**Diplomarbeit**

# Methotrexate concentrations in third space fluids in comparison with serum concentrations in patients with osteosarcoma


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Graz, 02.03.2014 Claudia Heu

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# Zusammenfassung

Hintergrund:

Methotrexat als eines der für die Behandlung von Osteosarkomen eingesetzten Chemotherapeutika erzeugt dosisabhängig Nebenwirkungen. Durch die Akkumulation in Geweben, Körperhöhlen und so genannten Dritträumen (periprothetischen Seromen) kommt es zur verzögerten Elimination und dem Auftreten toxischer Effekte. Um dies zu vermeiden, werden periprothetische Serome in unserer Abteilung trotz erhöhtem Infektionsrisiko punktiert. Ziel unserer Studie ist, Konzentrationen der Punktate mit Konzentrationen der Blutspiegel zu vergleichen, um eventuell Drittraum-Effekte fassbar zu machen.

Methodik:

In einer monozentrischen Datenanalyse wurden retrospektiv zwischen 1991 und 2011 45 mit Endoprothesen versorgte und mit hoch-dosiertem Methotrexat behandelte Osteosarkom Patientinnen und Patienten hinsichtlich der Methotrexat Konzentrationen in periprothetischen Seromen und korrespondierenden Blutspiegeln verglichen. Die statistische Auswertung erfolgte anonymisiert mit Wilcoxon Vorzeichen-Rang-Test im SPSS (für Mac, 20.0).

Ergebnisse:

112 Dritträume wurden bei 18 der 45 Patientinnen und Patienten punktiert. (Median 5 Punktionen pro Patient, Bereich 1-20 Punktionen pro Patient). Die Punktatmenge war in 101 Fällen dokumentiert und reichte von 5-420ml (Median 150ml). Methotrexat Konzentrationen wurden in 61,11% aller Punktionen bestimmt.

Die 24 Stunden Methotrexat Konzentrationen in periprothetischen Seromen waren zwischen 1,49-42,97 (Median 14,86) Mal höher als entsprechende Blutspiegel Konzentrationen. Die 48 und 72 Stunden Methotrexat Konzentrationen in periprothetischen Seromen zeigten im Median ebenfalls Erhöhungen: 8,50 (Bereich 1,36-52,56) und 2,66 (Bereich 0,66–5,82) Mal höhere Werte als im korrespondieren Blutspiegel. Speziell die 24 Stunden Messungen der Methotrexat Konzentrationen in periprothetischen Seromen erreichten toxische Werte bis 170,74µmol/L (Median 109,83µmol/L, Bereich 4,91-170,71µmol/L) im Vergleich zu 3,09µmol/L im Median vom Blutspiegel (Bereich 0,37-45,0µmol/L, p=0,001 (Wilcoxon Vorzeichen-Rang-Test)). Ähnlich statistisch signifikante Differenzen wurden bei 48 ($p<0,001$) und 72 Stunden ($p=0.015$) beobachtet.

Schlussfolgerungen:
Diese Studie zeigt, dass die Methotrexat Konzentrationen in periprothetischen Seromen signifikant höher sind als in korrespondierenden Blutspiegeln. Der Konzentrationsunterschied ist hinweisend auf einen Drittraum-Effekt. Aus diesem Grund sollten periprothetische Serome punktiert werden, um Toxizitäten durch erhöhte Methotrexat Konzentrationen in jedem Fall zu vermeiden.

Schlüsselwörter:
Dritträume, periprothetische Serome, hoch dosiertes Methotrexat, Drittraum-Effekte, Osteosarkom

# Abstract

Background:

Besides surgery, high-dose Methotrexate is a mainstay of osteosarcoma treatment. However, it is associated with severe adverse effects, which are partly dose-dependent. Methotrexate is known to accumulate in tissues, cavities and so-called third spaces (periprosthetic seromas) leading to local toxicity and delayed elimination. To avoid this, considerable periprosthetic seromas are punctured in our department. We compared the concentrations of Methotrexate in serum and periprosthetic seromas to describe a potential toxic risk based on a third space effect.

Methods:

In 45 osteosarcoma patients who were treated with endoprosthesis and high-dose Methotrexate, we retrospectively analysed Methotrexate concentrations in periprosthetic seromas and serum. Differences were assessed by means of a Wilcoxon test.

Results:

112 periprosthetic seroma punctures were performed in 18 of 45 patients. Methotrexate concentrations were determined in 61.1% of all punctures. At 24 hours the periprosthetic seroma concentrations were 1.49-42.97 (median 14.86, $p=0.001$) and at 48 hours 1.36-52.56 (median 8.50, $p<0.001$) higher than the corresponding serum levels. At 72 hours the periprosthetic seroma concentrations were in median 2.66 ($p=0.015$, range 0.66-5.82) times higher than the corresponding serum levels. At 24 hours, highly toxic concentrations were observed in seromas (median 109.83, range 4.91-170.71µmol/L).

Conclusions:

Methotrexate concentrations of periprosthetic seromas are significantly higher than corresponding serum levels possibly contributing to a third space effect. To avoid severe adverse effects puncture of these effusions should be considered.



Key words:

Third space, periprosthetic seroma, high-dose Methotrexate, third space effect, Osteosarcoma

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# Abbreviations

| | |
|---|---|
| A / DOX | Doxorubicin (=Adriamycin, A) |
| AP | Alkaline phosphatase |
| BSA | Body surface area |
| CNB | Core needle biopsy |
| COG | North American Children's Oncology Group |
| COSS | German – Austrian – Swiss – Cooperative Osteosarcoma Study Group |
| CRP | C-reactive protein |
| CT | Computerized tomography |
| DDP/ CDDP /P | Cisplatin (=Cis – diaminedichloroplatinum II, CDDP; Platinol, P) |
| DHF | Dihydrofolate |
| DHFR | Dihydrofolate reductase |
| DNA | Desoxyribunucleic acid |
| E / ETO | Etoposide |
| EFS | Event - free survival |
| EGF | Epidermal growth factor |
| EOI | European Osteosarcoma Intergroup |
| FNAB | Fine needle aspiration biopsy |
| GFR | Glomerular filtration rate |
| HDMTX | High-Dose Methotrexate |
| HER2/erbB2 | factor of human epidermal growth factor receptor 2, a proto-oncogene |
| HGF | Hepatocyte growth factor |
| I / IFO / IFX | Ifosfamide |
| ICD-O | International Classification of Diseases for Oncology |
| IFNα | Pegylated interferon α-2b |
| K7M2 | metalloproteinase |
| LDH | Lactate dehydrogenase |
| LV | Leucovorin |
| M / MTX | Methotrexate (=Amethopterin) |
| MIF | Migration inhibitory factor |

| | |
|---|---|
| MRI | Magnet resonance imaging |
| NSAIDs | Nonsteroidal anti-inflammatory drugs |
| OS | Overall survival |
| PET | Positron emission tomography |
| PRL | Prolactin |
| RB gene | Rentinoplastoma protein gene, a tumour suppressor gene |
| RG | Regression grade by Salzer - Kuntschik |
| SOX9 | Gene in humans |
| SSG | Scandinavian Sarcoma Group |
| THF | Tetrahydrofolate |
| TNF | Tumour necrosis factor |
| TNM | Classification of primary tumour, regional lymph nodes and distant metastasis |
| WBC | White blood cell |
| WHO | World Health Organisation |
| κB RANK-ligand | Receptor activator of nuclear factor κB, a membrane protein of osteoclasts |

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# 1 Introduction

An osteosarcoma is the most common primary malignancy of the bones that mainly occurs in children and adolescents. It is an aggressive disease with a poor prognosis and a three-year overall survival (OS) of approximately 60-90% (1,2). The treatment requires a multidisciplinary collaboration of oncologists, radiologists, pathologists and surgeons.

Currently, the treatment consists of a wide, if possible en-block, resection of the tumour and a multi-agent chemotherapy. The most frequently used antineoplastic agents are Doxorubicin, Cisplatin, Methotrexate, Ifosfamide and Etoposide. The cytotoxic agents are administered according to the EURAMOS-1 trial. This is a collaboration of different osteosarcoma study groups with the aim of improving the treatment for patients with osteosarcoma (1,3,4).

However, the Methotrexate (MTX) used in osteosarcoma treatment is associated with severe adverse effects, which are partly dose-dependent. Among other toxicities (see Appendix 1), MTX particularly tends to accumulate in tissues, cavities and so-called third spaces. Those third spaces are assumed to be responsible for an increased incidence of toxicities. Several studies report that third spaces can result in delayed elimination of MTX and even in drug related deaths (4–7), but to our knowledge there is no one with a third space in form of a periprosthetic seroma. Most third spaces concern ascites, pleural effusions or oedematous tissues (4,6,8–11).

We therefore conducted a retrospective, monocentric study analysing MTX concentrations in patients treated because of an osteosarcoma, both in serum and in those with a periprosthetic seroma. Patients who received a limb salvage surgery with endoprosthesis could only be included. We compared the serum MTX levels among themselves and with MTX concentrations in periprosthetic seromas, our so-called third spaces.

The aim of our study was to verify a potential toxic risk based on a third space effect, emanating from the periprosthetic seroma. Furthermore we want to confirm the necessity of punctures despite the increased risk of infections in immunodeficient patients during chemotherapy.

# 2 Background

The following section provides an overview of osteosarcomas and their treatment. Bone tumours are rare and difficult to diagnose. In many cases, the diagnostic steps take several weeks until the patient is allocated to a reference centre. It is therefore important to know about clinical appearance, grading and staging in order to avoid wasting time unnecessarily. The chapter titled “diagnosis” deals with investigations to be performed as well as the typical radiological signs of osteosarcomas. The section “background information” also contains general information about epidemiology and aetiology. Furthermore, the classification of bone tumours of the World Health Organisation (WHO) and histological subtypes are explained. There are several systems of classification, but none of them is perfect and therefore generally accepted.

## *2.1 Osteosarcoma*

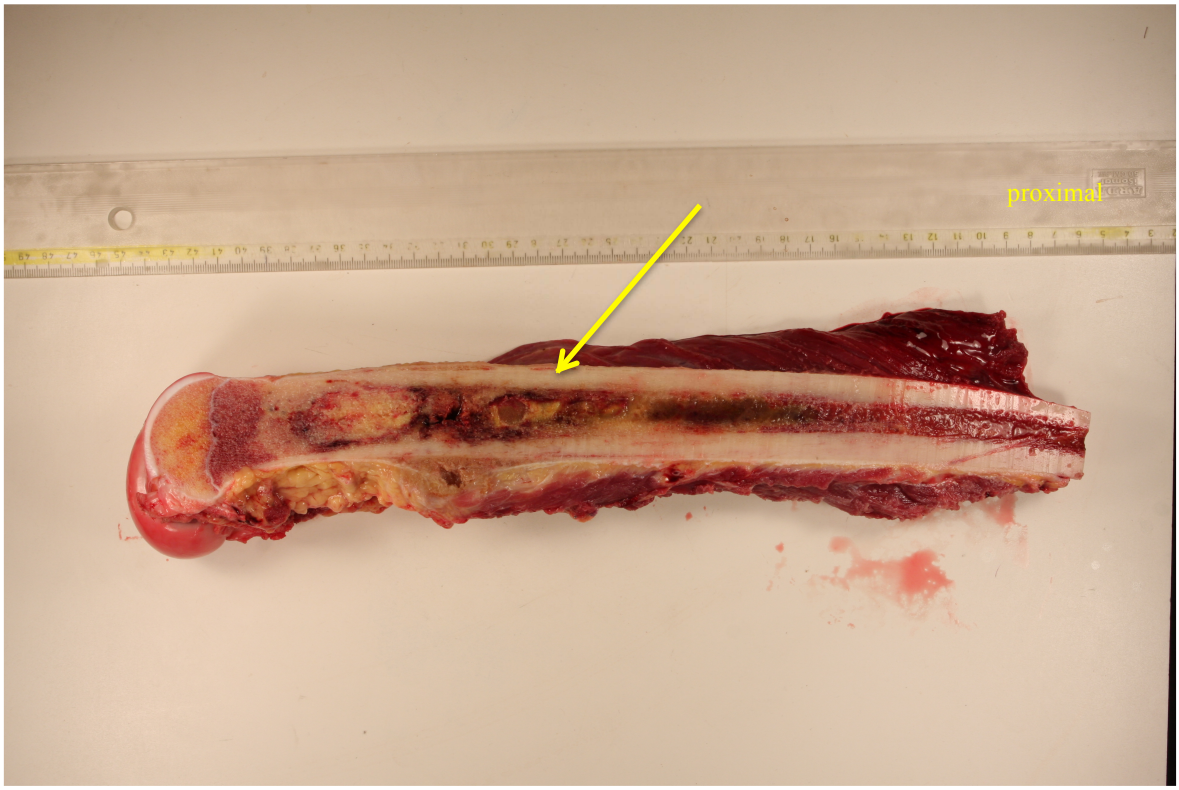



Figure 1 Resection specimen of a left distal femur of a 15-year-old male with an osteosarcoma.

The Department of Orthopaedics and Orthopaedic surgery at the Medical University of Graz is a reference centre for bone tumours. Together with the Division of Clinical Paediatric Oncology and the Division of Clinical Oncology about 90 patients with primary malignant bone and soft tissue tumours are treated annually.

Figure 1 shows a specimen after surgery of an osteosarcoma in the left distal femur of a 15-year-old male. The surgery criteria and methods are explained later in chapter 2.2.3, however, at this point it should be considered that surgery alone leads to a poor outcome for patients with osteosarcoma. Furthermore, it should be taken into account that, despite improved surgical methods, the limb salvage surgery is only recommended if the tumour can be removed as a single block within safety margins, otherwise an amputation might be advantageous.

Figure 2 shows a specimen after surgery of an osteosarcoma of the left proximal tibia of a 10-year-old male. The tumour was removed en-block with a closed capsule and wide margins. In this patient a limb salvage surgery with endoprosthesis was performed. Figure 3 shows the intraoperative implanting of an endoprosthesis.

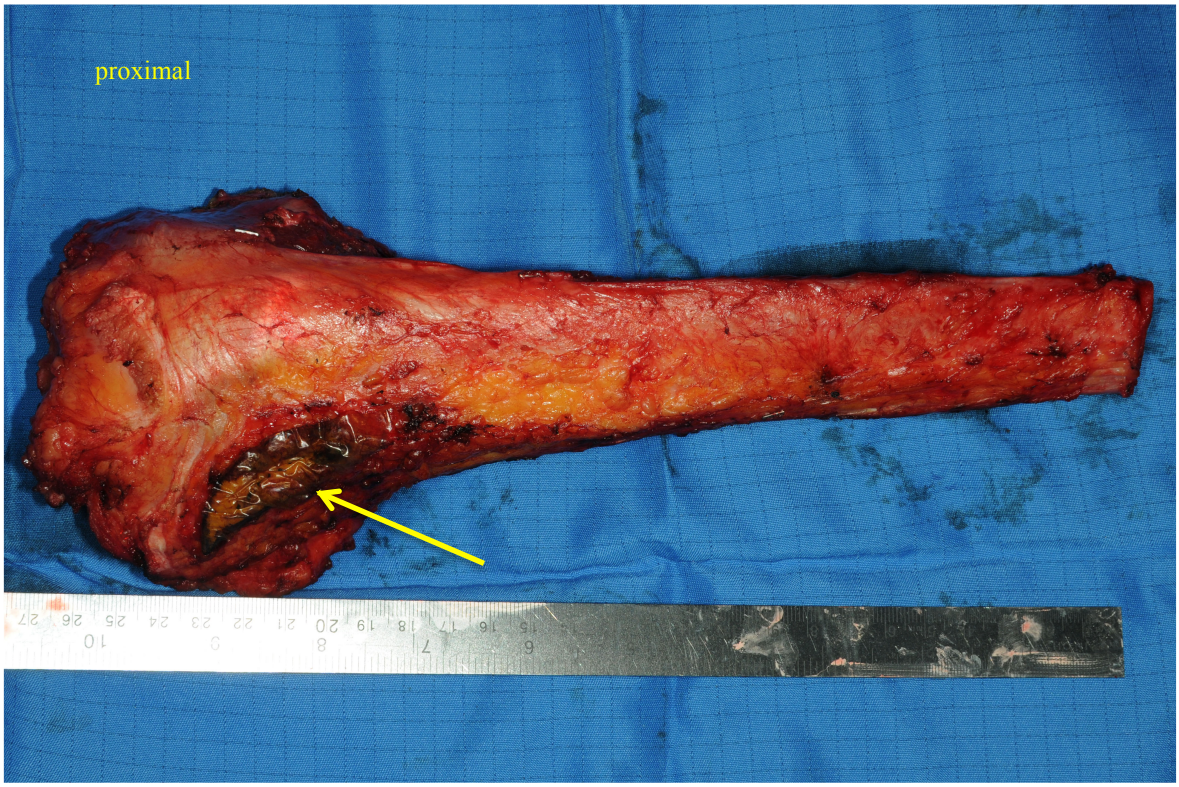



Figure 2 Resection specimen of a left proximal tibia of a 10-year-old male with an osteosarcoma.

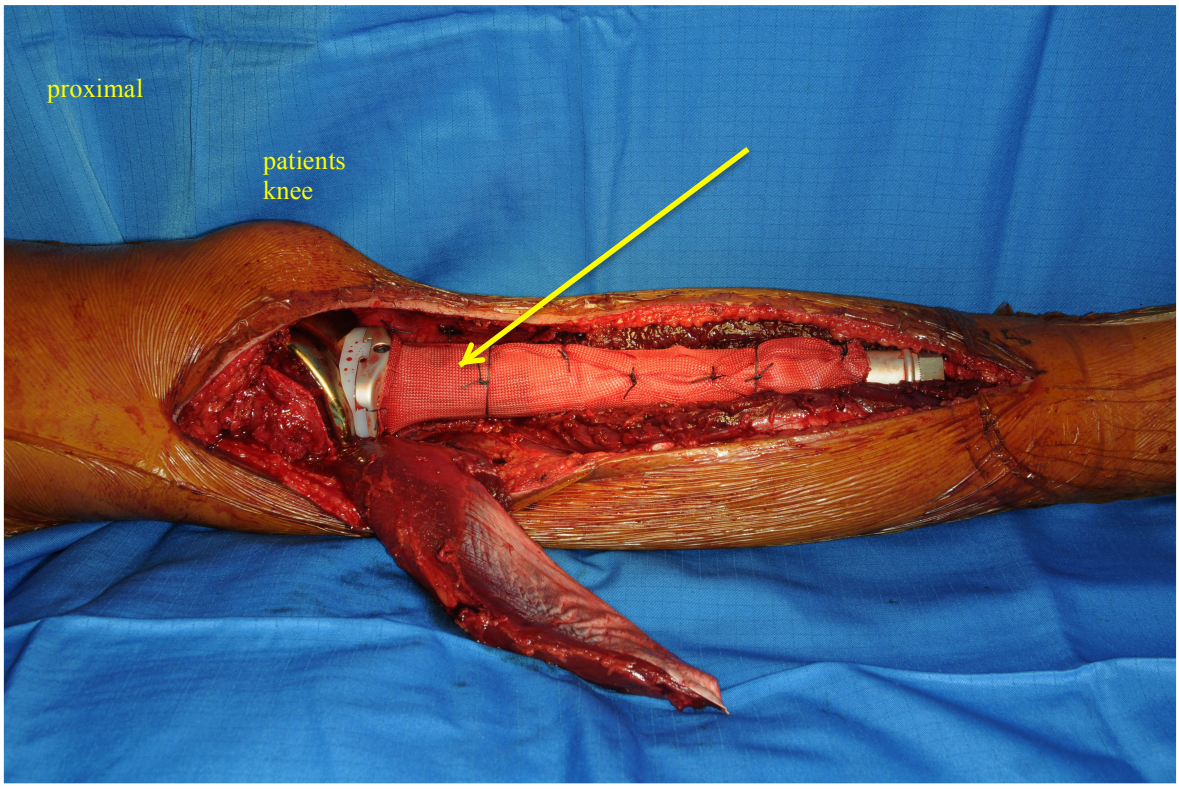



Figure 3 Intraoperative implanting of an endoprosthesis after removing the left proximal tibia of the 10-year-old boy from Figure 2.

Osteosarcomas occur rarely, but the research group in Graz is a part of the EURAMOS-1 trial to investigate whether it is feasible to improve the outcome for patients with osteosarcoma. The effort for improvement requires the cooperation of surgeons, oncologists, radiologists and pathologists.

Figure 4 shows a pathologist examining the bone tumour of Figure 2, the left proximal tibia of the 10-year-old boy and the extent of the osteosarcoma. A lesion that reaches into the metaphysial bone marrow is also visible in this image. Figure 5 shows an enlarged image of this lesion.

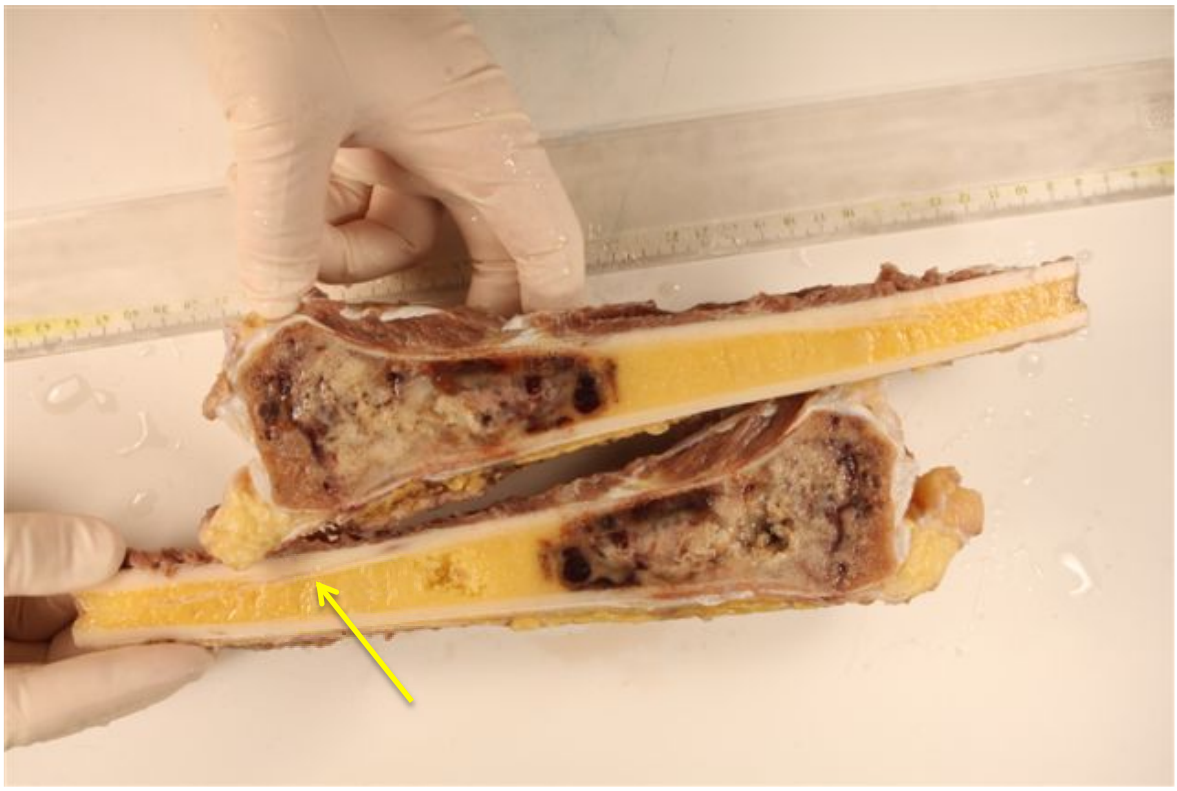


Figure 4 Pathological examination of the left proximal tibia of the 10-year-old-boy from Figure 2 with an osteosarcoma.

