoma and good histologic response to preoperative MAP: first results of the EURAMOS-1 good response randomized controlled trial. *J Clin Oncol.* 2015;33:2279-2287. Erratum in: *J Clin Oncol.* 2016;34:4059.
26. Anninga JK, Gelderblom H, Fiocco M, et al. Chemotherapeutic adjuvant treatment for osteosarcoma: where do we stand? *Eur J Cancer.* 2011;47:2431-2445.
27. Ferrari S, Bielack SS, Smeland S, et al. EURO-B.O.S.S.: a European study on chemotherapy in bone-sarcoma patients aged over 40: outcome in primary high-grade osteosarcoma. *Tumori.* 2018;104:30-36.
28. Gaspar N, Occean BV, Pacquement H, et al. Results of methotrexate-etoposide-ifosfamide based regimen (M-EI) in osteosarcoma patients included in the French OS2006/sarcome-09 study. *Eur J Cancer.* 2018;88:57-66.