Diplomarbeit

OSTEOPOROSIS – A LATE EFFECT AFTER CHEMOTHERAPY FOR BONE SARCOMA

eingereicht von

Ulrike Michaela Pirker-Frühauf

Mat.Nr.: 0014245

zur Erlangung des akademischen Grades

Doktorin der gesamten Heilkunde

(Dr. med. univ.)

an der

Medizinischen Universität Graz

ausgeführt an der

Universitätsklinik für Orthopädie und Orthopädische Chirurgie

unter der Anleitung von

a.o. Prof. Dr. Andreas Leithner

Graz, am 11.01.2011

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Graz, am 11.01.2011

ACKNOWLEDGEMENTS

First of all I would like to thank my advisor and intermittent head of the Department of Orthopaedics and Orthopaedic surgery at the Medical University of Graz, a.o. Professor Andreas Leithner, who gave me the chance to join his tumour team during the period of writing my master thesis. His inspiring scientific approach and his trust in this project encouraged me to sight medicine from a complete new point of view and aroused my interest in further scientific work. My deep gratitude also belongs to a.o. Professor Barbara Obermayer-Pietsch, Division of Endocrinology and Metabolism (Department of Internal Medicine, Medical University of Graz), who generously supported our investigation and was an extraordinary helpful advisor concerning the complex evaluation of our laboratory findings. I also want to express my thanks to Dr. Franz Quehenberger, Institute of Medical Informatics, Statistics and Documentation (Medical University of Graz), for his statistical analysis of our compiled data and his patient explanations. Last but not least I want to give credit to Karin Novotny, Dr. Jörg Friesenbichler and Dr. Thomas Lovse for their kind assistance.

ZUSAMMENFASSUNG

Das chemotherapeutische Konzept von an Ewing Sarkom bzw. Osteosarkom erkrankten Patienten unterscheidet sich im Wesentlichen im Einsatz von hochdosiertem Methotrexat, welches für seine osteoreduktive Wirkung im Langzeitverlauf, vor allem bei Kindern und jungen Erwachsenen, bekannt ist. Neuesten Studien zufolge konnte jedoch auch bei Ewing Sarkompatienten, deren Behandlung nicht auf Methotrexat basiert, eine Verminderung der Knochendichte gemessen werden. Daher starteten wir eine kollektive Nachuntersuchung, zu der wir alle an unserer Klinik bezüglich oben genannter Tumore behandelten und seit 1998 datenbanktechnisch erfassten Patienten zur Teilnahme einluden, um etwaige Fälle von Osteopenie/Osteoporose sowie damit assoziierten Frakturen zu eruieren.

An unserer Messung nahmen 43 Patienten teil – 18 Ewing Sarkome, davon zehn Männer und acht Frauen, mit einem Durchschnittsalter von 26 Jahren ± 7.61 SD (12 bis 44), und 25 Osteosarkome, 16 männlich und neun weiblich, mit einem Durchschnittsalter von 27 Jahren ± 10.34 SD (7 bis 49). Das durchschnittliche Zeitintervall zwischen Tumordiagnose und Nachuntersuchung betrug bei Ewing Sarkompatienten 8 Jahre (± 4.17 SD) und 7 Jahre (± 4.73 SD) bei der Osteosarkomgruppe. Die Untersuchung der Knochendichte erfolgte mittels Densitometrie von Lendenwirbelsäule und Femur, welche durch eine gezielte Serumanalyse sowie einen Fragebogen hinsichtlich Lebensstil und Ernährungsgewohnheiten ergänzt wurde.