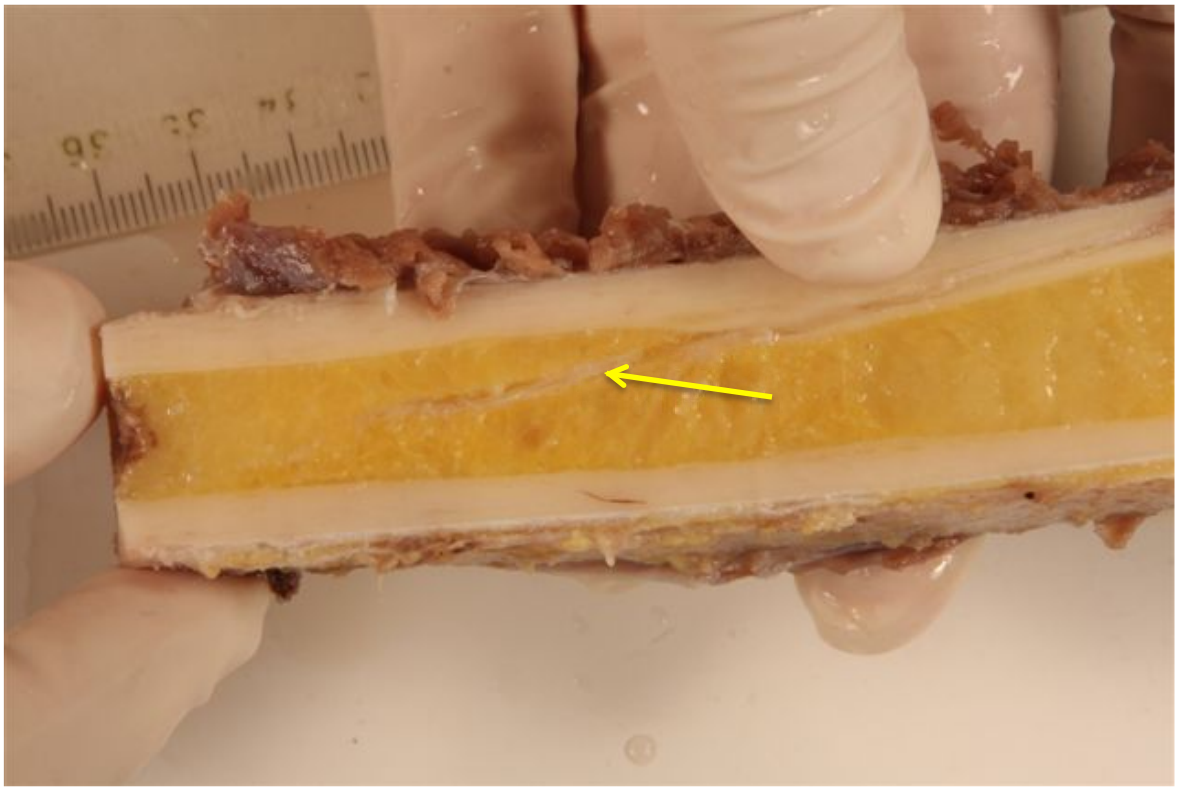


Figure 5 Enlarged image of Figure 4. Skip lesion of the 10-year-old-boy from Figure 2 with an osteosarcoma of the left tibia.

### 2.1.1 Classification

The WHO distinguishes bone tumours according to histological and genetic type. Besides osteogenic tumours, the WHO-list of malignant bone tumours includes Ewing sarcomas, tumours of the cartilage, fibrogenic tumours, fibrohistiocytic tumours, haematopoitic tumours, notochordal tumours, vascular tumours, smooth muscle tumours, lipogenic tumours and miscellaneous tumours. A general overview of all bone tumours that have been established so far is given in Table 2.

Osteosarcomas are the most common primary malignant bone tumours, accounting for approximately 35% in Europe and North America. Second most common malignant bone tumour is the chondrosarcoma (25%), followed by Ewing sarcoma (16%). With regard to osteosarcomas, a distinction is made between central (medullary) and surface (peripheral) cancers. Each group has its pathologic subtypes, shown in Table 1 (12–16).

The most common type of osteosarcomas is the medullary conventional high-grade central osteosarcoma, which occurs in eight to nine out of ten cases. It is characterised by areas of necrosis, atypical mitosis and destruction of the cartilage due to the production of malignant osteoid tissue. The production of osteoid tissue is described as an osteoblastic subtype. Figure 6 shows the microscopic histology. Other frequent subtypes, classified in terms of predominant matrix, are chondroblastic (Figure 7) and fibroblastic (Figure 8) osteosarcomas (12–15).

| | |
|---|---|
| Medullary osteosarcoma | Conventional high-grade central osteosarcoma |
| | Telangiectatic osteosarcoma |
| | Intraosseous well-differentiated (low-grade) osteosarcoma |
| | Small cell osteosarcoma |
| Peripheral osteosarcoma | Parosteal (juxtacortical) well-differentiated (low-grade) osteosarcoma |
| | Periosteal osteosarcoma low- to intermediate-grade osteosarcoma |
| | High-grade surface osteosarcoma |

Table 1 Osteosarcoma subtypes divided into central and surface tumours (12–15).

| **Cartilage tumours** | ICD-0 |
|---|---|
| Osteochondroma | 9210/0 |
| Chondroma | 9220/0 |
| Enchondroma | 922 |
| Periosteal chondroma | 922 |
| Multiple chondromatosis | 922 |
| Chondroblastoma | 9230/0 |
| Chondromyxoid fibroma | 9241/0 |
| Chondrosarcoma | 9220/3 |
| Central, 1° and 2° | 922 |
| Peripheral | 922 |
| Dedifferentiated | 924 |
| Mesenchymal | 924 |
| Clear cell | 924 |
| **Osteogenic tumours** | |
| Osteoid osteoma | 9191/0 |
| Osteoblastoma | 9200/0 |
| Osteosarcoma | 9180/3 |
| Conventional | 9180/3 |
| Chondroblastic | 9181/3 |
| Fibroblastic | 9182/3 |
| Osteoblastic | 9180/3 |
| Teleangiectatic | 9183/3 |
| Small cell | 9185/3 |
| Low grade central | 9187/3 |
| Secondary | 9180/3 |
| Parosteal | 9192/3 |
| Periosteal | 9193/3 |
| High grade surface | 9194/3 |
| **Fibrogenic tumours** | |
| Desmoplastic fibroma | 8823/0 |
| Fibrosarcoma | 8810/3 |
| **Fibrohistiocytic tumours** | |
| Benign fibrous histiocytoma | 8830/0 |
| Malignant fibrous histiocytoma | 8830/3 |
| **Primitive neuroectodermal tumours** | |
| Ewing sarcoma | 9260/3 |

| **Haematopoietic tumours** | ICD-0 |
|---|---|
| Plasma cell myeloma | 9732/3 |
| Malignant lymphoma, NOS | 9590/3 |
| **Giant cell tumours** | |
| Giant cell tumour | 9250/1 |
| Malignancy in giant cell | 9250/3 |
| **Notochordal tumours** | |
| Chordoma | 9370/3 |
| **Vascular tumours** | |
| Haemangioma | 9120/0 |
| Angiosarcoma | 9120/3 |
| **Smooth muscle tumours** | |
| Leiomyoma | 8890/0 |
| Leiomyosarcoma | 8890/3 |
| **Lipogenic tumours** | |
| Lipoma | 8850/0 |
| Liposarcoma | 8850/3 |
| **Neural tumours** | |
| Neurilemmoma | 9560/0 |
| **Miscellaneous tumours** | |
| Adamantioma | 9261/3 |
| Metastatic malignancy | |
| **Miscellaneous lesions** | |
| Aneurysmal bone cyst | 3364/0 |
| Simple cyst | 3340/0 |
| Fibrous dysplasia | 7491/0 |
| Osteofibrous dysplasia | 9262/0 |
| Langerhans cell histiocytosis | 9751/1 |
| Erdheim – Chester disease | 7792/0 |
| Chest wall hamartoma | 7558/0 |
| **Joint lesions** | |
| Synovial chondromatosis | 9220/0 |

Table 2 WHO classification of bone tumours and International Classification of Diseases for Oncology (ICD-0) (12).

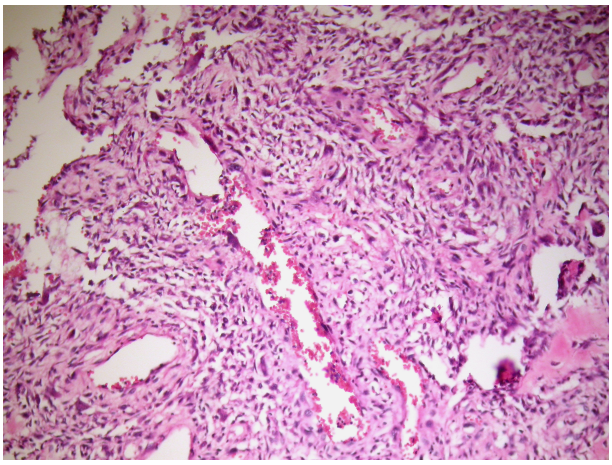


Figure 6 Osteoblastic osteosarcoma of the right distal femur with predominantly osteoid matrix in a 13-year-old girl.

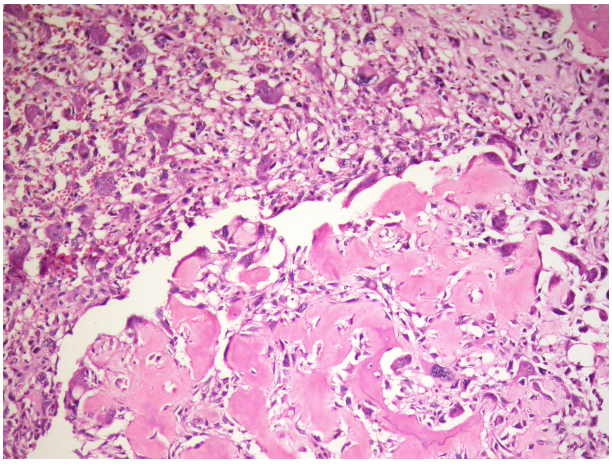


Figure 7 Chondroblastic osteosarcoma of the left proximal tibia with predominantly chondroid matrix in a 10-year-old boy.

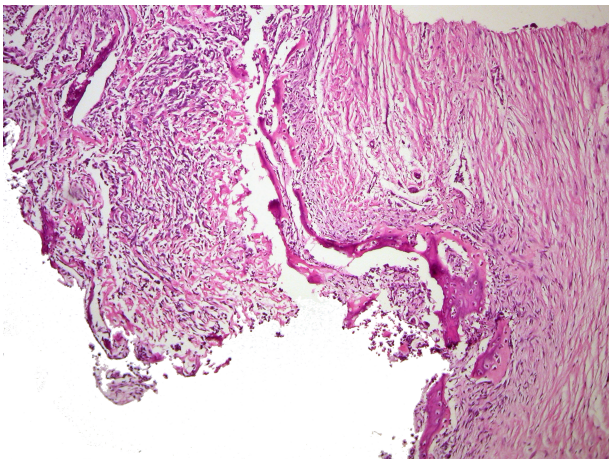


Figure 8 Fibroblastic osteosarcoma of the left calcaneus with predominantly spindle cell matrix in a 34-year-old woman.

### 2.1.2 Epidemiology

Osteosarcomas are the most common primary malignant bone tumours, which mainly occur in children, adolescents, and young adults (age group <24 years). A second peak of incidence is observed in elderly people (age group >60 years), mainly in patients with abnormal bone structure, with Paget's disease or after radiotherapy .

The incidence per million varies, depending on the consulted study, from 3.5 to 5.1, with a slight bias towards males. Children under the age of five years have an incidence below 1 per million, which increases to 8 to 11 per million between the ages of fifteen to nineteen. The male to female ratio is 1.4:1 in adolescents and adults (1,15,18,19). Interestingly, younger girls are more susceptible than boys up to the age of about 13 years (1,14,15,18,20).

Further differences in incidence can be observed in regard to different ethnicities. The greatest incidence in patients under 24 years can be found in Asian and

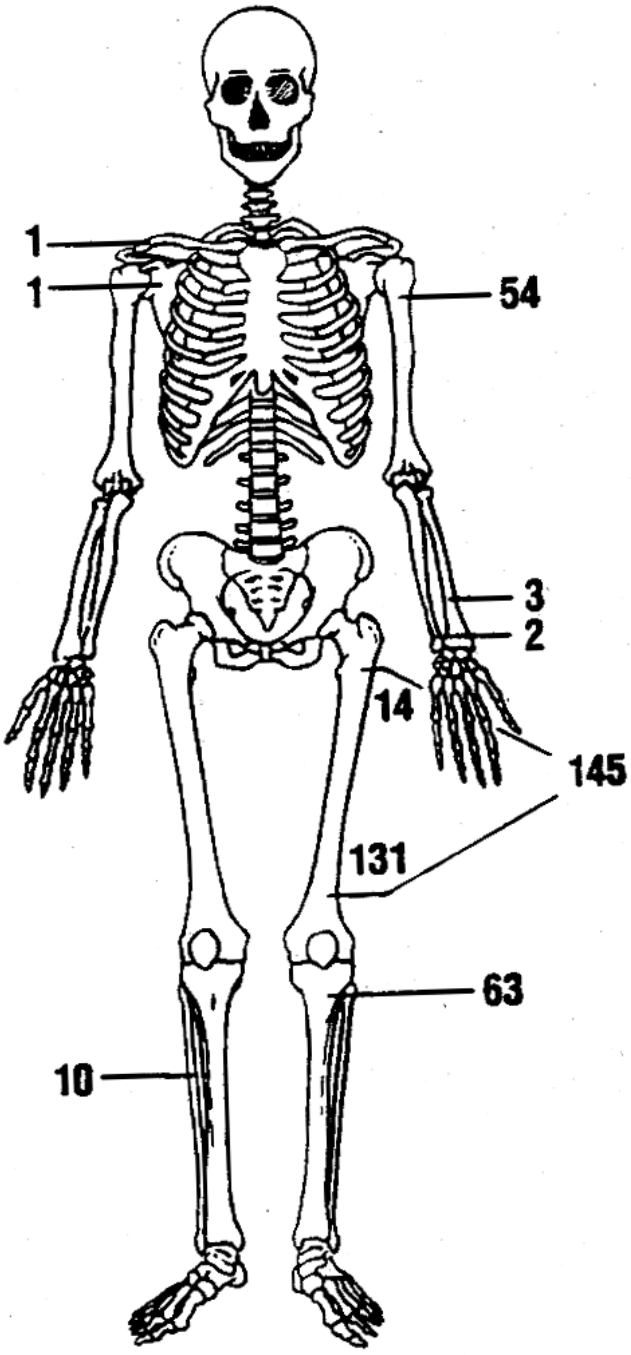



Figure 9 Localisation of 279 primary osteosarcoma in a study of Glasser et al. (1992).
The numbers next to the skeleton term the frequency of occurrence of osteosarcomas in every bone (17).

Pacific Islanders, while the incidence in elderly patients is higher among the Caucasian population. As a secondary malignancy the osteosarcoma has its highest incidence in Blacks (1,2,13–15,18,21,22).

Osteosarcomas can occur in every bone of the skeleton. Figure 9 provides an overview of the most frequent sites of osteosarcomas. The areas, which are typically affected in children and young adults, are those of rapid growth, namely the metaphysis of the long bones. Osteosarcomas have a predilection for the region around the knee. Conventional high-grade central osteosarcomas arise particularly in the distal femur and the proximal tibia, followed by the proximal humerus. Low-grade osteosarcomas are more likely seen in head and neck cancer. The anatomic site distribution in elderly patients is variable and also includes axial skeleton and unspecific bone sites such as the pelvis, ribs or neck (1,13–15).

### 2.1.3 Aetiology

Osteosarcomas are derived from primitive bone-forming mesenchyme, most common in a period of rapid growth (23–26), but also in full-grown adults (12,15,23,25,26). This suggests that the development of osteosarcomas may in some cases (children, adolescents) be related to progressive and fast length growth. Furthermore, chronic inflammatory conditions, like osteomyelitis or bone infarcts, are thought to be potential aetiological factors (12,13,15). Other conditions suspected for an osteosarcoma are metallic implants (e.g. aluminium, chromium, cobalt, methyl-methacrylate, nickel, titanium, and polyethyelene) or previous radiotherapy. Studies have been published where radiation is documented in approximately 3% of patients who developed an osteosarcoma (12,13,15). Premalignant diseases, multiple exostoses, fibrous dysplasia and Paget's disease are associated with increased risk for osteogenic tumours in patients older than 40 years (12,13,15).

Most cases of osteosarcomas occur sporadically, becoming suspicious through persisting load-independent pain and swelling. A few cases are associated with heritable germ line abnormalities such as the Li-Fraumeni syndrome, Werner syndrome, Rothmund-Thomson syndrome, Bloom syndrome and hereditary retinoblastoma (14,26). The causative role of osteosarcomas is still inconclusive. There are some genetic predisposing factors like the loss of heterozygotsity of the RB gene, the HER2/erbB-2 expression, the κB RANK-ligand expression, the presence of K7M2 cell line, potential biomarkers as MIF and chronic low

TNF or the SOX9 up-regulation, but to this day, the aetiology has not been entirely clarified. Table 3 explains the predisposing factors (13,16,20,27–29).

| Genetic factors and their explanation | |
|---|---|
| RB gene | Retinoblastoma protein gene, a tumour suppressor gene |
| HER2/erbB-2 | Factor of human epidermal growth factor receptor 2, a proto-oncogene |
| κB RANK-ligand expression | Receptor activator of nuclear factor κB, a membrane protein of osteoclasts |
| K7M2 | Metalloproteinase, occurs in metastatic osteosarcomas |
| MIF | Migration inhibitory factor |
| TNF | Tumour necrosis factor |
| SOX9 | Gene in humans |

Table 3 Explanation of genetic factors.

### 2.1.4 Grading and staging

Even for experienced clinicians, radiologists or pathologists, it is sometimes difficult to recognize and assess bone tumours. Therefore osteosarcoma study groups recommend that patients with suspected bone malignancies should be referred to a bone sarcoma centre for treatment (14,30).

Despite the difficulties in assessing bone tumours, the histological features, the degree of differentiation and the local spread are useful criteria for treatment. Grading and staging are used both for predicting the biological behaviour and for assessing a prognosis and are indispensible information before surgery (14,16,30).

#### 2.1.4.1 Grading

Grading is based on histological features, like cellularity, and correlates with prognosis. The most important criteria for grading are the relative amounts of cells compared to the matrix, nuclear contours, enlargement and hyperchromasia of the nuclei. Mitotic figures and necrosis are additional features that correlate with grade. Figure 10 shows a

microscopic image of a conventional high-grade osteosarcoma with typical signs of malignancy: abnormal mitosis and bizarre and hyperchromatic nuclei.

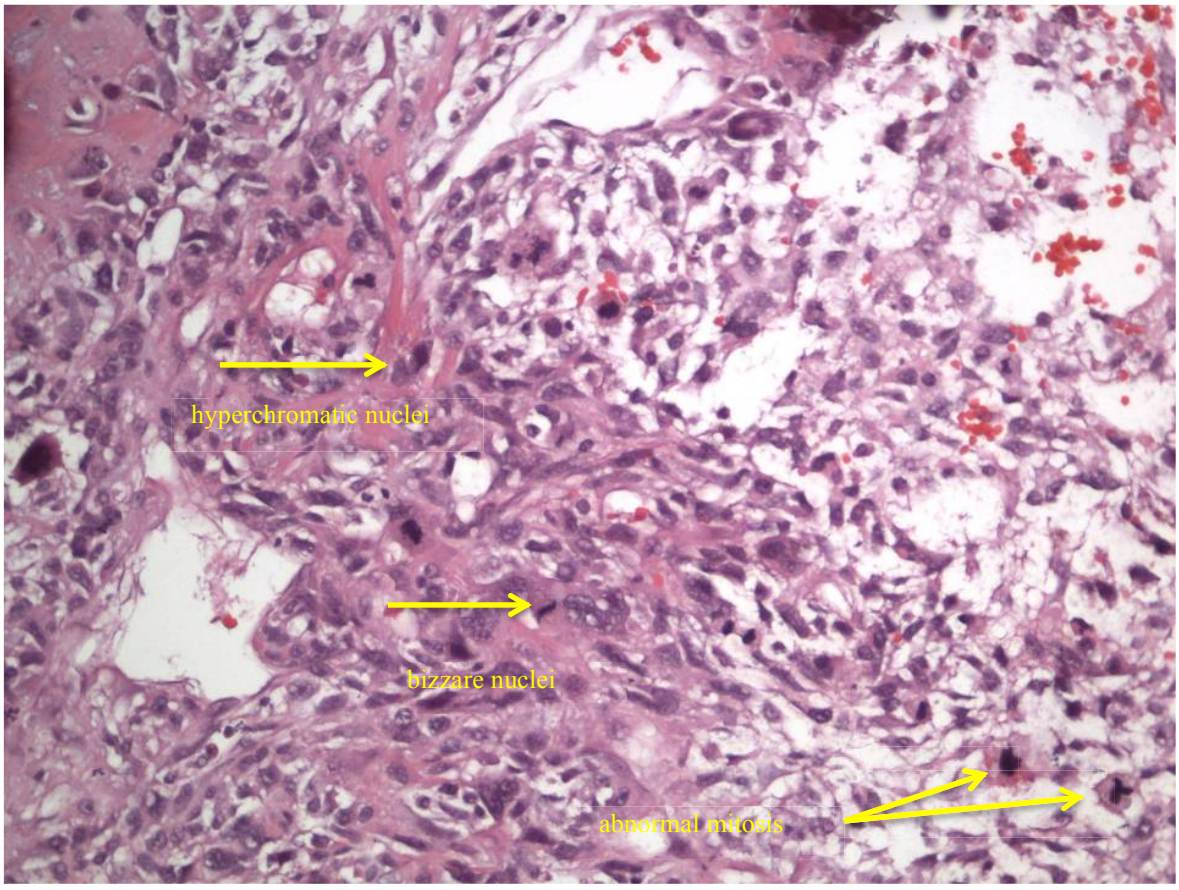



Figure 10 Microscopic pathology of a conventional osteosarcoma of the left distal femur in a 10-year old male.

High-grade tumours (G2, G3 in a three grading system or G3, G4 in a four grading system) are associated with bad prognoses and have to be distinguished from low-grade tumours (G1 in a three grading system and G1, G2 in a four grading system) that have better prognoses (12,15). The oncological and surgical grading scale is not identical with histopathologic grading, leading to differences in the grading classification.

### 2.1.4.2 Staging

"Staging incorporates the degree of differentiation as well as local and distant spread, in order to estimate a patient's prognosis"(12,15). Osteosarcomas and other bone tumours should not be classified by the universal TNM staging system because they very rarely metastasize in lymph nodes. Nevertheless, a TNM classification for bone tumours exists, as shown in Table 4 and Table 5.

| T – primary tumour | |
|---|---|
| TX | Primary tumour cannot be assessed |
| T0 | No evidence of primary tumour |
| T1 | Tumour 8 cm or less in greatest dimension |
| T2 | Tumour more than 8 cm in greatest dimension |
| T3 | Discontinuous tumours in the primary bone site |
| N – regional lymph nodes | |
| NX | Regional lymph nodes cannot be assessed |
| N0 | No regional lymph node metastasis |
| N1 | Regional lymph node metastasis |
| M – Distant metastasis | |
| MX | Distant metastasis cannot be assessed |
| M0 | No distant metastasis |
| M1 | Distant metastasis |
| M1a | Lung |
| M1b | Other distant sites |
| G – Histologic grade | |
| GX | Grade cannot be assessed |
| G1 | Well differentiated-low grade |
| G2 | Moderately differentiated-low grade |
| G3 | Poorly differentiated-high grade |
| G4 | Undifferentiated-high grade |

Table 4 TNM Classification of bone tumours (12).

| Stage | T - primary tumour | N - regional lymph nodes | M - distant metastasis | Grade |
|---|---|---|---|---|
| I A | T1 | N0, NX | M0 | Low grade |
| I B | T2 | N0, NX | M0 | Low grade |
| II A | T1 | N0, NX | M0 | High grade |
| II B | T2 | N0, NX | M0 | High grade |
| III | T3 | N0, NX | M0 | Any grade |
| IV A | Any T | N0, NX | M1a | Any grade |
| IV B | Any T | N1 | Any M | Any grade |

Table 5 TNM staging group of bone tumours, also called SNOMED (12).

The system devised by Enneking et al. in 1985/1986, shown in Table 6, offers a more compatible system for surgery. The general staging of musculoskeletal tumours according to Enneking, as shown in Table 7, is applied internationally. It is based on grade, local extent of the tumour and the presence of metastases.

| Stage | Grade | Extent | Metastasis |
|---|---|---|---|
| I A | G1 | T1 | M0 |
| I B | G1 | T2 | M0 |
| II A | G2 | T1 | M0 |
| II B | G2 | T2 | M0 |
| III | G1-2 | T1-2 | M1 |

Table 6 Surgery staging described by Enneking et al. (31).

| Stage | Grade | Extent | Metastasis |
|---|---|---|---|
| I A | G1 | T1 | M0 |
| I B | G1 | T2 | M0 |
| II A | G2 | T1 | M0 |
| II B | G2 | T2 | M0 |
| III A | G1, G2 | T1 | M1 |
| III B | G1, G2 | T2 | M1 |

Table 7 International staging described by Enneking et al. (31).

The grading system is different for benign and malign tumours. Benign tumours are always G0, divided into inactive (stage 1), active (stage 2) or aggressive (stage3) tumour behaviour. The grading system for malignant tumours takes into account whether they are low-grade tumours (stage I), high-grade tumours (stage II) or any grade with metastases (stage III).

The extent of the tumour is classified as intracompartmental (T1, tumour remains in place) or extracompartmental (T2, tumour extends into nearby structures).

The presence of metastases is classified as M0, if no spread occurred and as M1, if malignant daughter cells exist. About 15-20% of all patients already have metastases at the time of diagnosis. In most cases, the lungs are affected by metastatic disease (95%), followed by local recurrence (50%) (12,15,21).

### 2.1.5 Clinical appearance

The clinical symptoms of bone tumours are non-specific. The most common and earliest clinical presentation is pain. Patients may report intermittently occurring, dull pain, which is worse at night. Often a history of trauma is associated with non-mechanical pain or swelling.

Initially the pain may be perceived as neuralgia-like, later it subsequently intensifies. During disease progression the complaints become excruciating and intolerable, hence the need for adequate pain medication. Symptoms like paraesthesia and paralysis can occur when the tumour presses on nerves. The duration of symptoms varies between weeks and several months, with an average duration of approximately three months before the diagnosis is made (12,14,15,30).

Another cardinal symptom is swelling. Swelling leads to the diagnosis, when an extraosseous part of the tumour or soft tissue masses are involved. Benign bone tumours tend to extend slowly, wherefore a long duration without any complaints is possible. Malignant tumours grow rapidly and frequently cause early complaints, like pain or swelling. Considerable swelling should be described according to the consistency (hard vs. soft, coarse vs. elastic), the mobility of the tumour to surrounding tissues (relocatable vs. fixed), the dimensions (in centimetres) and the appearance (intact skin vs. shining, livid or ulcerated skin).

The more pronounced the symptoms (hard consistence, little mobility, large dimensions and alteration in skin appearance) are the more likely it is that these factors are criteria for

malignancy. Large tumour masses or reactive synovitis in a joint can be responsible for limitation in movement (12).

In less than 1% of cases, a pathologic fracture occurs as a primary symptom of bone tumour. Later, in advanced tumour stages, fractures may occur alongside more general symptoms like fever, exhaustion and weight loss persist too.

### 2.1.6 Diagnosis

Figure 11 shows an algorithm for diagnosing bone tumours, translated from Leithner et al. (16,32).

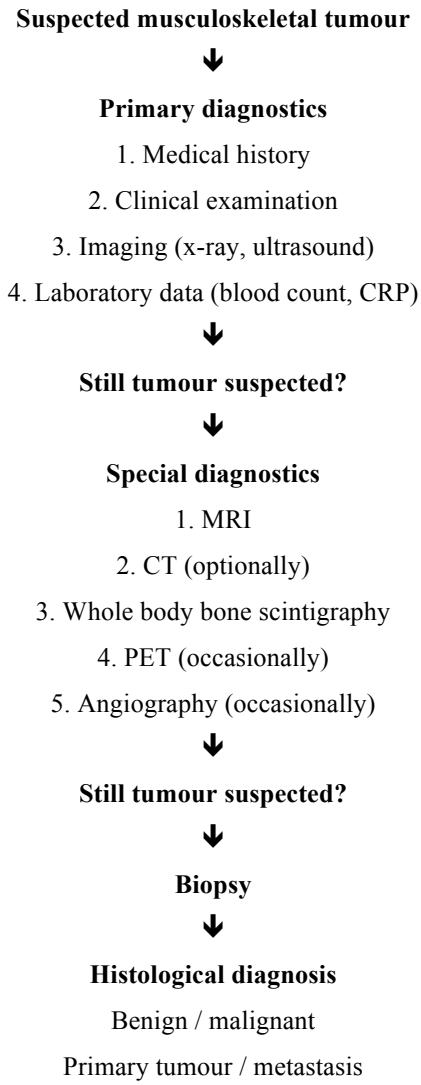



Figure 11 Diagram of the simplified diagnostic algorithm recommended by Leithner et al. (2007), translated version

#### 2.1.6.1 Primary diagnostics

In a first step, the patient's medical history, including start, manner and duration of symptoms has to be assessed. Knowing about prior diseases, familiar predispositions, unwanted weight loss, paleness, loss in performance and night sweats might also be helpful in diagnosing bone tumours. Secondly, a clinical examination involving the inspection, palpation and the relation to the area surrounding the suspected tumour must be performed. Symptoms that might cause suspicion during an inspection include swelling, rubor, vascular markings or shining, livid or ulcerated skin. The palpable size of the tumour mass has to be documented in centimetres. During clinical examination, the examiner should also take note of a potential limitation of movement, intra-articular effusions or sensomotoric deficiencies. Furthermore, the whole body should be inspected, not only the affected region. Some symptoms might lead to diagnosis, if they are detected, like café-au-lait spots in neurofibromatosis. Even if there are no screening tests for musculoskeletal tumours available, some general blood parameters could be useful in diagnosis: red and white blood cell count, C-reactive protein (CRP), alkaline phosphatase (AP), lactate dehydrogenase (LDH), calcium etc. (16,32–34).

#### 2.1.6.2 Special diagnostics

If there is still a suspicion of bone tumour after physical examination, a first investigation should consist of conventional radiographs in two planes. X-rays with anterior-posterior projection and a lateral radiograph, like in Figure 12 and Figure 13, are common. The diagnosis of an osteosarcoma is characteristically suspected by radiographic changes of the affected bone. Lodwick et al. summarized the radiological characteristics of nearly all bone tumours (35).

On x-ray, osteosarcomas can appear as osteoblastic (Figure 14), osteolytic (Figure 15) or mixed (Figure 16) lesions. Parameters to determine tumour aggressiveness are anatomical location, size, composition of matrix, pattern of bone destruction and periosteal reaction (12,14,15,30).

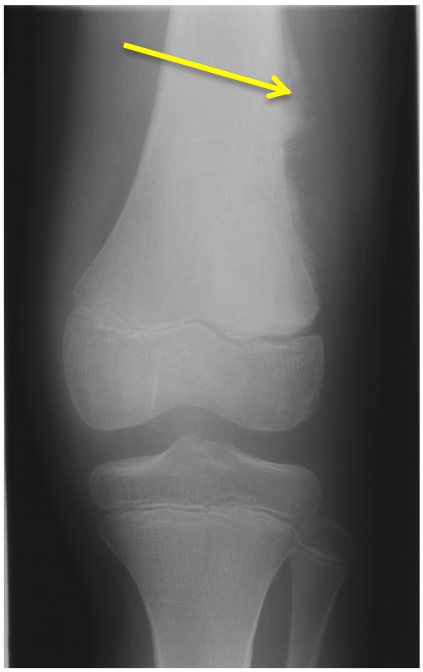


Figure 12 X-ray of a left knee, a-p radiograph of 10-year-old boy with osteosarcoma.

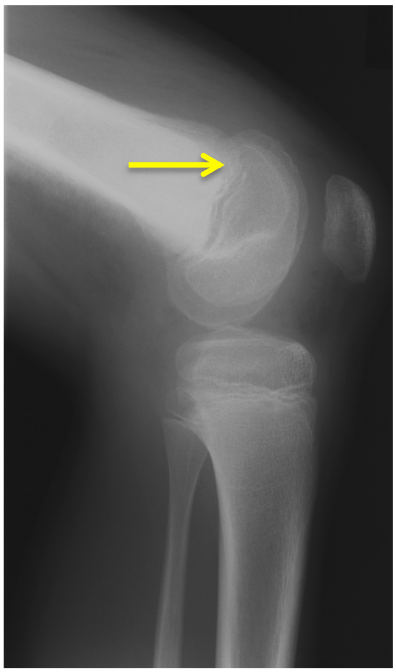


Figure 13 X-ray of a left knee, lateral radiograph of 10-year-old boy with osteosarcoma.

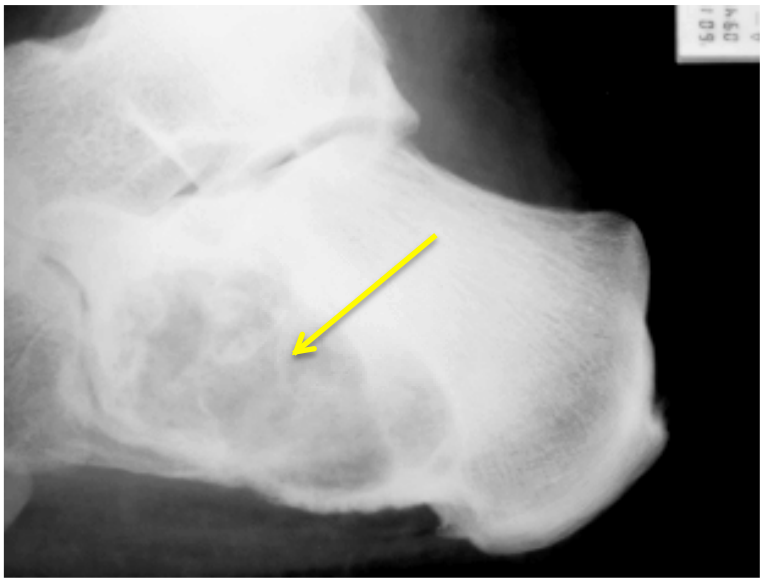


Figure 14 Osteoblastic osteosarcoma of the left calcaneus of a 34-year-old woman.

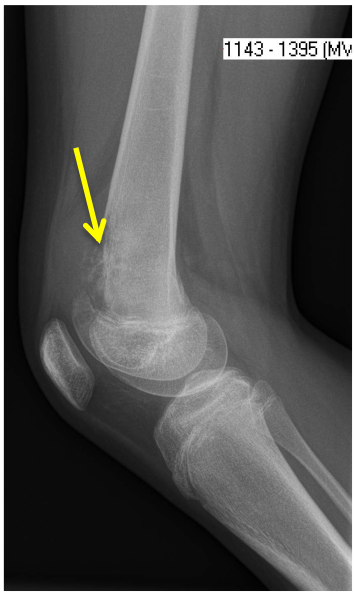



Figure 15 Osteolytic osteosarcoma of the right distal femur of a 13-year-old girl.

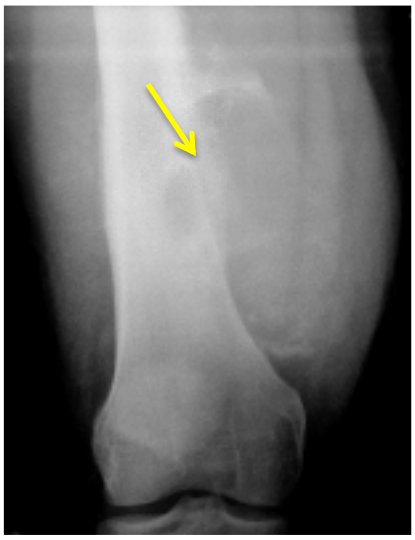


Figure 16 Mixed osteosarcoma of the distal femur of a 15-year-old male.

Some bone tumours more commonly develop in certain bones as well as in particular anatomical regions; e.g. osteosarcomas' most frequent site is the distal femur, followed by the proximal tibia. Bone tumours most often occur eccentrically, in the long bones of the extremities (12,14,20).

Of course the age of the patient is crucial, since age-dependent differences in prevalence exist. Bone tumours at the age of five to fifteen years are certainly either osteosarcomas (35%) or Ewing sarcomas (16%). Bone tumours amongst the elderly, however, are most commonly metastases (1,12,18).

The radiologically determinable size of a tumour can be indicative of its dignity. Small tumour dimensions (<6 cm) are likely benign, whereas larger dimensions can either be benign or malignant. The quicker and larger a tumour appears within a short period of time, the more likely it is to be malignant (12,15,21,36).

The type of matrix in bone tumours can show radiological differences and may point to a specific diagnosis. E.g. chondroblastic osteosarcomas appear to be white due to the mineralized and non-mineralized chondroid matrix, whereas osteoblastic osteosarcomas seem to have a more granular structure. Most tumours appear radiolucent, like lytic or mixed osteosarcomas, while some appear sclerotic or calcified (12,15,30).

The radiological pattern of bone destruction indicates the aggressiveness of a tumour. A geographic pattern (Type 1) of bone destruction is characterised by lytic areas. It is divided into three subtypes: Type 1A exhibits a rim of sclerosis between lytic areas, whereas Type 1B shows no signs of sclerosis but a clear separation from normal bone structure. Type 1C is characterised by a less clear separation from normal bone. Type 2 refers to a moth-eaten pattern, which has multiple holes in the bone. It is more often aggressive than Type 1. Type 3 is the permeative pattern. It is the most aggressive pattern, characterised by rapid progression and diffuse lytic areas. Type 2 and Type 3 occur in malignant tumours, such as Ewing sarcoma or osteosarcoma, but also in some benign lesions (12,30).

The radiological pattern of periosteal formation reflects the rate of growth of the tumour. Slowly growing tumours build a thick layer of bone, while multiple periosteal layers are formed by fast and slow growth. The extension of the tumour through the cortex, perpendicular formations and fast growth suggest malignancy. A frequently described radiological sign for periosteal reaction is the Codman triangle (Figure 17). It occurs within benign and malignant tumours when the periosteum disrupts during steps of growth (12,14,30).

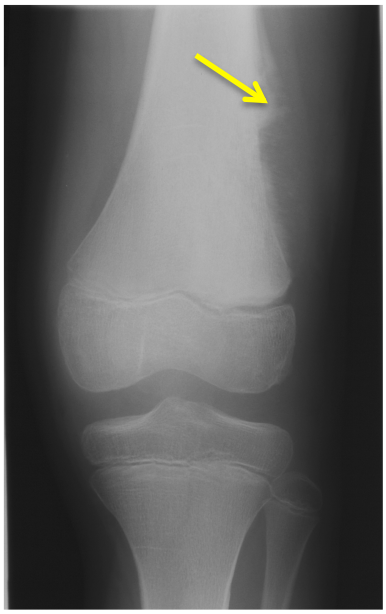


Figure 17 Codman triangle in an osteosarcoma of a 10-year-old boy, a-p radiograph of the left femur and knee.

Furthermore, the interface between the tumour and its surrounding area should be noted. In case of poorly defined lesions with a broad zone of transition it is likely a malignant tumour. An ossification of the surrounding tissues, a so-called “sunburst pattern”, is a characteristical radiological sign for osteosarcoma, but not specific. However, a sclerotic rim occurs often in slow growing, likely benign tumours (12,15,30).

Despite a lot of radiological sings indicating a malignancy, further imaging techniques are necessary to assess the extent of the tumour in the bone and the surrounding area. Magnetic resonance imaging (MRI) is particularly important for the assessment of intraosseous extension and the extension to muscles, important neurovascular structures, subcutaneous fat and involved joints (Figure 18). Because of the accuracy in accessing the extension, staging is based on MRI. The advantages of MRI are the high contrast in tissues without radiation and the possibility of three-dimensional images due to layering (Figure 19). MRI is able to detect possible skip lesions or local metastases. Therefore, prior definitive surgery a contrast-enhanced MRI is mandatory. (12,14,16,30,32,34)

A computerized tomography (CT) of the affected bone can be conducted in order to assess the size, the periosteal reaction, the pattern of destruction, the ossification process and the involvement of soft tissue. Nevertheless, CT of the affected bone is not the method of choice due to the high radiation. CT is essential in visualizing the cortical bone structures and the presence of metastases, especially those of the chest, which appear as calcified nodules (Figure 20, Figure 21). (16,32,34)

A further technique for detecting metastases is a radionuclide bone scan. The most common radionuclide is technetium 99m. Its uptake visualizes the extent of the primary tumour and helps to define a safe margin by the detection of skip lesions (Figure 22). The local uptake provides information about biological activity of the tumour, but nothing about its dignity. (16,34) Other imaging techniques, like positron emission tomography (PET), angiography and whole body MRI are still under evaluation (12,14–16,30,34).

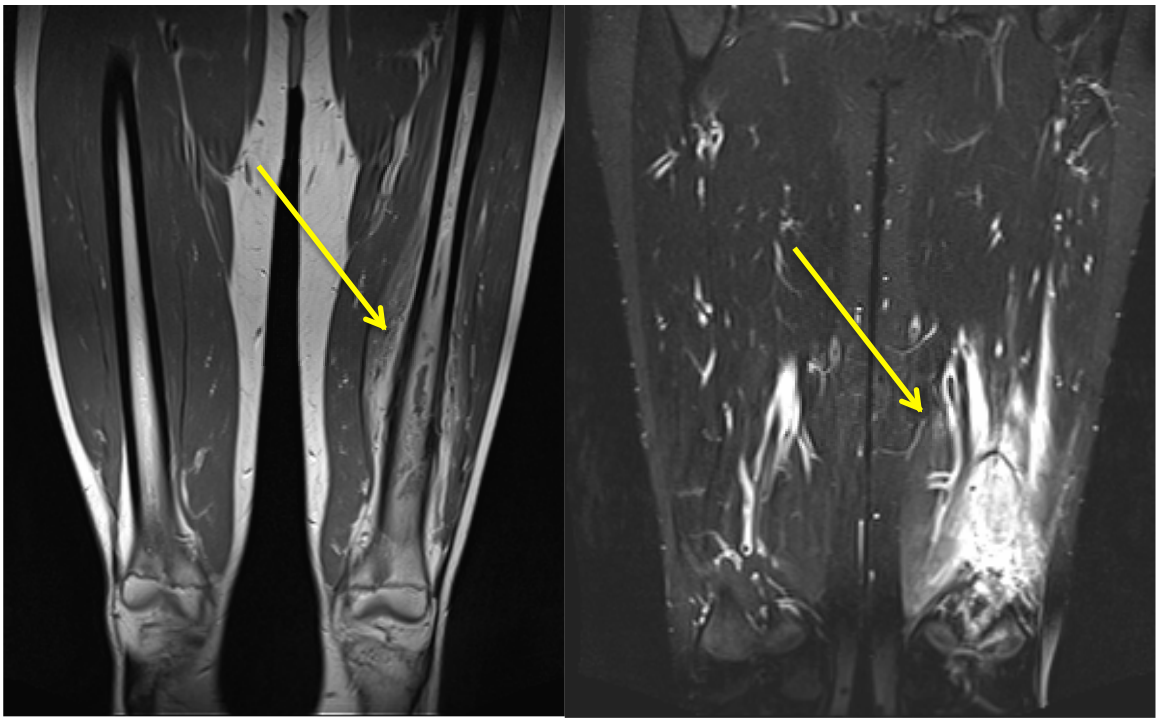


Figure 18 Coronary MRI of both legs, osteosarcoma located at the left distal femur in a 15-year-old boy.
T2 weighted, native on the left side and T2 weighted, fat suppressed with contrast enhancement on the right side.