Die Auswertung der Densitometrie auf Basis des alters- und geschlechtsbezogenen Z Score Systems ergab eine pathologische Knochendichteminderung in 58% (37% Osteopenie, 21% Osteoporose) der Studienteilnehmer an zumindest einem der Messpunkte (LWS oder Femur). Zusätzlich frakturierten zwei Ewing Sarkom- (2/18) und fünf Osteosarkompatienten (5/25) atraumatisch im proximalen Femur 30 und 72 Monate nach Diagnosestellung, im distalen Femur nach 29 und 72 Monaten, in der proximalen

Tibia nach 29, 32 und 192 Monaten sowie digital nach 36 Monaten (ein Patient mit Ewing Sarkom erlitt zugleich eine Fraktur des distalen Femur sowie der proximalen Tibia). In der Laborauswertung zeigte sich neben Normabweichungen sämtlicher knochenstoffwechselbezogener Parameter ein Vitamin D-Mangel bei 88% der Probanden, kombiniert mit einer genetischen Prädisposition für die Entwicklung einer Lactoseintoleranz bei 37%. Die Ergebnisse von Densitometrie und Labor zeigten keinen signifikanten Unterschied zwischen der Ewing Sarkom- und Osteosarkomgruppe.

Unser Studienergebnis weist darauf hin, dass ein möglicher Knochensubstanzverlust infolge Chemotherapie nicht zwingend auf die Anwendung von Hochdosis-Methotrexat im Zuge der Osteosarkomtherapie beschränkt ist, sondern in einem ebenso großen Ausmaß auch Ewing Sarkompatienten betreffen kann. Neben dem Einfluss der Chemotherapie spielen auch viele andere Faktoren, wie z.B. therapieinduzierte Übelkeit und eine möglicherweise damit verbundene Mangelernährung sowie lange Phasen der Immobilisation, eine wesentliche Rolle. Kombiniert mit saisonalem/permanentem Vitamin D- und Calciummangel sowie einer genetischen Prädisposition für Lactoseintoleranz, können all diese Faktoren den knochenschädlichen Einfluss der Chemotherapie potenzieren. Eine suffiziente Versorgung dieser Patienten mit Vitamin D und Calcium könnte ein erster Schritt in Richtung nachhaltiger Osteoporoseprävention sein.

ABSTRACT

High-dose methotrexate, a standard agent in the therapy protocols for osteosarcoma, has long been suspected to have a negative long-term effect on bone metabolism and bone mineral density (BMD), especially in children and young adults. Recent literature questioned this association as also the BMD of Ewing's sarcoma patients treated without methotrexate is known to be decreased. We therefore wanted to evaluate the skeletal status of our patients concerning bone loss and laboratory alterations as well as skeletal related events like osteopenia-/ osteoporosis-associated fractures after chemotherapeutic treatment.

Our series of measurements included 43 patients – 18 Ewing´s sarcoma, ten male and eight female, with a mean age of 26 years ± 7.61 SD (12 to 44), and 25 osteosarcoma, 16 male and nine female, with a mean age of 27 years ± 10.34 SD (7 to 49). The mean time between diagnosis and investigation was 8 years (± 4.17 SD) in Ewing´s sarcoma and 7 years (± 4.73 SD) in osteosarcoma. We were using dual-energy x-ray absorptiometry (DEXA) for measuring bone mineral density in the femur and the lumbar spine in addition to laboratory examinations and a lifestyle questionnaire.

Referring to the age and gender matched Z-score we found a reduction in BMD in 58% (37% osteopenia, 21% osteoporosis) of our study participants in at least one of the measured localisations (lumbar spine or femur). Additionally in two Ewing´s sarcoma (2/18) and five osteosarcoma patients (5/25) non-trauma-associated fractures occurred after chemotherapeutic treatment localised in the proximal femur 30 and 72 months after tumour diagnosis, the distal femur after 29 and 72 months, the proximal tibia after 29, 32 and 192 months and digital after 36 months (one Ewing´s sarcoma patient suffered from fractures affecting both – the distal femur and the proximal tibia). Concerning laboratory our patients presented numerous dysbalances in bone metabolism combined with a vitamin D deficiency in 88% and a genetic predisposition for lactose intolerance in 37%. Densitometry results as well as laboratory findings did not significantly vary between Ewing´s

sarcoma and osteosarcoma patients although the Ewing´s sarcoma group tended to show lower bone mineral contents.