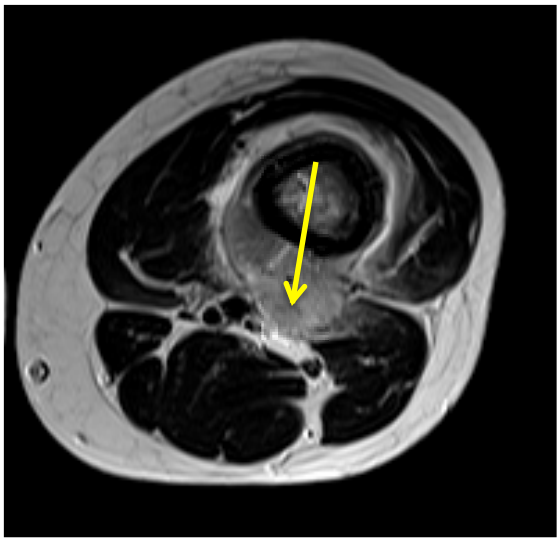


Figure 19 Axial T2 weighted MRI of the same patient (Figure 18) with an osteosarcoma of the left distal femur.
Major blood vessels and nerval structures are not infiltrated.

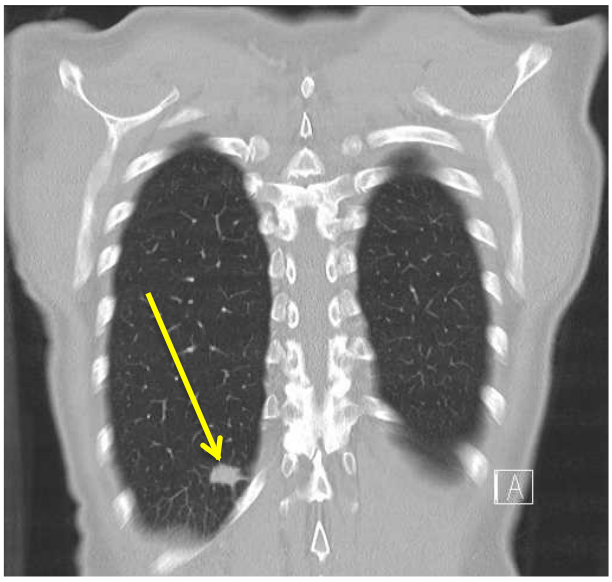



Figure 20 Metastasis of the right lung in a 62-year-old woman with an osteosarcoma.

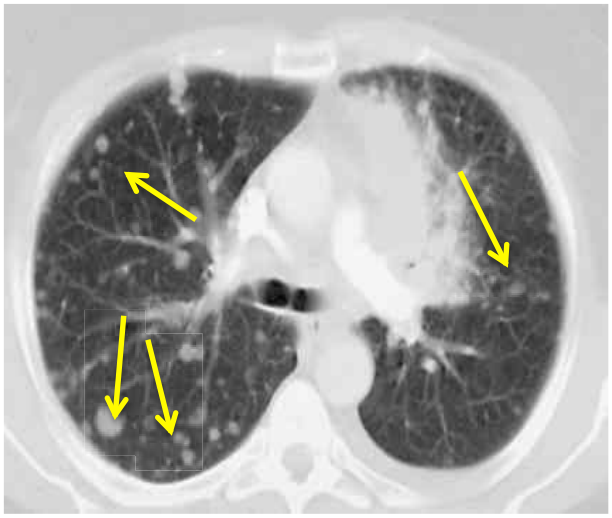


Figure 21 Multiple metastases of both lungs in the same patient (Figure 20), several months later.

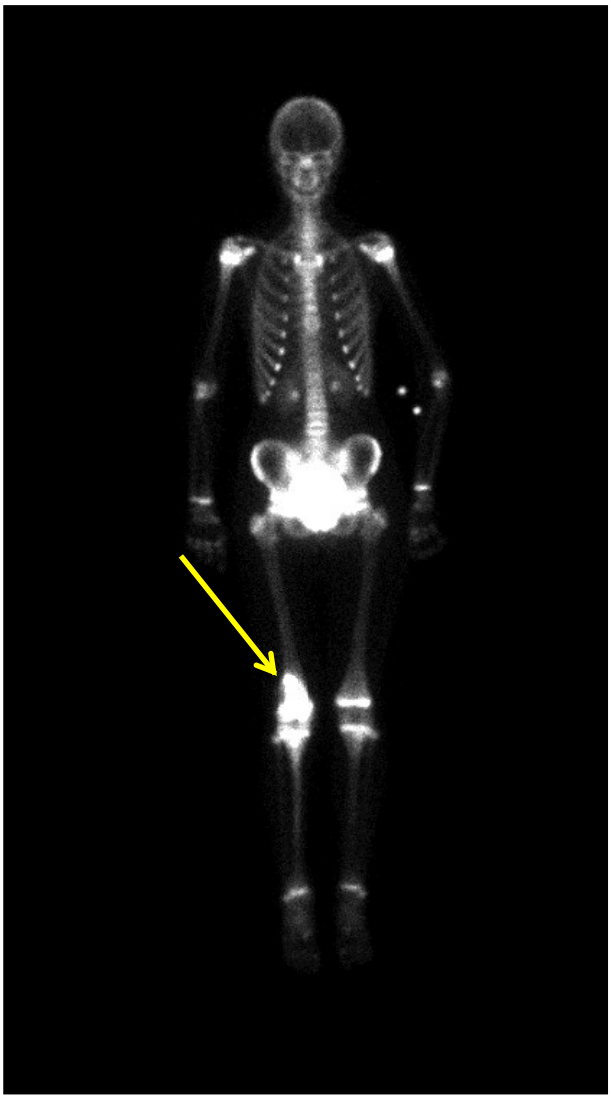


Figure 22 Radionuclide bone scan with an osteosarcoma of the right distal femur in a 13-year-old girl.

Until today, no specific serum test results indicating a bone tumour have been discovered. Hogendoorn et al. describe an elevation of AP and / or LDH in a considerable number of patients with Ewing sarcoma and osteosarcoma. Those increased parameters are used during follow-up examinations, because they seem to be negative prognostic factors (14).

### 2.1.6.3 Biopsy

The only way to verify a bone tumour is histological determination after biopsy. (12,14–16,32,34) The biopsies should be taken from the suspicious site detected by x-ray, MRI or CT. It is recommended that the surgeon, preferably an orthopaedic oncologist, performing the final operation should also perform the biopsy, ideally after involving pathologists and radiologists to ensure the adequate localisation for biopsy (14,16,30,32,34). The biopsies should minimally contaminate the normal tissue, especially when a limb salvage surgery is

planned. Otherwise contaminations might lead to local recurrences and an amputation might be indicated. Therefore, an exact imaging (containing x-rays in two planes, MRI and/or CT, radionuclide bone scan or PET) and planning the surgery prior performing biopsy are mandatory. The different options to get histological specimens are either an open (surgical, excisional or incisional) or a closed (percutaneous, e.g. core needle) biopsy. (14,16,30,32,34)

The closed biopsy, e.g. core needle biopsy (CNB) or fine needle aspiration biopsy (FNAB) are performed under sterile conditions with 22-25-gauge needles in general or local anaesthesia. It is the most widely used technique in homogenous tumours. Furthermore, the closed biopsy "is faster, simpler and cheaper compared to open biopsy" (32). The most important thing of closed biopsy is an exactly documentation and / or mark of the biopsy canal, because the biopsy tract always has to be considered as contaminated and has to be resected in case of malignancy. Nevertheless, the decision, whether for open or closed technique, should be made individually. The operability of an open biopsy depends on the location and local expertise. In each case of open biopsy, a small longitudinal incision should be performed to minimise the contamination of the surrounding tissue. The advantage of open biopsy is the representative amount of tissue, which cannot be reached with closed techniques. Especially in case of inconclusive correlation of histology and clinical presentation an open biopsy is recommended. The most important things for a successful biopsy are: no hurry, no contamination of surrounding structures, no operation without preoperative imaging, consider later treatment, gain enough tissue, avoid haematoma and do not forget a drain out of the biopsy tract (32,34). An excision biopsy is only indicated in evident benign tumours. Otherwise excision biopsies are contraindicated because the adequate oncologic-surgical margin cannot be adhered to (14–16,32,34).

After a biopsy, the specimens should either be fixed in 10% neutral buffered formalin or they should be snap-frozen (-70°C) for future studies or - in the definite case of a surgery - for determining the resection margin. The formalin is suitable for nearly all bone specimens; only the Ewing sarcoma should be fixed in absolute alcohol. The routine way to fix the specimen is decalcification with acid solutions. Unfixed specimens have to be received by the laboratory immediately (within half an hour).

Every specimen, regardless of whether it is fixed or not, must be interpreted by an experienced pathologist. The pathologists need further clinical features to allocate the tumour to a type / subtype according to the latest WHO criteria (Table 8). Every bone tumour should finally be classified by using the SNOMED or ICD-0 codes (12,14,30).

| | |
|---|---|
| Clinical features associated with bone tumour | Age, sex, racial background of the patient |
| | Anatomical Site |
| | Nature and duration of symptoms (pain, swelling, previous trauma) |
| | Pre-existing or concomitant skeletal disease, familial history |
| | Occupational and treatment history, systemic disease, test results |

Table 8 Clinical features for pathologists in bone tumour diagnosis (12,30).

#### 2.1.6.4 Differential diagnosis

Osteosarcomas need to be distinguished from other bone tumours, which are listed in Table 2. The most important differential diagnoses to osteosarcomas are the Ewing sarcoma (ES) and the chondrosarcoma.

##### *2.1.6.4.1 Ewing Sarcoma*

The ES is the second most common primary malignancy of the bone, beginning from small cells of neuroectodermal tissue. The ES, like osteosarcomas, occurs most commonly in children and young adolescents (<20 years), but also in adults (14,37,38). Just like osteosarcomas, the occurrence of ES is slightly biased towards males (1.5:1 male:female ratio). In contrast to osteosarcomas, however, aetiological factors for ES are known; these include chimeric transcripts of the EWS genes with other genomic transcripts, like the FLI1, the ERG2 gene (both are human genes for transcription) or the DKK2 gene (a key player in stem cell signalling) (14,37,38).

According to different studies, the incidence for ES is approximately 0.3/100.000/year in Caucasians. Blacks and Asians hardly ever suffer from ES. The most affected bones of the skeleton are the long bones of the extremities (50%), followed by pelvic bones (25%). The ribs or the vertebral column are less commonly affected, but also mentioned in several studies. Figure 22 and Figure 23 show an x-ray and a pathologic specimen of an ES (14).

The prognosis for patients with ES is similar to that of osteosarcomas: OS of approximately 70% in localized disease and about 30% in metastatic disease (37–40). A large tumour mass, older age, axial localisation and poor response to chemotherapy (less than 90 % necrosis after primary chemotherapy) are bad prognostic factors for ES, as well as for osteosarcomas. Interestingly, the gender of patients seems to be of importance for the prognosis. Females have a lower OS (17 %-30 %) than males (45 %-48 %) (37,39).

Metastases are present in about 25% of all patients at the time of diagnosis, whereby the median delay in diagnosis is 18 months. The occurrence of metastases is related to metaphyseal tumours. The ES metastasises most frequently in the bones and lungs (38,39,41).

Nevertheless, the prognosis has improved through the use of aggressive multi-agent chemotherapy (5-year EFS 30-60%). The total treatment time is 35 weeks. In this time Vincristine, DOX, IFO (VAI) and P, DOX, IFO (PAI) are administered. Surgery of the primary tumour is scheduled for week 9. Like patients with osteosarcoma, patients with inoperable ES receive radiotherapy (40,42). Also a few cases with operable ES receive radiotherapy as a preoperative treatment.

#### *2.1.6.4.2 Chondrosarcoma*

Amongst the elderly, bone metastases have to be distinguished from primary bone tumours. The third most common primary bone tumour is the chondrosarcoma. It occurs mainly in-between the third and sixth decade of life and is equally frequent among males and females with an incidence of 0.1/100.000/year (14,43).

The most common localisation of chondrosarcomas is in the centre of diametaphyseal bones (proximal femur, proximal humerus, distal femur), but it also occurs in the pelvis (Figure 24), ribs and scapula (14,43).

The treatment of chondrosarcomas differs according to histological subtypes and grades. The most common subtype is the conventional chondrosarcoma, followed by mesenchymal and clear cell chondrosarcomas. The majority of chondrosarcomas are low-grade (G1). The 5-year OS is approximately within 75% and 83% (14,43).

Other, non-tumour-related differential diagnoses can be found with x-ray. Patterns of bone destruction can occur due to infections, like osteomyelitis, or after a trauma. Another osteolytic differential diagnosis is the aneurysmal bone cyst. Osteoblastic lesions are osteoid osteoma and osteoblastoma.

### 2.1.7 Prognostic factors

Osteosarcomas are rare, representing less than 1% of all malignant cancers diagnosed in the United States. Even today, they remain an aggressive disease with a poor prognosis (event-free survival (EFS) around 50-60%) (2,29).

Of course, the patient's prognosis can only be made during the later course of the disease, when the quality of surgical resection and the response to chemotherapy can be evaluated. Despite the improvement of therapy, approximately 90% of all patients die from progressive osteosarcoma. The most common reason for death during the therapy is pancytopenia, later, the deaths in remission are caused by cardiomyopathy or second malignant tumours (13,20).

The most important predictor of the outcome in primary localised osteosarcoma is the patient's response to pre-operative chemotherapy. Interestingly, Bielack et al. claim that the date of surgery does not really matter in prognosis (13). They found no difference in EFS between patients with primary chemotherapy and those with delayed chemotherapy. The EURAMOS-1 trial provides surgery in week 11, after six cycles of chemotherapy (15).

Bielack et al. discovered that a large tumour size, the male sex, older age, proximal localisation of the tumour, higher AP and / or LDH values and the presence of metastases are bad prognostic factors (13). The quicker and larger a tumour appears, the more likely it is to be an aggressive and malignant disease, associated with poor prognosis. Nevertheless, there is no correlation between tumour size and response to chemotherapy. Therefore, the response to chemotherapy is repeatedly confirmed as being the most important prognostic factor (13). An investigation of the connection between the duration of symptoms and the outcome showed no statistically significant correlation, but a long history confers a higher risk of poor response (13). The pre-diagnostic symptoms and previous malignancies have no correlation to prognosis (13).

With regard to the patients' sex, to be female seems to be a good prognostic factor. Up to now, scientific obvious explanations for correlation between sex and distribution or between sex and prevalence are not available. Leithner et al. published that hormones might be the reason why girls are affected earlier and why they have better response to chemotherapy (19). Furthermore, it has been observed that females have fewer relapses and better overall survival, which is why being female is correlated with a good prognosis (13,19,20).

Different studies associate the age of 40 years and older with a poor prognosis. Additionally, a proximal or axial tumour site is considered to be a bad prognostic factor. Among osteosarcomas of the extremities, those of the humerus have the worst prognosis, while those of the tibia have the best (13,20).

Hogendoorn et al. described an elevation of AP and / or LDH as negative prognostic factors (14). Furthermore, there are several molecules and biomarkers that potentially predict metastatic spread (low TNF-level, epidermal growth factor (EGF), hepatocyte growth factor (HGF), prolactin (PRL)), leading to increased risk of death (27). In general, the presence of primary metastases leads to a poor prognosis; however, the presence of lung metastases alone is better than the additional occurrence of other metastases (13,20,21,36).

## *2.2 Treatment*

Up to the 1970s, surgery was the only treatment for patients with osteosarcoma. The five-year OS at this time was about 20% (15,21). With the appearance of chemotherapeutic agents in this disease, complete regression of bone tumour was described for the first time. The combination of surgery and chemotherapy improved the three-year OS up to 60% (1,3,4,24,25).

### 2.2.1 Chemotherapy

Pessimism concerning the chemotherapeutic agents arose because of treatment-induced adverse effects, such as gastrointestinal dysfunction and damage of the bone marrow system. Even drug-related deaths and long-term nephrotoxicities were noted. The need for more experienced treatment schedules resulted in the modification of conventional regimes according to the Osteogenic Sarcoma Study Group (15,21).

Up to now, the pre-operative and post-operative application of multi-agent chemotherapy, as well as the improvement of surgical techniques and radiotherapies, have increased a three-year OS to approximately 60-90%, depending on factors like age, sex, location and response to chemotherapy (1,3,4,22).

Doxorubicin (DOX; Adriamycin, A), Cisplatin (DDP; Cis–diaminedichloroplatinum II, CDDP; Platinol, P), Methotrexate (MTX; Amethopterin), Ifosfamide (IFX; IFOS, IFO), Etoposide (ETO; VP-16, VePesid) and Pegylated interferon α-2b (IFNα; Peg-Intron) are the most active chemotherapeutic agents used in all countries participating in EURAMOS-1. Currently, the EURAMOS-1 trial represents a collaboration between four major research groups:

- North American Children's Oncology Group (COG),
- German-Austrian-Swiss-Cooperative Osteosarcoma Study Group (COSS),
- European Osteosarcoma Intergroup (EOI) and the
- Scandinavian Sarcoma Group (SSG).

The advantage of such a huge collaboration is the ability to conduct large trials, which aids therapeutic development and makes the investigation of new agents possible.

The aim of the EURAMOS-1 trial is to optimize therapy for patients with osteosarcoma, by using an open label as well as randomized controlled clinical trials of parallel groups,

which may require refinements of traditional clinical regimes. Together with the WHO the EURAMOS collaborators "prevent and treat these disabling disorders" (12,15).

#### 2.2.1.1 EURAMOS-1 schedule

EURAMOS-1 tries to improve the EFS and the quality of life for patients with osteosarcoma. All patients receive 10 weeks of chemotherapy with MAP (M (=MTX), A (=DOX) and P (=DDP)). The MAP trial is the first chemotherapy cycle and it is the same for every patient. The treatment regime and dosage of the cytotoxic agents are summarised in Table 9.

| Treatment regimen | | | |
|---|---|---|---|
| A (DOX) | Adriamycin, Doxorubicin | 37.5 mg/m$^2$/day × 2 | Σ 75mg/m$^2$ |
| Week 1, 6, 12 + MAP / MAPinf: 17, 22, 26 or MAPIE: 20, 28, 36 | | | |
| E (ETO) | Etoposide | 100 mg/m$^2$/day × 5 | Σ 500mg/m$^2$ |
| Only MAPIE: Week 16, 24, 32 | | | |
| P (DDP) | Cisplatin | 120 mg/m$^2$/course | Σ 120mg/m$^2$ |
| Week 1, 6, 12 + MAP / MAPinf: 17 or MAPIE: 28 | | | |
| I (IFO 14g) | Ifosfamide 14g | 2800 mg/m$^2$/day × 5 | Σ 14000mg/m$^2$ |
| I (IFO 9g) | Ifosfamide 9g | 3000 mg/m$^2$/day × 3 | Σ 9000mg/m$^2$ |
| Only MAPIE: 16, 20, 24, 32, 36 | | | |
| M (MTX) | Methotrexate with leucovorin rescue | 12000 mg/m$^2$ | Σ 12000mg/m$^2$ |
| Week 4, 5, 9, 10 + MAP / MAPinf: 15, 16, 20, 21, 24, 25, 28, 29 or MAPIE: 15, 19, 23, 27, 31, 35, 39, 40 | | | |
| ifn (IFNα) | Pegylated interferon α-2b (Peg-Intron) | 0.5 – 1.0 µg/kg s.c. once weekly | |
| Only MAPinf: 30, 104 | | | |

Table 9 Treatment regimes used in EURAMOS-1 with abbreviations and dosage of all i.v. drugs except Pegylated interferon α-2b.

After those 10 weeks surgery should be conducted and the response to chemotherapy should be assessed. Patients are randomized between good and poor responders. Good responders will have less than 10% viable tumour cells after the first chemotherapy cycle, whereas poor responders will have more than 10% viable tumour cells (15).

#### *2.2.1.1.1 Response to chemotherapy*

The treatment trial provides another randomization of good and poor responders: Good responders are further randomized between MAP and MAPifn and poor responders are randomized between MAP and MAPIE. An overview of all randomization opportunities of the EURAMOS-1 treatment trial is shown in Figure 23. In significantly good responders that should maintain remission, Pegylated interferon α-2b is used additionally. This regime is called the MAPinf trial.

At least pre-operative therapy in good responders has increased the three-year EFS up to 75%, compared to 45-55% for poor responders (13,15,20). The aim is that patients with poor response should reach a similar outcome to that reported in good responders. For this reason, Ifosfamide and Etoposide are administered (MAPIE trial).

In case of metastatic disease, which concerns approximately 15 to 20% of all osteosarcoma patients at the time of diagnosis, high-dose Ifosfamide and Etoposide are also administered. The two-year EFS in those patients is similar to patients with poor chemotherapy response, about 45% (15,18,20).

Figure 23 EURAMOS scheduled treatment dates.

### 2.2.1.2 Methotrexate

MTX is a folic acid antagonist that inhibits dihydrofolate reductase (DHFR). Normally, this enzyme reduces dihydrofolate (DHF) to its active form, tetrahydrofolate (THF), which is an essential cofactor in DNA, purine, pyrimidine and protein synthesis. MTX binds the DHFR, blocks the recycling of DHFR and diminishes reduced folate pools (8,25,44–46).
Intracellular MTX is metabolized to polyglutamate forms, which directly block the synthesis of nucleotides. Those polyglutamate forms also increase the intracellular drug accumulation and drug retention (4,15,46). The intracellular manner of MTX and the competition for transport between MTX and other folates make it possible to override its effect. This fact is used in supportive care measures to minimize the adverse effects of MTX (4,7,46,47).
Finally, MTX induces cytotoxic cell damage in the S phase of the cell- cycle, (5,48) therefore it is used as a widely effective anti-metabolite cancer therapy against osteosarcoma and also lymphocytic leukaemia, Non-Hodgkin-Lymphoma, choriocarcinoma and cancer in the neck, head or breast (4,5,25,46,48,49).

#### *2.2.1.2.1 Administration*

In osteosarcoma treatment MTX is administered as High-Dose Methotrexate (HDMTX). The EURAMOS-1 schedule provides HDMTX ($12g/m^2$) intravenously over four hours in weeks 4, 5, 9, 10, 15, 16, 20, 21, 24, 25, 28 and 29 (MAP or MAPifn trial) or in weeks 4, 5, 9, 10, 15, 19, 23, 27, 31, 35, 39 and 40 (MAPIE trial).
Before each chemotherapeutic cycle, some mandatory requirements have to be fulfilled to reduce the risk of complications and adverse effects during the chemotherapeutic administration. First of all, the body surface area (BSA) must be calculated according to the actual height and weight of the patient. All chemotherapeutic agents are administered following the BSA. There are different formulae for adults and children (Formula 1, Formula 2)

$$BSA\ [m^2] = \frac{weight^{0.425}\ [kg] \times height^{0.725}\ [cm] \times 71.84\ [m^2/kg \times cm]}{10000}$$

Formula 1 Body surface area for adults.
http://www.cato.eu/de/koerperoberflaeche.html
Downloaded on 2014-01-24

$$BSA\ [m^2] = \sqrt{\frac{weight\ [kg] \times height\ [cm]}{3600}}$$

Formula 2 Body surface area for children.
http://www.cato.eu/koerperoberflaeche-kinder.html
Downloaded on 2014-01-24

Furthermore, an entire clinical examination must be performed to exclude general infections, serous effusions / other third spaces and suspicious appearances of the skin (paleness as a hint to anaemia, jaundice as a hint to liver dysfunction, oedema as a hint to kidney dysfunction etc.).

The administration of HDMTX is permitted when blood count, differential white count (WBC), blood chemistry, glomerular filtration rate (GFR), Bilirubin and transaminases of the liver are within the range, shown in Table 10 (15).

| Requirements for HDMTX administration | |
|---|---|
| Neutrophils | $\geq 0.25 \times 10^9$/L |
| WBC | $\geq 1.0 \times 10^9$/L |
| Platelets | $\geq 50 \times 10^9$/L |
| Bilirubin | $\leq 1.25 \times$ ULN |
| Transaminases | Elevated during the therapy, but no hint to hepatic failure |
| GFR | $\geq$ 70mL/min/1.73m$^2$ |
| Urinary pH | > 7.0 prior to administration |

Table 10 Minimum requirements for HDMTX administration (15).

### 2.2.1.3 Supportive Care

The kidneys are the principal routes of excretion of MTX. The doses as such are fatal and require supportive care measures. These include the administration of Leucovorin (LV, folic acid) together with adequate fluid, electrolytes and bicarbonates.

LV rescue supplies the product of inhibited enzymes and is administered at the end of MTX infusion (50). LV is given intravenously or orally (15 mg/m$^2$) and started 24-28 hours after the MTX infusion for three consecutive days. In the event of vomiting or increased serum MTX concentrations, the administration of LV is continued until the serum MTX level is considered as safe (<0.1µmol/L) (15).

Fluids (Glucose 4%, Sodium chloride 0.18%, Potassium chloride 20mmol/L, Sodium bicarbonate 50mmol/L) must be given with an infusion rate of 3 L/m$^2$/24 hours to maintain the urine output and to alkalinise the urine pH (5,25,44,45,49,51). The urinary pH must be >7.0 prior to the administration of MTX (15).

The monitoring of serum MTX levels is routinely performed 24-28 hours after the start of the MTX infusion and then daily, to assess the efficacy of supportive care measures. In order to minimize the adverse effects and to be considered safe, the serum MTX concentrations should be <0.1µmol/L (15). The serum MTX levels are determined by fluorescence polarisation immunoassay and should not exceed the upper serum limit, shown in Table 11 (4,8,10,44,46,49,51–54).

| Time | MTX serum concentration |
|---|---|
| At 24 h | <20µmol/L |
| At 48 h | <2µmol/L |
| At 72 h | <0.2µmol/L |

Table 11 The upper limit of serum MTX at 24, 48 and 72 hours.

Other preventive measures include the avoidance of drug interaction and the drainage of third spaces (4,46). The EURAMOS-1 trial recommends carefully monitoring patients' bodily fluids, but it does not separately mention drainage. Drug interactions with aminoglycosides, cisplatin and Nonsteroidal anti-inflammatory drugs (NSAIDs) lead to the disturbance of renal function and should therefore be avoided.

Furthermore, all patients must be treated freehandedly with antiemetic drugs and antibiotic coverage in case of nausea, vomiting or fever. Anaemia and thrombocytopenia can require transfusions. Other undesirable effects must be treated early, corresponding to clinical signs.

#### 2.2.1.4 Toxicities

It is common knowledge that the duration of exposure and the drug concentration appear to be critical factors in development of toxicity (3,4,24,44). However, the higher response and dose intensification of chemotherapeutic agents is responsible for severe adverse effects, including haematological and non-haematological toxicities.

The most common haematological toxicities are neutropenia (occurred in 67-90%) and thrombocytopenia (33%). The non-haematological toxicities concern the kidneys, liver, lung, bone marrow, skin and mucosa. Other common adverse effects are mild to moderate fatigue, anorexia and rigors (15). Especially the administration of MTX causes severe adverse effects, which are partly dose-dependent and potentially life-threatening. Every adverse effect requires prompt intervention (15).

Nausea and vomiting occur immediately (within two days) after the administration of MTX in about 20-40% of all treated patients (occasional adverse effect) and count as minimal systemic toxicities, according to different studies (3,4,11,46,48,55). An acute hepatic toxicity is described in many cases and concerns 98% of all patients. The transaminase values of GPT, GGT and Bilirubin are most commonly increased (46,49).

Other toxicities including allergic reactions, tenderness of the skin, ocular irritation, dizziness and malaise can also occur after receiving the drug. However, these toxicities count as rare. Diarrhoea, stomatitis and myelosuppression occur occasionally within a few weeks from start of the treatment and concern approximately 20% of the patients treated with MTX. Within the same timeframe, serious toxicities (renal failure, lung damage, neurotoxicity, permanent liver dysfunction) can occur too, but they are rare (15,46,49,55).

Moreover, MTX is known as a foetal and teratogenic toxic agent. This has to be considered when MTX is administered to young and fertile patients.

Another point to consider is that MTX tends to accumulate in tissues and cavities and induces third spaces. The third spaces lead to local, sometimes even systemic, toxicity and delayed elimination (4,6,8,10,45,53,54,56,57). Currently, there is hardly any information

about third space effect in patients with periprosthetic seromas and its relevance for treatment.

### 2.2.2 Third spaces

Until today, several different studies investigating third spaces, also called malignant effusions, third space fluids or effusion spaces, have been published. Third spaces are suspected to cause inter-individual variations in the pharmacokinetics of MTX. The third space compartments, primarily pleural effusions, ascites and oedematous tissues are supposed to be responsible for prolonged excretion and increased risk of toxicities (7,10,54,56,57).

The content of proteins and the product of permeability in third spaces are responsible for the precipitate of chemotherapeutic agents. The product of permeability constant to lipid solubility indicates the therapeutic efficacy and the development of a third space effect.

### 2.2.3 Surgery

As previously mentioned, surgery was the only treatment for osteosarcomas up to the 1970s. Whereas previously an amputation was the surgical method of choice, different types of reconstruction are feasible today. Nowadays, limb salvage surgery is implemented in two-thirds of all treated patients (13). The type of reconstruction depends on both the patient and surgeon. In every case the risks and benefits must be assessed in comparison with alternative treatment options (14,15,58).

In an ideal case, every patient with suspected bone tumour is referred to a reference centre. First of all adequate pre-operative staging and biopsy must be taken to verify osteosarcoma. After histological verification of an osteosarcoma, chemotherapy can be initiated and definitive surgery can be planned. Surgery of the primary tumour is provided for week 11 according to the EURAMOS-1 trial. If possible, a wide or radical (en-block) resection should be performed. EURAMOS-1 refers to Enneking's classification (Table 12).

| Type | Dissection |
|---|---|
| Intralesional | Within the lesion |
| Marginal | Through the pseudocapsule or reactive tissue |
| Wide | Lesion (including biopsy scar), pseudocapsule and / or reactive zone and an unviolated cuff of normal tissue completely surrounding the mass removed as a single block |
| Radical | Entire anatomic compartment containing the tumour removed as one block |

Table 12 Adapted from Enneking (1980): A system for the surgical staging of musculoskeletal tumours (15,31,58).

#### 2.2.3.1 Limb salvage surgery

While most often a limb salvage surgery can be performed, sometimes a mutilating surgery becomes necessary when a safe removal of the tumour is not possible. The indications for limb salvage surgery depend on the patients' age, the size and response of the tumour to the pre-operative chemotherapy, its localisation and local extent.

Small tumours with good pre-operative chemotherapy response are particularly suited for limb salvage surgery. Several x-rays (including sizing and allocation of the skeletal age) and MRIs of the affected site must be taken to determine the extent and estimated type and size of endoprosthesis before surgery. Additionally, bone scans are recommended to identify skip lesions.

The reconstruction with endoprosthesis is the most common option, especially in tumours of the distal femur, proximal femur, proximal humerus and proximal tibia (if extensor mechanism is reconstructed). In young children (<age of 10) with bone tumours, the use of extendable endoprosthesis has been recommended. Those special prostheses with lengthening function have a higher risk for complications (15). Furthermore, more revisions are necessary in younger patients with endoprostheses (15).

In case of poor response to pre-operative chemotherapy, a limb salvage surgery must be undertaken with particular caution (13–15). In general, for limb salvage surgery, some things should be considered: a limb salvage surgery could only be performed, if the tumour can be resected as a single block, without contamination of surrounding tissues and the reconstruction with endoprosthesis is possible and likely to be successful. Patients should

be aware of the risk–benefit ratio, assessing it together with an experienced oncological and surgical team (12,15,58).

#### 2.2.3.2 Amputation

A marginal or intralesional resection should be avoided in surgery any way possible. When this is not possible, there is a major of local recurrence and death (13–15). In such cases an amputation seems to be the method of choice (14,15,20,58). The inability to remove the tumour without leaving residual disease is an obvious indication (14,15,20).
Furthermore, an extensive involvement of neurovascular structures might lead to a better outcome with amputation (13,15,21). The extent of amputation depends on the intraosseus tumour involvement and the availability of sufficient closure with skin (15,21). A safety margin must be adhered to, in other cases a disarticulation of involved joints has to be done. The safety margin is determined by pre-operative imaging and intraoperative monitoring of the tumour (15).
Of course, attention has to be paid to the patient's preference, but individual wishes may compete with optimal surgical treatment. It must be clear that residuals worsen the patients' EFS and OS (14,15).

#### 2.2.3.3 Resection or radiotherapy

Tumours of the axial skeleton require comprehensive pre-operative staging in order to decide whether they are resectable or not. The operability of a tumour depends on amenability of the tumour site. A tumour is operable when it can be removed completely and potentially curative (13,14).
The aim of surgery is to remove the tumour completely while adhering to the safest oncological margins; nevertheless the curative intent cannot always be achieved. In those cases of inoperable sites or with inadequate margins, there is the opportunity of radiotherapy (13–15).
When radiotherapy is indicated, it is realised additionally to chemotherapy. The outcome of patients treated with radiotherapy and chemotherapy is worse to those with surgery (<20% long term survival) (14,15). Therefore, several experienced surgeons should assess pre-operative images before assuming inoperability (14,15).

### 2.2.4 Metastatic disease

Patients with metastases are treated with curative intent as long as a resection of all metastatic deposits is possible. Even when several regions and different sites are affected, those patients can have a similar prognosis to those with localised disease, when a complete surgical removal of all deposits can be achieved. This applies particularly to patients who have pulmonary metastases at the time of diagnosis. About 15-20% of all patients already have metastases at the time of diagnosis. The lungs are affected most commonly by metastatic disease (95%, Figure 24), followed by local recurrence (50%) (14,15).

The surgery of the pulmonary metastases should be realised between weeks 11 and 20 according to the EURAMOS-1 trial. The collaborators of EURAMOS recommend a bilateral thoracotomy, although x-ray and CT suggest unilateral disease. Experience shows that surgeons can detect very small mutations since they are more sensitive than usual radiological methods. Additionally, thoracosopic techniques should be avoided, because they cannot replace surgeons' hands.

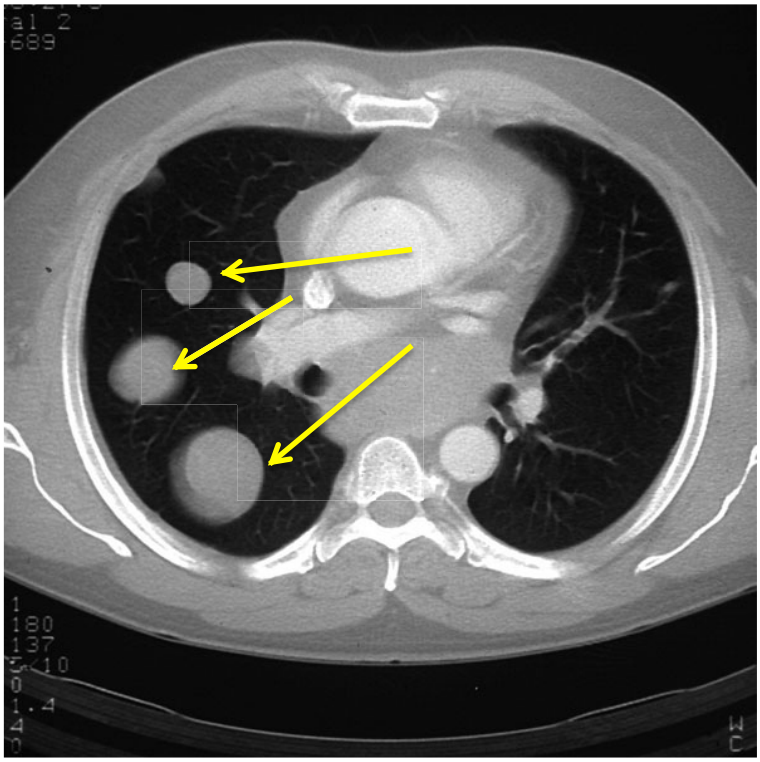


Figure 24 Lung metastases in the right lung in a 17-year-old man, one year after diagnosis of an osteosarcoma

# 3 Materials and methods

## *3.1 Patients*

We performed a monocentric, retrospective analysis of 104 consecutive patients, who had been treated between 1991 and 2011 for histologically confirmed high-grade osteosarcoma at the Medical University of Graz (Department of Orthopaedic Surgery and Division of Clinical Paediatric Oncology, Department of Paediatrics or Division of Clinical Oncology, Department of Internal Medicine).

59 patients had to be excluded, thus 45 patients were analysed. The reasons for exclusion were diverse: seventeen patients (16.35%) had surgeries without implanting an endoprosthesis and four patients (3.85%) had inoperable tumours. Further fourteen cases (31.11%) were excluded because of missing HDTMX data. Twenty-four patients (23.07%) had only surgery in the reference centre in Graz but the chemotherapy at their home hospital. Because of the monocentric study design these patients had to be excluded too.

Patients who had a limb salvage surgery with endoprosthesis and chemotherapy with HDMTX were included. It should be mentioned at this point that our institutions are accurate with the EURAMOS-1 schedule. Nevertheless, the trial provisions could not always be adhered to because of individual adverse events. If patients did not receive HDMTX 12 times, as required in EURAMOS-1 trial, we nevertheless, decided to include them because the aim of our study was a comparison of MTX levels in serum and periprosthetic seromas, so the number of MTX administrations in each patient played a minor role for our question. Thirteen patients (28.89%) did not receive HDMTX 12 times because of drug-related toxicities (e.g. pancytopenia), disease-related deaths or other unknown reasons. Two patients died before finishing the chemotherapy because of their tumour progression. One of them died after the eighth administration, the other one after the tenth cycle. Three patients did not get the last two administrations, one patient did not get the last four administrations and one patient only got half of the 12 cycles because of severe chemotherapy-induced adverse effects. Six patients had ten HDMTX administrations, the reason for that is from a present-day perspective inexplicable.

On the other hand, we also included further MTX administrations than the required 12 of the EURAMOS-trial: in patients with metastatic disease or in administration schedules of prior therapy trials (e.g. COSS-86, COSS-91 and COSS-96). Five patients had six additional HDMTX administrations and one patient four additional administrations.

All in all, we analysed 514 episodes of HDMTX in 45 patients instead of 540 HDMTX episodes. We also included patients older than 40 years, because of our small number of patients that were children or adolescents. This including criterion yielded two patients additionally, who received their therapy according to the EURO-BOSS trial.
All patients' data were anonymised with ongoing numbers for statistical analysis.
Furthermore, the ethical committee of the medical University of Graz gave their consent for the conduction of this study.

## *3.2 Methods*

The aim of our study was to evaluate MTX concentrations in possible periprosthetic seromas in comparison to the corresponding MTX concentrations in serum. Because of the potential risk based on a third space effect emanating from the periprosthetic seromas, we punctured patients with periprosthetic seromas in our department despite the increased risk of infections in immunodeficient patients.
Both in serum and in periprosthetic seromas the MTX concentrations were determined by fluorescence polarisation immunoassay, for that reason a comparison is feasible. In this study we compared the concentrations routinely measured after 24, 48 and 72 hours in serum with the corresponding concentrations in periprosthetic seromas.
The date of HDMTX administration and the date of taking the blood samples were noted on the laboratory document, so that a chronological correlation can be detected easily. In the case of MTX determination from a periprosthetic seroma, the date and the additional information from where the specimen was taken could also be read from the laboratory document.
The difficulty in this study lay in finding the necessary information, which stretched back up to twenty years. As a first step we searched for patients whose therapy was based on aggressive surgery, meaning a wide, en-block resection and implantation of an endoprosthesis and a multi-agent chemotherapy including HDMTX, DOX, P, ETO, IFO and IFNα.
Furthermore, the cytotoxic agents have to be administered in the same way and dosage so that MTX concentrations become comparable. HDMTX was provided intravenously twelve times (with exceptions, see chapter 3.1 "Patients") with 12g/m$^2$ over four hours with an interval of at least three weeks in all patients. Supplementarily, all patients must receive an intravenous or oral Leucovorin (LV) rescue (initial dose of 15 mg/m$^2$ started 24-

28 hours after MTX infusion, later on according to the MTX serum level), hydration and alkalization to accomplish the provisions of the EURAMOS-1 schedule (see chapter 2.2.1.3 “Supportive care”).

The hydration was conducted with Glucose 4%, Sodium chloride 0.18%, Potassium chloride 20mmol/L and Sodium bicarbonate 50mmol/L. The infusion rate was approximately 3 L/$m^2$/24 hours, adjusted to urinary pH. The urinary pH must be >7.0 prior to the administration of MTX.

These criteria were the case if HDMTX was administered according to EURAMOS, EURO-BOSS, COSS-96, COSS-91 or COSS-86 regimens. Today the COSS-trials are resumed in the EURAMOS-1 collaboration. The EURO-BOSS trial was the corresponding treatment schedule for adults. In order to maintain an overview in this study, the EURAMOS-1 trial was explained (see chapter 2.2.1 “Chemotherapy” and chapter 2.2.1.1 “EURAMOS-1 schedule”).

As a second step, we looked for the MTX serum concentrations, which were routinely determined 24, 48 and 72 hours after starting the HDMTX infusion. According to the EURAMOS-1 trial, the serum drug monitoring should be performed every 24 to 28 hours until the MTX concentration was considered safe (<0.1µmol/L).

Moreover, we looked for patients who developed periprosthetic seromas. In our department, significant periprosthetic seromas were punctured before and/or after administration of HDMTX to avoid a third space effect. The periprosthetic seromas were verified by clinical examination and punctured under sterile conditions. If available, we noted the amount of punctured fluid and the HDMTX concentration of the periprosthetic seromas. Those periprosthetic seromas that were related to corresponding serum levels were included, those with no correlation to a prior HDMTX administration could not be compared.

Additionally, further information, like baseline patients’ characteristic, toxicities and renal parameters (creatinine), but also localisation, histological type / subtype and regression level of the tumour, relapses, metastases, revisions and deaths were recorded for descriptive statistical analysis. The information was obtained from the archives of our institutions and from our hospital information system MEDOCS.

### 3.2.1 Laboratory values

All test results, including blood count, blood chemistry, liver- and kidney parameters and drug monitoring were determined at the Department for Clinical and Chemical Laboratory Diagnostic, Medical University of Graz. The MTX concentrations in serum and periprosthetic seromas were measured by fluorescence polarisation immunoassay technology in the same way.

### 3.2.2 Statistical analysis

Patients' baseline characteristics were recorded with Microsoft Excel for Mac 2011, version 14.3.9. Every patients' identity was protected by using ongoing numbers.
Baseline characteristics included sex, age at the time of diagnosis, height, weight and BSA during the therapy, plus exact HDMTX dosage, creatinine value and adverse effects. The localisation of the tumour, affected side, histology and classification of osteosarcoma according to the regression grades defined by Salzer–Kuntschik et al. were chosen. Furthermore, surgical treatment with endoprosthesis, relapses, metastatic disease and surgical revisions were noted.
All HDMTX concentrations of every patient were analysed by determination of minimum, maximum and range. In another statistical analysis, we compared serum MTX concentrations routinely obtained 24, 48 and 72 hours after starting the HDMTX infusion according to the EURAMOS-1 trial with MTX concentrations of corresponding periprosthetic seromas, obtained six hours before to six hours after the respective serum concentration. Differences between concentrations of periprosthetic seromas and corresponding serum were assessed by means of a Wilcoxon test, using SPSS for Mac 20. Differences in serum concentrations between punctured and non-punctured patients were assessed by means of a Mann-Whitney-U-test using SPSS for Mac 20. The p value $<0.05$ was chosen to be significant. In addition, the Gaussian distribution was tested by using Kolmogorov-Smirnov and Shapiro-Wilk-test in SPSS.

# 4 Results

## *4.1 Baseline patients characteristics*

45 patients with limb salvage surgery and implantation of an endoprosthesis were treated with HDMTX because of an osteosarcoma. The patients had a median age of 14.4 years (range 4.9-45.6 years) at the time of diagnosis. With a number of 28 male and 17 female patients, there were significantly more male patients (Fisher exact test, p=0.034).
Table 13 and Table 14 show an overview of baseline characteristics: sex, age at the time of diagnosis, height, weight and BSA during the therapy plus HDMTX dosage and creatinine value. The included patients, who died during treatment or follow-up were listed too.

| 45 Patients | Number | Percentage |
|---|---|---|
| Male | 28 | 62.2 |
| Female | 17 | 37.8 |
| Punctured patients | 18 | 40.0 * |
| Punctured patients with periprosthetic seroma MTX concentrations | 11 | 61.11** |
| Deceased during treatment or follow-up | 19 | 42.2 |

Table 13 Patients baseline characteristics: sex, punctures and decedents.
* referred to all 45 patients
** referred to 18 punctured patients

| Baseline characteristics | Median (minimum-maximum) |
|---|---|
| Age | 14.4 years (4.9-45.6 years) |
| Height | 168.0cm (108.0-199.0cm) |
| Weight | 56.7kg (16.9-99.5kg) |
| BSA | 1.65$m^2$ (0.73-2.63 $m^2$) |
| HDMTX dosage | 19g (8.8-25g) |
| Creatinine during therapy | 0.71mg/dl (0.29-1.18mg/dl) |

Table 14 Patients baseline characteristics: age, height, weight, BSA, HDMTX dosage and creatinine.

Osteosarcomas most commonly affected the distal femur (29 cases, 64.4%), followed by the proximal tibia (12 cases, 26.7%) and humerus (four cases, 8.9%). Interestingly, almost twice as many of the cases (29 cases, 64.4 %) were located on the left side in comparison to the right side (16 cases, 35.6%, Fisher exact test p=0.011).

The pathologic subtypes of osteosarcomas could not be retrospectively identified in all patients, but the osteoblastic (thirteen cases) and chondroblastic (ten cases) osteosarcomas occurred more often than the fibroblastic (four cases). The most common histological type was the central, conventional high-grade (G3, G4) osteosarcoma, with regression grade (RG) 3 (15 cases, 33.3%) followed by RG 5 (14 cases, 31.1%). RG 2 and RG 4 were seen in five cases (11.1%) and eleven cases (24.4%), respectively.

Surgery for primary tumour was performed arround week 11. Afterwards, 17 patients (37.7%) had revisions because of elongation of the endoprosthesis, endoprosthesis changes or wound infections. Altogether 20 patients (44.4%) suffered from a systemically or local relapse, which in most cases presented as lung metastases (88.7%).

During chemotherapy all patients had normal creatinine levels (median 0.71 mg/dl, range 0.29-1.18 mg/dl). Observed toxicities included mucositis (20%), nausea / vomiting (30%), fatigue (4.4%), anorexia (6.5%), deafness (8%), leukopenia (15%), thrombocytopenia (13.5%), delayed elimination indicated by a third space effect emanating from periprosthetic seromas (40%). The percentages of the toxicities were referred to information from patients' documents. Excluding the patients who dropped out because of missed appointments, we knew about 19 disease-related deaths until December 2012 (42.2%).

All patients were registered to the respective osteosarcoma study group and fulfilled the criteria of the EURAMOS-1, COSS-86, COSS-91, COSS-96 and EURO-BOSS treatment trials, shown in Table 15, except for the age. In addition to children and adolescents, we included patients with an age of 40 years and older. Two adult patients (40 years and 46 years) fulfilled the remaining criteria and were be included.

| Patients selection criteria |
|---|
| Histological evidence of a high – grade osteosarcoma of the extremity or axial skeleton (including those arising as second malignancies) |
| Resectable disease (completely, wide) |
| Age ≤ 40 years* |
| Registration within 30 days of diagnostic biopsy |
| Start chemotherapy within 30 days of diagnostic biopsy |
| Neutrophils ≥ $1.5 \times 10^9$/L or WBC ≥ $3.0 \times 10^9$/L |
| GFR ≥ 70ml/min/1.73m$^2$ |
| Serum bilirubin ≤ 1.5 × ULN |
| Sufficient cardiac function: SF ≥ 28% or EF ≥ 50% |
| Adequate performance status (Karnofsky score ≥ 60, WHO ≤ 2) |
| Fit to undergo protocol treatment and follow-up |
| Written informed consent |

Table 15 EURAMOS-1 patient selection criteria.

## *4.2 HDMTX administration*

| Administration | Median (minimum-maximum) |
|---|---|
| 514 HDMTX administrations in 45 patients | |
| Administrations per patient | 12 (6-14) |

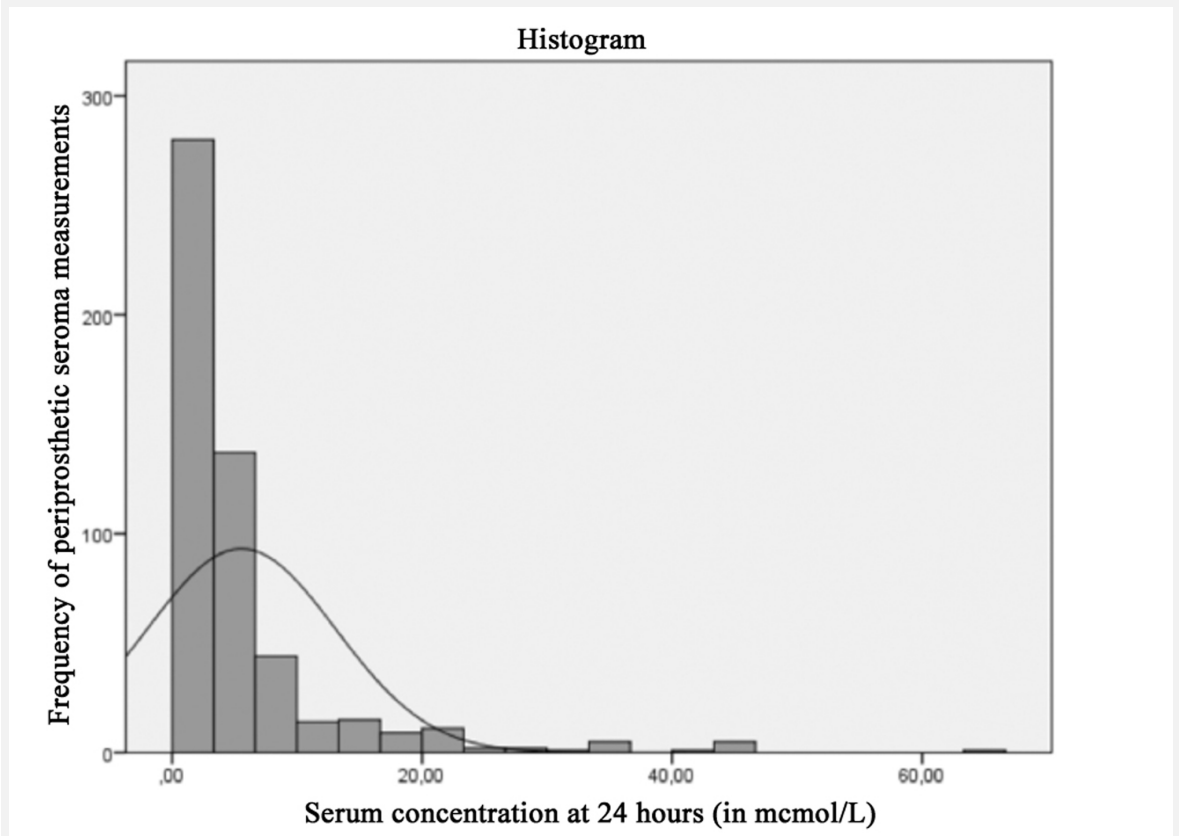
Table 16 Overview HDMTX administrations.

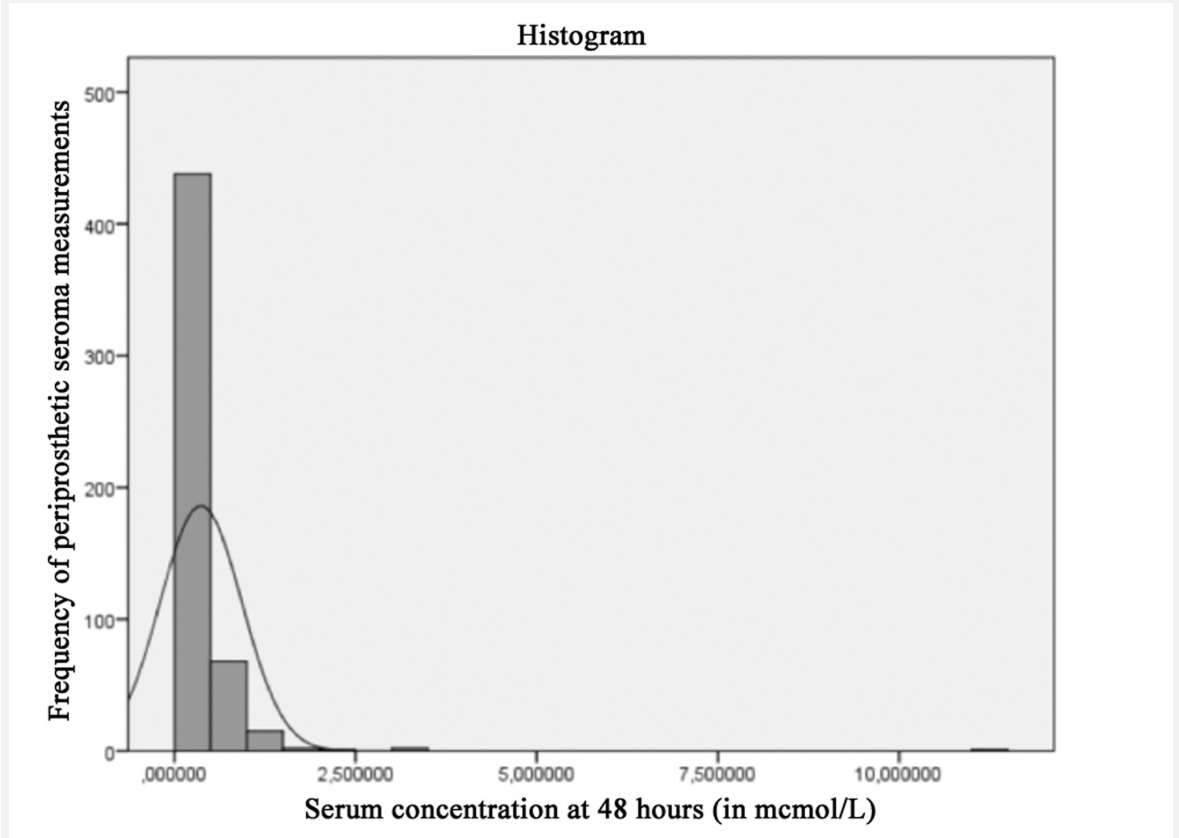
All patients received HDMTX intravenously over four hours in weeks 4, 5, 9, 10, 15, 16, 20, 21, 24, 25, 28 and 29 if they had a good response (MAP or MAPifn trial), if not, HDMTX was administered in weeks 4, 5, 9, 10, 15, 19, 23, 27, 31, 35 and 39 (MAP or MAPIE trial). Altogether we found 514 episodes of HDMTX (median 19g, range 8.8-25g according to 12g/m$^2$) to be evaluated (Table 16).

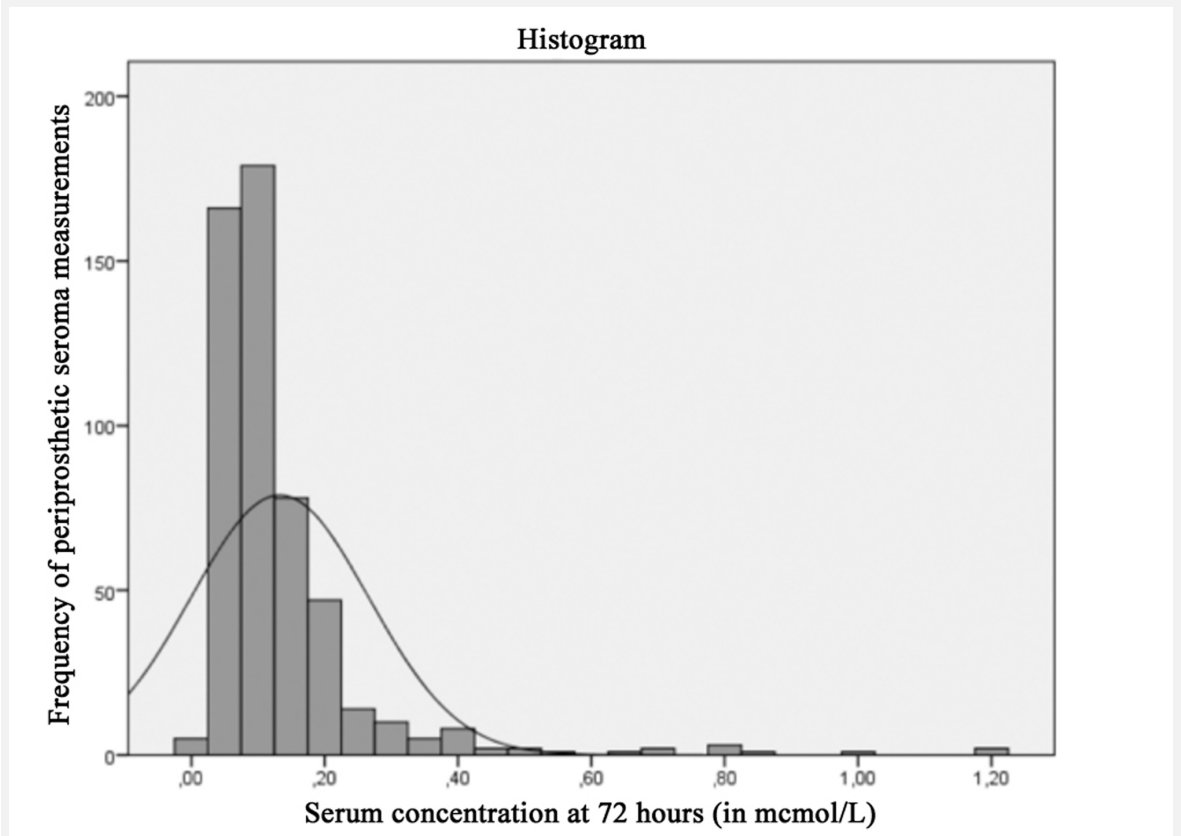
Not every patient received 12 cycles of HDMTX: Some had inacceptable adverse effects of MTX, others died before finishing the treatment.
Two patients died before finishing the chemotherapy because of their tumour progression. One of them died after the eighth administration, the other one after the tenth cycle. Three patients did not get the last two administrations, one patient did not get the last four administrations and one patient only got half of the 12 cycles because of severe chemotherapy-induced adverse effects (infections, thrombocytopenia, leukopenia). Six patients had only 10 HDMTX administrations for unknown reasons.

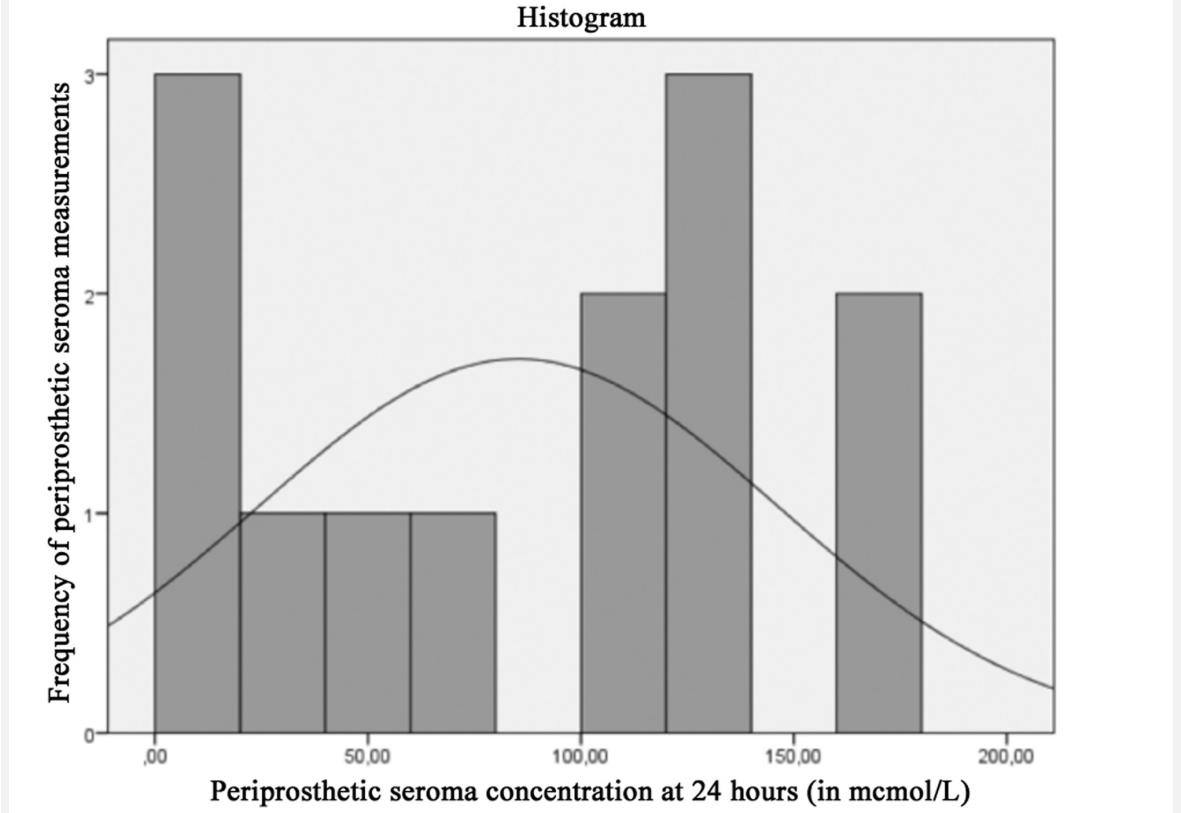
### 4.2.1 Distribution of MTX values

The Kolmogorov-Smirnov and Shapiro-Wilk test showed that the MTX concentrations are not normally distributed, both in serum concentrations and in periprosthetic seroma concentrations, shown in Table 17. All 514 episodes of HDMTX of all 45 patients were analysed, with regard to the routine time points of measurements (24, 48, 72 hours after MTX administration). In Figure 24, the x-axes show how often serum measurements or periprosthetic seroma measurements were performed. The y-axes show the respective concentrations at 24, 48 and 72 hours from start of MTX infusion in μmol/L. The line represents the Gaussian distribution.


Histogram
Frequency of periprosthetic seroma measurements
300
200
100
0
,00
20,00
40,00
60,00
Serum concentration at 24 hours (in mcmol/L)


Histogram
Frequency of periprosthetic seroma measurements
500
400
300
200
100
0
,000000
2,500000
5,000000
7,500000
10,000000
Serum concentration at 48 hours (in mcmol/L)


Histogram
Frequency of periprosthetic seroma measurements
200
150
100
50
0
,00
,20
,40
,60
,80
1,00
1,20
Serum concentration at 72 hours (in mcmol/L)


Histogram
Frequency of periprosthetic seroma measurements
3
2
1
0
,00
50,00
100,00
150,00
200,00
Periprosthetic seroma concentration at 24 hours (in mcmol/L)

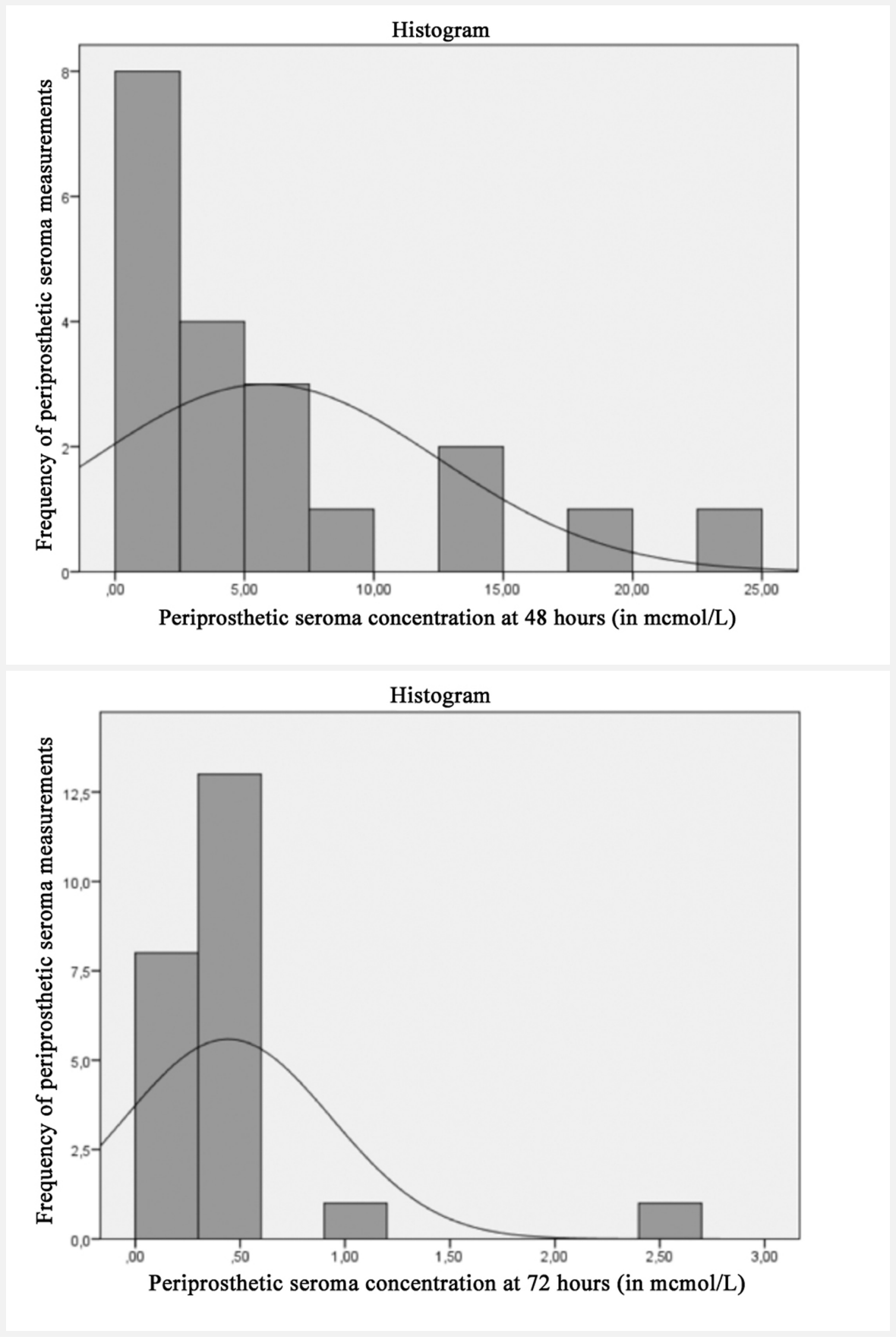



Figure 24 Distribution of MTX concentrations in serum and periprosthetic seromas at 24, 48 and 72 hours. after MTX infusion.

### 4.2.2 Comparison of MTX in serum and periprosthetic seromas

The MTX concentrations in serum and in periprosthetic seromas were within a wide range. As expected, the comparison of both serum concentrationsas well as periprosthetic seroma concentrations showed statistically significant differences between the three different time points. All observed values are summarized in Table 18.

| Time | Serum | Seroma | p-value |
|---|---|---|---|
| 24 h | 3.09 (0.37-45.0) | 109.83 (4.91-170.74) | =0.001 |
| 48 h | 0.26 (0.04-3.39) | 2.63 (0.85-24.36) | <0.001 |
| 72 h | 0.09 (0.01-1.21) | 0.38 (0.11-2.5) | =0.015 |

Table 17 MTX values (median, minimum and maximum) measured in serum and periprosthetic seromas (unit µmol/L) at 24, 48 and 72 hours from start of infusion

### 4.2.3 Periprosthetic seromas: Third space fluids

| Punctures | Median (minimum-maximum) |
|---|---|
| 112 punctures in 18 patients<br>71 punctures with HDMTX concentrations in 11 patients<br>41 punctures with HDMTX concentrations and serum levels | |
| Punctures per patient | 4.5 (1-20) |

Table 18 Overview periprosthetic seroma punctures

Periprosthetic seromas occurred in 18 patients (40.0%), which where punctured 112 times. The median of performed punctures per patient was 5 (range 1-20 punctures per patient).
The amount of punctured periprosthetic seromas was documented in 101 punctures, ranging from 5-420 ml (median 150 ml).
MTX concentrations were determined in 71 punctures, performed in 11 patients (61.1%). The interval to the corresponding HDMTX administration was available in 41 measurements. Forty-one periprosthetic seromas punctures were attributable to corresponding serum levels. In those the periprosthetic seroma punctures were 1.49-42.97 (median 14.86) times higher than the corresponding serum MTX concentration at 24 hours. At 48 hours the seroma punctures were 1.36-52.56 (median 8.50) higher than the

corresponding serum levels. At 72 hours the periprosthetic seroma concentrations were in median 2.66 (range 0.66-5.82) times higher than the serum measurements. Especially the 24 hours periprosthetic seroma concentration ranged up to highly toxic 170.74µmol/L (median 109.83µmol/L, range 4.91-170.71µmol/L) in comparison to a median value of 3.09µmol/L in serum (range 0.37-45.0µmol/L, Wilcoxon test; p=0.001). Similar statistically significant differences were observed at 48 (p<0.001) and 72 hours (p=0.015). Figure 25 shows the differences of periprosthetic seroma concentrations and concentrations in serum by using the median values of all 514 administrations and all 41 corresponding serum levels in a logarithmic scale.

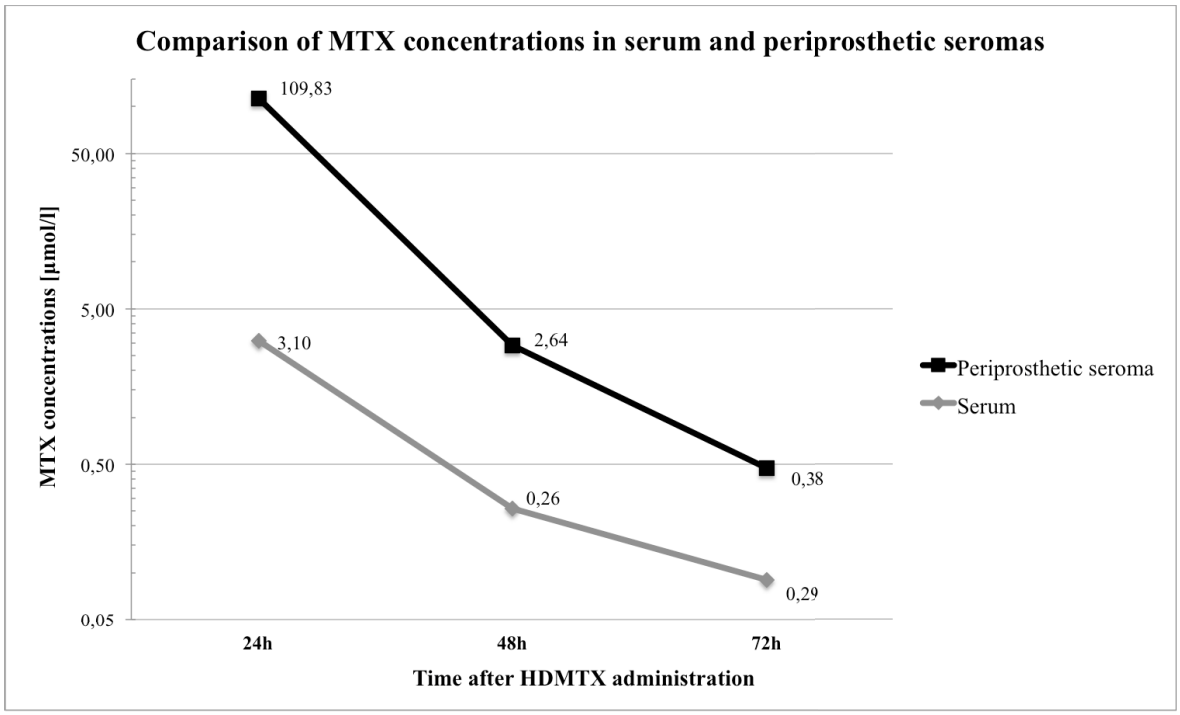



Figure 25 Comparison of MTX in serum and periprosthetic seromas in a logarithmic scale.

### 4.2.4 Differences between punctured and non-punctured patients

Figure 26 shows the serum concentrations at 24, 48 and 72 hours after HDMTX administration in patients who were punctured and those who were not. The Mann-Whitney-U-test showed no statistical differences at all three points of measurement (p=0.64 at 24 hours, p=0.57 at 48 hours and p=0.97 at 72 hours after administration of HDMTX).

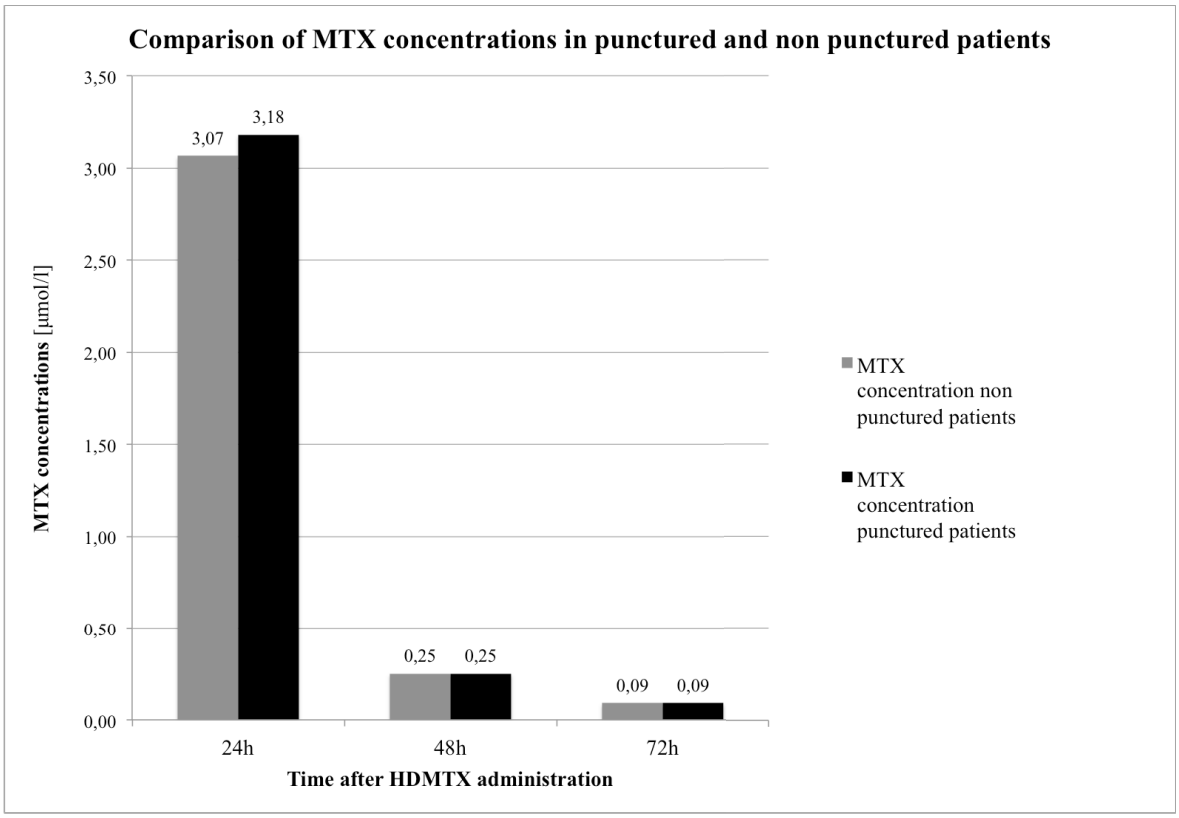



Figure 26 Comparison in MTX concentrations in serum between punctured and non-punctured patients.

# 5 Discussion

The aim of our study was to describe MTX concentrations in possible periprosthetic seromas after HDMTX administration for osteosarcoma and to compare MTX concentrations of these periprosthetic seromas with corresponding serum concentrations. Our results show, that MTX concentrations of periprosthetic seromas are statistically significant higher than corresponding serum MTX concentrations at all three times of routine measurements.

Osteosarcomas are an aggressive disease with poor prognosis and an EFS of approximately 50-60% (2,20). Collaboration of osteosarcoma study groups and the launch of adequate surgery and multi-agent chemotherapy improved the OS, even if it is not satisfactory at all. Despite the current state of knowledge about osteosarcoma, the survival rates did not further improve within the last two decades. Different studies confirm that further approaches are needed to determine more aetiological and prognostic factors (1,18,20,23).

In the course of their efforts to optimize the therapy, Whelan et al. suggest that patients who underwent limb salvage surgery had an improved five-year OS compared to those who were undergoing amputation (20). They report higher local recurrence rates, but a better overall survival.

In this present study we did neither analyse patients undergoing amputations nor EFS of osteosarcoma patients. We focused on patients who underwent limb salvage surgery with endoprosthesis and among those the prognosis is poor, despite surgery and chemotherapy.

The aim of our study was to evaluate a potential toxic risk based on a third space effect emanating from the periprosthetic seromas. Furthermore, we wanted to confirm the necessity of punctures despite the increased risk of infections in immunodeficient patients during chemotherapy.

Up to now it is widely established that the concentration as well as the duration of exposure of antineoplastic agents appear to be critical factors in the development of toxicities (3,4,24,44). Severe toxicities of MTX are potentially life-threatening and require prompt intervention. Different studies report approximately 6% of drug-related deaths (4–7,10,56).

In some studies, prolonged MTX concentrations, caused by renal failure, are considered to be responsible for toxicities (4–6,46,52,55,57), while others argue that renal dysfunction

alone, which occurs in only 1.8-2%, cannot entirely explain the origin of toxicities (4,49,51). Treon et al. suggest that even patients with normal renal function (normal serum creatinine and GFR >60ml/min) developed toxicities in their study. Their explanation is that the variations of toxicities apparently depend on age. Younger patients are more likely to develop mild gastrointestinal toxicities, while older patients suffer from serious toxicities (4). Other studies do not attach greater importance to age (46,52,57). They say that mild and moderate toxicities occur commonly, occasionally or rarely, regardless of age. These mild and moderate toxicities comprise nausea, vomiting, anorexia, diarrhoea, stomatitis, photosensitivity, myelosuppression and elevation of the transaminases (46,52,57).

Jaffe et al. suggest that the increased frequency of toxicities is associated with dehydration, infection, fever and the presence of third spaces. In their study they were not able to find significant complications with urine output alone, but with third spaces (55). Further studies also consider toxicities to be associated with third spaces, but they use different names for it, e.g. malignant effusions, pleural effusions, oedema, ascites or serous fluids. According to these studies, third spaces are responsible for the higher risk of developing toxicities and even for drug-related deaths (4,6,7,10,46,48,54,55,57).

Despite the fact that there are different opinions concerning distribution and elimination of MTX in third spaces, there is a recommendation to avoid MTX while third spaces are present or to puncture third spaces before administering MTX (54–56). The EURAMOS-1 trial recommends prompt intervention in case of severe toxicities; include provisions for adequate hydration, alkalinisation of the urine, further determinations of MTX serum levels and Leucovorin (LV) administration in case of delayed elimination. In case of third spaces, HDMTX administration should be avoided. Especially the pleural effusions after thoracotomies are highlighted, but management of periprosthetic seromas is not mentioned anywhere (15).

Third spaces are considered to alter drug pharmacokinetics of MTX and to contribute to inter-individual variations in distribution and elimination. A delayed elimination increases the risk of toxicities, especially in patients with third spaces (7,44,59). Moreover, an altered distribution gives the indication for prolonged therapeutic drug monitoring. It is supposed that drug concentrations of third spaces exceeds that of serum (8,11). Li et al. even consider third spaces as potentially life-threatening (53).

One explanation for the alteration of drug pharmacokinetics and the variations in distribution and elimination might be the slow release of the drug from third spaces. A slow release causes an increase in the steady state volume of distribution and in prolonged half-life (46,53,56).
Chabner et al. and Evans et al. describe a two compartment model of distribution, while Wan et al. and Pauley et al. talk about a triphasic decline of MTX (8–10,56,59). Currently, we do not know whether the distribution follows a two or three compartment model, but we know that the decline is different in third spaces. Chan et al. criticise that no accurate measurements for third space effects are available (6).

The rate of change in the third space is a function of the effusion-volume, infusion-rate and the concentration between third space and serum (48). Further parameters influencing third space concentrations are changes in osmotic pressure and the placement of drug carrier complexes. Through the content of proteins in cavities, third spaces become regions for sequestering chemotherapeutic agents. Apart from this, the product of permeability constant to lipid solubility is also important for the therapeutic efficacy of third spaces (7,53). To summarize it can be noted that any pharmacokinetic change may influence third spaces and may contribute to a third space effect. Hall et al. and Bird et al. confirm the effect of MTX in third spaces. They used MTX intra-articularly in patients with knee afflictions and found systemic reactions (60–62).
Otherwise there are also studies that found no statistical difference between distribution and elimination (54) or reduced response of MTX in patients with third spaces (8). Jaffe et al. even assert that any dose escalation does not enhance the therapeutic efficacy (3).

Despite various attempts to explain the importance and effects of third spaces on MTX it can be resumed that third spaces result in prolonged half-life and decreased clearance of cytotoxic MTX, reflected by changes in diffusion and permeability (5,7,25,57). Our data confirms the hypothesis that third spaces may cause an increased risk for toxicities.
In our present study we analysed third spaces in terms of periprosthetic seromas, an investigation that, to our knowledge, was conducted for the first time. The strength of this study is the comparison of periprosthetic seromas and serum measurements. However, there are some limitations, mostly arising from the retrospective study design.
We have to assume that the routine monitoring 24, 48 and 72 hours from the start of infusion were realised within the period of service (+/- four to six hours). Further, it has to

be mentioned that punctures were not performed exactly to the same time points as measurements of serum concentrations. There were both serum and seroma measurements not performed exactly 24, 48 and 72 hours after HDMTX administration, from which different MTX values might have been occurred. A bias, that arose due to the retrospective study design. Nevertheless, our measurements were close to the defined routine measurements (24, 48 and 72 hours from start of infusion) and always determined in the same way. Therefore there is, of course, any doubt upon the results of our study.
The periprosthetic seroma punctures, which were related to an MTX serum level, show an obvious result. We found significant higher concentrations and concentrations in the third spaces than in serum, possibly contributing to a third space effect.
Information in all other toxicities, which were analysed were taken from patients' documents. It should be considered that due to the retrospective study design and the fact that probably not every adverse effect was documented the percentage of severe toxicities might be higher. Nevertheless, the cases in which adverse effects were noted were analysed and the results were similar to those of other studies. (3,4,11,46,48,55)

At this time we cannot predict which patients will develop periprosthetic seroma that might act as a third space. What we know is that the concentrations of those third spaces are significantly higher than in corresponding serum levels.
Furthermore, we agree with those who observed a wide variation in serum levels (51,52) just as in third spaces (5,7,8,10,11,50,56,59). We also found a wide variation among the serum values, but no statistical difference between those patients with puncture of periprosthetic seromas and those without. This might be explained by the non-randomized character of the study. In out centre, all patients with significant periprosthetic seromas are punctured. So, in this retrospective study we compared patients with significant periprosthetic seroma and puncture and patients without periprosthetic seroma (and therefore without puncture). On the other hand, our data shows that puncture of significant periprosthetic seromas does not decrease the therapeutic level of MTX in serum in comparison to patients without periprosthetic serum (and therefore without puncture).
The statistical difference between periprosthetic seroma punctures and serum indicate the feasibility of punctures to avoid local toxicity and delayed elimination and support punctures despite the increased risk of infections.

## *5.1 Conclusion*

In this study we demonstrated that MTX concentrations in periprosthetic seromas were significantly higher than in corresponding serum levels and reached highly locally toxic levels at 24, 48 and 72 hour intervals. Relating to this we recommend the puncture of periprosthetic seromas before MTX courses in order to avoid delayed elimination and possible toxic side effects.

# 6 Bibliography


1. Mirabello L, Troisi RJ, Savage SA. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the Surveillance, Epidemiology, and End Results Program. Cancer. 2009 Apr 1;115(7):1531–43.
2. Troisi R, Stephansson O, Jacobsen J, Tretli S, Sørensen HT, Gissler M, et al. Perinatal characteristics and bone cancer risk in offspring - a Scandinavian population-based study. Acta Oncol Stockh Swed. 2013 Dec 9;
3. Jaffe N, Prudich J, Knapp J, Wang YM, Bowman R, Cangir A, et al. Treatment of primary osteosarcoma with intra-arterial and intravenous high-dose methotrexate. J Clin Oncol Off J Am Soc Clin Oncol. 1983 Jul;1(7):428–31.
4. Treon SP, Chabner BA. Concepts in use of high-dose methotrexate therapy. Clin Chem. 1996 Aug;42(8 Pt 2):1322–9.
5. Ellithy MMA, El Baghdady NSEDM, El Wakeel LM, Abdeltawab KA, Badary OA. An open-label phase I pilot study of continuous intrapleural infusion of escalated doses of methotrexate in malignant pleural mesothelioma. Am J Clin Oncol. 2012 Oct;36(5):514–8.
6. Chan H, Evans WE, Pratt CB. Recovery from toxicity associated with high-dose methotrexate: prognostic factors. Cancer Treat Rep. 1977 Aug;61(5):797–804.
7. Chu BC, Howell SB. Pharmacological and therapeutic properties of carrier bound methotrexate against tumor confined to a third space body compartment. J Pharmacol Exp Ther. 1981 Nov;219(2):389–93.
8. Chabner BA, Stoller RG, Hande K, Jacobs S, Young RC. Methotrexate disposition in humans: case studies in ovarian cancer and following high-dose infusion. Drug Metab Rev. 1978;8(1):107–17.
9. Chabner BA, Young RC. Threshold methotrexate concentration for in vivo inhibition of DNA synthesis in normal and tumorous target tissues. J Clin Invest. 1973 Aug;52(8):1804–11.
10. Pauley JL, Panetta JC, Schmidt J, Kornegay N, Relling MV, Pui C-H. Late-onset delayed excretion of methotrexate. Cancer Chemother Pharmacol. 2004 Aug;54(2):146–52.
11. Jones RB, Collins JM, Myers CE, Brooks AE, Hubbard SM, Balow JE, et al. High-volume intraperitoneal chemotherapy with methotrexate in patients with cancer. Cancer Res. 1981 Jan;41(1):55–9.

12. World Health Organization. WHO Classification of Bone Tumours [Internet]. http://w2.iarc.fr. [cited 2014 Jan 13]. Available from: http://www.iarc.fr/en/publications/pdfs-online/pat-gen/bb5/bb5-classifbone.pdf
13. Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, 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 Feb 1;20(3):776–90.
14. Hogendoorn PCW, Athanasou N, Bielack S, Alava ED, Tos APD, Ferrari S, et al. Bone sarcomas: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2010 May 1;21(suppl 5):v204–v213.
15. EURAMOS clinical trial protocol. A randomized trial of the European and American Osteosarcoma Study Group to optimize treatment strategies for resectable osteosarcoma based on histological response to pre-operative chemotherapy [Internet]. The European and American Osteosarcoma Study Group. 2011 [cited 2014 Nov 1]. Available from: http://www.ctu.mrc.ac.uk/euramos/
16. Leithner A, Windhager R. Diagnostik von Tumoren des Stütz- und Bewegungsapparates. Wien Med Wochenschr. 2007 Jan;157(1-2):21–6.
17. Glasser DB, Lane JM, Huvos AG, Marcove RC, Rosen G. Survival, prognosis, and therapeutic response in osteogenic sarcoma. The Memorial Hospital experience. Cancer. 1992 Feb 1;69(3):698–708.
18. Gatta G, Botta L, Rossi S, Aareleid T, Bielska-Lasota M, Clavel J, et al. Childhood cancer survival in Europe 1999-2007: results of EUROCARE-5-a population-based study. Lancet Oncol. 2014 Jan;15(1):35–47.
19. Leithner A, Pirker-Frühauf U, Wimber C. Genderperspektiven in der Therapie von Knochen- und Weichteiltumoren unter besonderer Berücksichtigung des Osteosarkoms. Gender-Unterschiede in der Orthopädie . bis an die Knochen: eine Einführung. Wien: Facultas; 2012. p. 228–33.
20. Whelan JS, Jinks RC, McTiernan A, Sydes MR, Hook JM, Trani L, et al. Survival from high-grade localised extremity osteosarcoma: combined results and prognostic factors from three European Osteosarcoma Intergroup randomised controlled trials. Ann Oncol. 2012 Jun 1;23(6):1607–16.
21. Friedman MA, Carter SK. The therapy of osteogenic sarcoma: current status and thoughts for the future. J Surg Oncol. 1972;4(5):482–510.
22. Daw NC, Neel MD, Rao BN, Billups CA, Wu J, Jenkins JJ, et al. Frontline

treatment of localized osteosarcoma without methotrexate: results of the St. Jude Children's Research Hospital OS99 trial. Cancer. 2011 Jun 15;117(12):2770–8.
23. Guillon MAM, Mary PMJ, Brugière L, Marec-Bérard P, Pacquement HD, Schmitt C, et al. Clinical characteristics and prognosis of osteosarcoma in young children: a retrospective series of 15 cases. BMC Cancer. 2011;11:407.
24. Van Dalen EC, van As JW, de Camargo B. Methotrexate for high-grade osteosarcoma in children and young adults. Cochrane Database Syst Rev. 2011;(5):CD006325.
25. Frei E 3rd, Jaffe N, Tattersall MH, Pitman S, Parker L. New approaches to cancer chemotherapy with methotrexate. N Engl J Med. 1975 Apr 17;292(16):846–51.
26. Calvert GT, Randall RL, Jones KB, Cannon-Albright L, Lessnick S, Schiffman JD. At-risk populations for osteosarcoma: the syndromes and beyond. Sarcoma. 2012;2012:152382.
27. Cates JMM, Friedman DB, Seeley EH, Dupont WD, Schwartz HS, Holt GE, et al. Proteomic analysis of osteogenic sarcoma: association of tumour necrosis factor with poor prognosis: Proteomic analysis of osteosarcoma. Int J Exp Pathol. 2010 Mar 26;91(4):335–49.
28. Lee JA, Jung JS, Kim DH, Lim JS, Kim MS, Kong C-B, et al. RANKL expression is related to treatment outcome of patients with localized, high-grade osteosarcoma. Pediatr Blood Cancer. 2011 May;56(5):738–43.
29. Zhu H, Tang J, Tang M, Cai H. Upregulation of SOX9 in osteosarcoma and its association with tumor progression and patients' prognosis. Diagn Pathol. 2013 Nov 4;8(1):183.
30. Mangham DC, Athanasou NA. Guidelines for histopathological specimen examination and diagnostic reporting of primary bone tumours. Clin Sarcoma Res. 2011 Jul 25;1:6.
31. Enneking WF. A system of staging musculoskeletal neoplasms. Clin Orthop. 1986 Mar;(204):9–24.
32. Leithner A, Maurer-Ertl W, Windhager R. Biopsy of Bone and Soft Tissue Tumours: Hints and Hazards. In: Tunn P-U, editor. Treatment of Bone and Soft Tissue Sarcomas [Internet]. Springer Berlin Heidelberg; 2009 [cited 2014 Feb 9]. p. 3–10. Available from: http://link.springer.com/chapter/10.1007/978-3-540-77960-5_1
33. Windhager R, Kastner N, Leithner A. Benigne Knochentumoren und tumorähnliche Läsionen. Monatsschr Kinderheilkd. 2006 Jan;154(1):20–31.

34. Leithner A, Windhager R. Leitlinien der Biopsie von Knochen- und Weichteiltumoren. Orthop. 2007 Feb;36(2):167–75.
35. Lodwick G, Wilson A, Farrell C, Virtama P, Dittrich F. Radiology: Determining growth rates of focal lesions of bone from radiographs. [Internet]. 1980 [cited 2014 Feb 9]. Available from: http://han.medunigraz.at/han/pubmed/pubs.rsna.org/doi/abs/10.1148/radiology.134.3.6928321?url_ver=Z39.88-2003&rfr_id=ori:rid:crossref.org&rfr_dat=cr_pub%3dpubmed
36. Bieling P, Rehan N, Winkler P, Helmke K, Maas R, Fuchs N, et al. Tumor size and prognosis in aggressively treated osteosarcoma. J Clin Oncol. 1996 Mar 1;14(3):848–58.
37. De Ioris MA, Prete A, Cozza R, Podda M, Manzitti C, Pession A, et al. Ewing Sarcoma of the Bone in Children under 6 Years of Age. Perez-Martinez A, editor. PLoS ONE. 2013 Jan 31;8(1):e53223.
38. Hauer K, Calzada-Wack J, Steiger K, Grunewald TGP, Baumhoer D, Plehm S, et al. DKK2 Mediates Osteolysis, Invasiveness, and Metastatic Spread in Ewing Sarcoma. Cancer Res. 2012 Nov 30;73(2):967–77.
39. Sari N, Togral G, Cetindag M, Güngör B, Ilhan I. Treatment results of the Ewing sarcoma of bone and prognostic factors. Pediatr Blood Cancer. 2010 Jan;54(1):19–24.
40. Elomaa I, Blomqvist CP, Saeter G, Åkerman M, Stenwig E, Wiebe T, et al. Five-year results in Ewing's sarcoma. The Scandinavian Sarcoma Group experience with the SSG IX protocol. Eur J Cancer. 2000 May;36(7):875–80.
41. Brotzmann M, Hefti F, Baumhoer D, Krieg AH. Do Malignant Bone Tumors of the Foot Have a Different Biological Behavior than Sarcomas at Other Skeletal Sites? Sarcoma. 2013;2013:1–8.
42. Rosenthal J, Pawlowska AB. High-dose chemotherapy and stem cell rescue for high-risk Ewing's family of tumors. Expert Rev Anticancer Ther. 2011 Feb;11(2):251–62.
43. Mavrogenis AF, Angelini A, Drago G, Merlino B, Ruggieri P. Survival analysis of patients with chondrosarcomas of the pelvis: Chondrosarcomas of the Pelvis. J Surg Oncol. 2013 Jul;108(1):19–27.
44. Pinedo HM, Chabner BA. Role of drug concentration, duration of exposure, and endogenous metabolites in determining methotrexate cytotoxicity. Cancer Treat Rep. 1977 Jul;61(4):709–15.
45. Ambinder EP, Perloff M, Ohnuma T, Biller HF, Holland JF. High dose methotrexate followed by citrovorum factor reversal in patients with advanced cancer. Cancer. 1979 Apr;43(4):1177–82.

46. Jolivet J, Cowan KH, Curt GA, Clendeninn NJ, Chabner BA. The pharmacology and clinical use of methotrexate. N Engl J Med. 1983 Nov 3;309(18):1094–104.
47. Howell SB, Krishan A, Frei E 3rd. Cytokinetic comparison of thymidine and leucovorin rescue of marrow in humans after exposure to high-dose methotrexate. Cancer Res. 1979 Apr;39(4):1315–20.
48. Howell SB, Chu BB, Wung WE, Metha BM, Mendelsohn J. Long-duration intracavitary infusion of methotrexate with systemic leucovorin protection in patients with malignant effusions. J Clin Invest. 1981 Apr;67(4):1161–70.
49. Holmboe L, Andersen AM, Mørkrid L, Slørdal L, Hall KS. High dose methotrexate chemotherapy: pharmacokinetics, folate and toxicity in osteosarcoma patients. Br J Clin Pharmacol. 2012 Jan;73(1):106–14.
50. Ferrari S, Orlandi M, Avella M, Caldora P, Ferraro A, Ravazzolo G, et al. [Effects of hydration on plasma concentrations of methotrexate in patients with osteosarcoma treated with high doses of methotrexate]. Minerva Med. 1992 May;83(5):289–93.
51. Al-Turkmani MR, Law T, Narla A, Kellogg MD. Difficulty Measuring Methotrexate in a Patient with High-Dose Methotrexate-Induced Nephrotoxicity. Clin Chem. 2010 Nov 30;56(12):1792–4.
52. Roberts D, Kidd W, Pratt CB, Peck C, Grinnel J. Serum levels of methotrexate by the ligand-binding assay after “high-dose” therapy for osteosarcoma. Cancer. 1979 Sep;44(3):881–90.
53. Li J, Gwilt P. The effect of malignant effusions on methotrexate disposition. Cancer Chemother Pharmacol. 2002 Nov;50(5):373–82.
54. Dickgreber NJ, Sorensen JB, Paz-Ares LG, Schytte TK, Latz JE, Schneck KB, et al. Pemetrexed safety and pharmacokinetics in patients with third-space fluid. Clin Cancer Res Off J Am Assoc Cancer Res. 2010 May 15;16(10):2872–80.
55. Jaffe N, Frei E 3rd, Traggis D, Cassady JR, Watts H, Filler RM. High-dose methotrexate with citrovorum factor in osteogenic sarcoma--progress report II. Cancer Treat Rep. 1977 Jul;61(4):675–9.
56. Evans WE, Pratt CB. Effect of pleural effusion on high-dose methotrexate kinetics. Clin Pharmacol Ther. 1978 Jan;23(1):68–72.
57. Bissett D, Kaye SB, Kerr DJ. Can primary osteosarcoma act as a third space after high-dose methotrexate? Eur J Cancer Oxf Engl 1990. 1991;27(8):1060.
58. Cortes EP, Holland JF, Wang JJ, Sinks LF, Blom J, Senn H, et al. THE CLASSIC: Amputation and Adriamycin in Primary Osteosarcoma: Clin Orthop. 2005

Sep;&NA;(438):5–8.
59. Wan SH, Huffman DH, Azarnoff DL, Stephens R, Hoogstraten B. Effect of route of administration and effusions on methotrexate pharmacokinetics. Cancer Res. 1974 Dec;34(12):3487–91.
60. Hall GH, Jones BJ, Head AC, Jones VE. Intra-articular methotrexate. Clinical and laboratory study in rheumatoid and psoriatic arthritis. Ann Rheum Dis. 1978 Aug;37(4):351–6.
61. Hall GH, Head AC. Letter: Intra-articular methotrexate. Lancet. 1975 Aug 30;2(7931):409.
62. Bird HA, Ring EF, Daniel R, Bacon PA. Comparison of intra-articular methotrexate with intra-articular triamcinolone hexacetonide by thermography. Curr Med Res Opin. 1977;5(2):141–6.

# 7 Appendix

Common Terminology Criteria for Adverse Events v3.0 (CTCAE)

| Toxicity | Grade 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| **Allergy/immunology** | | | | | |
| Allergic reaction/ hypersensitivity (including drug fever) | Transient flushing or rash; drug fever <38°C (100.4°F) | Rash; flushing; urticaria; dyspnea; drug fever ≥38°C (.100.4°F) | Symptomatic bronchospasm, with or without urticaria; parenteral medication(s) indicated; allergy-related edema/angioedema; hypotension | Anaphylaxis | Death |
| **Auditory/ear** | | | | | |
| Hearing: Patients with/without baseline audiogram and enrolled in a monitoring program | Threshold shift or loss of 15 - 25dB relative to baseline, averaged at 2 or more contiguous test frequencies in at least one ear; or subjective change in the absence of a Grade 1 threshold shift | Threshold shift or loss of >25 - 90 dB, averaged at 2 contiguous test frequencies in at least one ear | Adult only: Threshold shift or loss of >25 - 90 dB, averaged at 3 contiguous test frequencies in at least one ear<br><br>Pediatric: Hearing loss sufficient to indicate therapeutic intervention, including hearing aids (e.g., ≥20 dB bilateral HL in the speech frequencies; >30 dB unilateral HL; and requiring additional speech-language related services) | Adult only: Profound bilateral hearing loss (>90 dB)<br><br>Pediatric: Audiologic indication for cochlear implant and requiring additional speech-language related services | |
| Remark: Pediatric recommendations are identical to adults, unless specified. For children and adolescents (≤18 years of age) without a baseline test, pre-exposure/pre-treatment hearing should be considered to be <5 dB loss. | | | | | |
| **Blood/bone marrow** | | | | | |
| Hemoglobin (Hgb) | < LLN - 10.0 g/dl<br>< LLN - 6.2mmol/L<br>< LLN - 100 g/L | <10.0 - 8.0 g/dl<br><6.2 - 4.9 mmol/L<br><100 - 80 g/L | <8.0 - 6.5 g/dl<br><4.9 - 4.0mmol/L<br><80 - 65 g/L | <6.5 g/dl<br><4.0mmol/L<br><65 g/L | Death |
| Leukocytes (total WBC) | < LLN - 3000/$mm^3$<br>< LLN - 3.0 x $10^9$ /L | <3000 - 2000/$mm^3$<br><3.0 - 2.0 x $10^9$ /L | <2000 - 1000/$mm^3$<br><2.0 - 1.0 x $10^9$ /L | < 1000/$mm^3$<br>< 1.0 x $10^9$ /L | Death |

| Toxicity | Grade 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| **Blood/bone marrow** | | | | | |
| Neutrophils/ granulocytes (ANC/AGC) | < LLN - 1500/$mm^3$<br>< LLN - 1.5 x $10^9$ /L | <1500 - 1000/$mm^3$<br><1.5 - 1.0 x $10^9$ /L | <1000 - 500/$mm^3$<br><1.0 - 0.5 x $10^9$ /L | < 500/$mm^3$<br>< 0.5 x $10^9$ /L | Death |
| Platelets | < LLN - 75000/$mm^3$<br>< LLN - 75.0 x $10^9$ /L | <75000 - 50000/$mm^3$<br><75.0 - 50.0 x $10^9$ /L | <50000 - 25000/$mm^3$<br><50.0 - 25.0 x $10^9$ /L | < 25000/$mm^3$<br>< 25.0 x $10^9$ /L | Death |
| **Cardiac arrhythmia** | | | | | |
| Cardiac arrhythmia - Other (specify,-) | Mild | Moderate | Severe | Life-threatening; disabling | Death |
| **Cardiac general** | | | | | |
| Left ventricular systolic dysfunction | Asymptomatic, resting ejection fraction (EF) <60 - 50%; shortening fraction (SF) <30-24% | Asymptomatic, resting EF <50 - 40%<br>SF <24 - 155% | Symptomatic CHF responsive to intervention; EF <40 - 20%<br>SF <15% | Refractory CHF or poorly controlled; EF <20%; intervention such as ventricular assist device, ventricular reduction surgery, or heart transplant indicated | Death |
| **Constitutional symptoms** | | | | | |
| Fatigue (asthenia, lethargy, malaise) | Mild fatigue over baseline | Moderate or causing difficulty performing some ADL | Severe fatigue interfering with ADL | Disabling | _ |
| Fever (in the absence of neutropenia, where neutropenia is defined as ANC < 1.0 x $10^9$/L) | 38.0 - 39.0°C (100.4 - 102.2°F) | >39.0 - 40.0°C (102.3 - 104.0°F ) | > 40.0°C (>104.0°F ) for ≤24hrs | > 40.0°C (>104.0°F ) for >24hrs | Death |
| Rigors, chills | Mild | Moderate, narcotics indicated | Severe or prolonged, not responsive to narcotics | _ | _ |
| Weight loss | 5 - <10% from baseline; intervention not indicated | 10 - <20% from baseline; nutritional support indicated | ≥20% from baseline; tube feeding or TPN indicated | _ | _ |

| Toxicity | Grade 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| **Endocrine** | | | | | |
| Thryoid function, high (hyperthyroidism, thyrotoxicosis) | Asymptomatic, intervention not indicated | Symptomatic, not interfering with ADL; thyroid suppression therapy indicated | Symptoms interfering with ADL; hospitalization indicated | Life-threatening consequences (e.g., thyroid storm) | Death |
| Thyroid function, low (hypothyroidism) | Asymptomatic, intervention not indicated | Symptomatic, not interfering with ADL; thyroid replacement indicated | Symptoms interfering with ADL; hospitalization indicated | Life-threatening myxedema coma | Death |
| **Gastrointestinal** | | | | | |
| Diarrhea | Increase of < 4 stools per day over baseline; mild increase in ostomy output compared to baseline | Increase of 4-6 stools per day over baseline; IV fluids indicated<24 hrs; moderate increase in ostomy output compared to baseline; not interfering with ADL | Increase of ≥7 stools per day over baseline; incontinence; IV fluids ≥24 hrs; hospitalization; severe increase in ostomy output compared to baseline; interfering with ADL | Life-threatening consequences(e.g. hemodynamic collapse) | Death |
| Remark: Diarrhea includes diarrhea of small bowel or colonic origin, and/or ostomy diarrhea | | | | | |
| Mucositis/stomatitis (clinical exam)<br>-Select:<br>- Anus<br>- Esophagus<br>- Large Bowel<br>- Larynx<br>- Oral Cavity<br>- Pharynx<br>- Rectum<br>- Small Bowel<br>- Stomach<br>- Trachea | Erythema of the mucosa | Patchy ulcerations or pseudomembranes | Confluent ulcerations or pseudomembranes; bleeding with minor trauma | Tissue necrosis; significant spontaneous bleeding; life-threatening consequences | Death |

| Toxicity | Grade 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Mucositis/stomatitis (functional/ symptomatic)<br>-Select:<br>- Anus<br>- Esophagus<br>- Large Bowel<br>- Larynx<br>- Oral Cavity<br>- Pharynx<br>- Rectum<br>- Small Bowel<br>- Stomach<br>- Trachea | Upper aerodigestive tract sites: Minimal symptoms, normal diet; minimal respiratory symptoms but not interfering with function<br><br>Lower GI sites: Minimal discomfort, intervention not indicated | Upper aerodigestive tract sites: Symptomatic but can eat and swallow modified diet; respiratory symptoms interfering with function but not interfering with ADL<br><br>Lower GI sites: Symptomatic, medical intervention indicated but not interfering with ADL | Upper aerodigestive tract sites: Symptomatic and unable to adequately aliment or hydrate orally; respiratory symptoms interfering with ADL<br><br>Lower GI sites: Stool incontinence or other symptoms interfering with ADL | Symptoms associated with life-threatening consequences | Death |
| Typhlitis (cecal inflammation) | Asymptomatic, pathologic or radiographic findings only | Abdominal pain; mucus or blood in stool | Abdominal pain, fever, change in bowel habits with ileus; peritoneal signs | Life-threatening consequences (e.g. perforation, bleeding, ischemia, necrosis); operative intervention indicated | Death |
| Vomiting | 1 episode in 24 hrs | 2 - 5 episodes in 24 hours; IV fluids indicated < 24 hrs | ≥6 episodes in 24 hrs; IV fluids, or TPN indicated ≥24 hrs | Life threatening consequences | Death |
| **Hemorrhage/bleeding** | | | | | |
| Hemorrhage, GU - Bladder | Minimal or microscopic bleeding, intervention not indicated | Gross bleeding, medical intervention, or urinary tract irrigation indicated | Transfusion, interventional radiology, endoscopic, or operative intervention indicated; radiation therapy (i.e. hemostasis of bleeding site) | Life-threatening consequences; major urgent intervention indicated | Death |

| Toxicity | Grade 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| **Infection** | | | | | |
| Febrile neutropenia (fever of unknown origin without clinically or microbiologically documented infection) (ANC < 1.0 x $10^9$/L, fever ≥38.5 °C) | - | - | Present | Life-threatening consequences (e.g., septic shock, hypotension, acidosis, necrosis) | Death |
| Infection (documented clinically or microbiologically) with Grade 3 or 4 neutrophils (ANC < 1.0 x $10^9$/L) | _ | Localized, local intervention indicated | IV antibiotic, antifungal, or antiviral intervention indicated; interventional radiology or operative intervention indicated | Life-threatening consequences (e.g., septic shock, hypotension, acidosis, necrosis) | Death |
| Infection with normal ANC or Grade 1 or 2 neutrophils | _ | Localized, local intervention indicated | IV antibiotic, antifungal, or antiviral intervention indicated; interventional radiology or operative intervention indicated | Life-threatening consequences (e.g. septic shock, hypotension, acidosis, necrosis) | Death |
| **Metabolic/laboratory** | | | | | |
| AST, SGOT (Serum glutamic oxaloacetic transaminase) | > ULN - 2.5 x ULN | > 2.5 - 5.0 x ULN | > 5.0 - 20.0 x ULN | > 20.0 x ULN | _ |
| Bilirubin (hyperbilirubinemia) | > ULN - 1.5 x ULN | > 1.5 - 3.0 x ULN | > 3.0 - 10.0 x ULN | > 10.0 x ULN | _ |
| Creatinine | > ULN - 1.5 x ULN | > 1.5 - 3.0 x ULN | > 3.0 - 6.0 x ULN | > 6.0 x ULN | Death |
| Remark: Adjust to age-appropriate levels for pediatric patients | | | | | |
| Glomerular filtration rate (GFR) | <75 - 50% LLN | <50 - 25% LLN | <25% LLN, chronic dialysis not indicated | Chronic dialysis or renal transplant indicated | Death |
| Phosphate, serum-low (hypophosphatemia) | <LLN - 2.5 mg/dL<br><LLN - 0.8 mmol/L | <2.5 - 2.0 mg/dL<br><0.8 - 06 mmol/L | <2.0 - 1.0 mg/dL<br><0.6 - 0.3 mmol/L | <1.0 mg/dL<br><0.3 mmol/L | Death |

| Toxicity | Grade 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| **Neurology** | | | | | |
| Confusion | Transient confusion, disorientation or attention deficit | Confusion or disorientation, or attention deficit interfering with function, but not interfering with ADL | Confusion or delirium interfering with ADL | Harmful to others or self; hospitalization indicated | Death |
| Mood alteration -select<br>- agitation<br>- anxiety<br>- depression<br>- euphoria | Mild mood alteration not interfering with function | Moderate mood alteration interfering with function, but not interfering with ADL; medication indicated | Severe mood alteration interfering with ADL | Suicidal ideation; or danger to self or others | Death |
| Neuropathy- motor | Asymptomatic, weakness on exam/testing only | Symptomatic weakness interfering with function, but not interfering with ADL | Weakness interfering with ADL; bracing or assistance to walk (e.g. cane or walker) indicated | Life-threatening; disabling (e.g. paralysis) | Death |
| Neuropathy-sensory | Asymptomatic; loss of deep tendon reflexes or paresthesia (including tingling) but not interfering with function | Sensory alteration or paresthesia (including tingling), interfering with function, but not interfering with ADL | Sensory alteration or paresthesia interfering with ADL | Disabling | Death |
| Somnolence/ Depressed level of consciousness | _ | Somnolence or sedation interfering with function, but not interfering with ADL | Obtundation or stupor; difficult to arouse; interfering with ADL | Coma | Death |
| Seizure | _ | One brief generalized seizure; seizure(s) well controlled by anticonvulsants or infrequent focal motor seizures not interfering with ADL | Seizures in which consciousness is altered; poorly controlled seizure disorder, with breakthrough generalized seizures despite medical intervention | Seizures of any kind which are prolonged, repetitive, or difficult to control (e.g. status epilepticus, intractable epilepsy) | Death |

| Toxicity | Grade 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| **Renal/Genitourinary** | | | | | |
| Cystitis | Asymptomatic | Frequency with dysuria; macroscopic hematuria | Transfusion; IV pain medications; bladder irrigation indicated | Catastrophic bleeding; major non-elective intervention indicated | Death |
| Urinary electrolyte wasting (e.g., Fanconi's syndrome, renal tubular acidosis) | Asymptomatic, intervention not indicated | Mild, reversible and manageable with replacement | Irreversible, requiring continued replacement | _ | _ |
| Renal failure | - | - | Chronic dialysis not indicated | Chronic dialysis or renal transplant indicated | Death |
| **Secondary malignancy** | | | | | |
| Secondary malignancy - possibly related to cancer treatment (specify, -) | - | - | Non-life-threatening basal or squamous cell carcinoma of the skin | Solid tumor, leukemia or lymphoma | Death |
| **Syndromes** | | | | | |
| Flu-like syndrome | Symptoms present but not interfering with function | Moderate or causing difficulty performing some ADL | Severe symptoms interfering with ADL | Disabling | Death |