Our results indicate that a reduction in bone mineral density in young childhood cancer survivors presents a possible threat not only in osteosarcoma patients treated with high-dose methotrexate, but also in patients with Ewing´s sarcoma. Young patients undergoing cancer treatment experience long time of hospitalisation, possible lack in nourishment due to chemotherapeutic nausea and contrary to healthy people of the same age are partially forced to inactivity in a life period of high importance for gaining their genetically programmed peak bone mass. When all this is cumulating with seasonal or permanent vitamin D insufficiency/deficiency in combination with calcium malnutrition plus a possible genetic determination for lactose intolerance it might potentiate the direct and indirect bone harming effects of cancer therapy on the skeletal system. Therefore preventive after-care treatment of childhood cancer survivors should be part of a sensitive patients´ management. Densitometry combined with laboratory examinations can provide us with easily achieved and patient sensitive information which does not only detect deficiencies in bone mineral density, but also gives important clinical information about the possible causes. Supporting young childhood cancer survivors with sufficient vitamin D and calcium might be the first step to prevent later osteoporosis which is likely to occur earlier and more seriously than in the average population.

TABLE OF CONTENTS

ABBREVIATIONS

Alb	Albumin
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
bALP (=BAP)	Bone specific alkaline phosphatase
BMD	Bone mineral density
BMI	Body mass index
Bu-Mel	Chemotherapeutic protocol including busulfan and melphalan
BUN	Blood urea nitrogen
C	Cervical vertebra
CESS	Cooperative Ewing´s Sarcoma Studies
cm	Centimetre
COLIA	Collagen Type I a1 S/s-allele
COSS	Cooperative Osteosarcoma Study
CT	Computed tomography
CTX	Chemotherapy
DEXA (=DXA)	Dual-energy x-ray absorptiometry
e.g.	Exepmpli gratia (for example)
EFT	Ewing family of tumours
EICESS	European Intergroup Cooperative Ewing's Sarcoma Study
EURAMOS	European and American Osteosarcoma Study
EURO-B.O.S.S.	EUROpean Bone Over 40 Sarcoma Study
Euro-E.W.I.N.G.	European Ewing Tumour Working Initiative of National Groups
FSH	Follicle stimulating hormone
fT3	Trijodthyronin
fT4	Thyroxin
GE	Total globulin
GGT (=γ-GT)	Gamma glutamyl transferase
hd-MTX	High-dose methotrexate
HGH	Growth hormone
i.e.	Id est
i.v.	Intravenous
IGF-1	Insulin-like growth factor 1
L	Lumbar vertebra
LH	Luteotrope hormone
max.	Maximum
Mb.	Morbus
min.	Minimum
MRI	Magnetic resonance imaging
MTX	Methotrexat
NSE	Neuron specific enolase
OC	Osteocalcin
OPG	Osteoprotegerin

PAS	Periodic acid-Schiff reaction
PINP	N-terminal telopeptide procollagen
PNET	Primitive neuroectodermal tumour
PTH	Parathyroid hormone
py(s)	Pack year(s)
QCT	Quantitative computed tomography
QUS	Quantitative ultrasound
RANKL	NF α B-receptor ligand
SHBG	Sexual hormone binding globulin
SNP	Single nucleotide polymorphism
SREs	Skeletal related events
TRAP	Tartratresistant acidic phosphatase
TSH	Thyroid stimulating hormone
UKCCSG	United Kingdom Children´s Cancer Study Group
UVB	Ultraviolet B wave
VAC	Chemotherapeutic protocol including vincristine, actinomycin-d and cyclophosphamide
VACA	Chemotherapeutic protocol including vincristine, actinomycin-d, cyclophosphamide, adriamycin
VAI	Chemotherapeutic protocol including vincristine, actinomycin-d and ifosfamide
VAIA	Chemotherapeutic protocol including vincristine, actinomycin-d, ifosfamide, adriamycin
vBMD	Volumetric bone mineral density
VIDE	Chemotherapeutic protocol including vincristine, ifosfamide, doxorubicin (=adriamycin) and etopside
WHO	World Health Organisation
ys	Years
ZOC	Zoledronic acid

LEGEND OF FIGURES

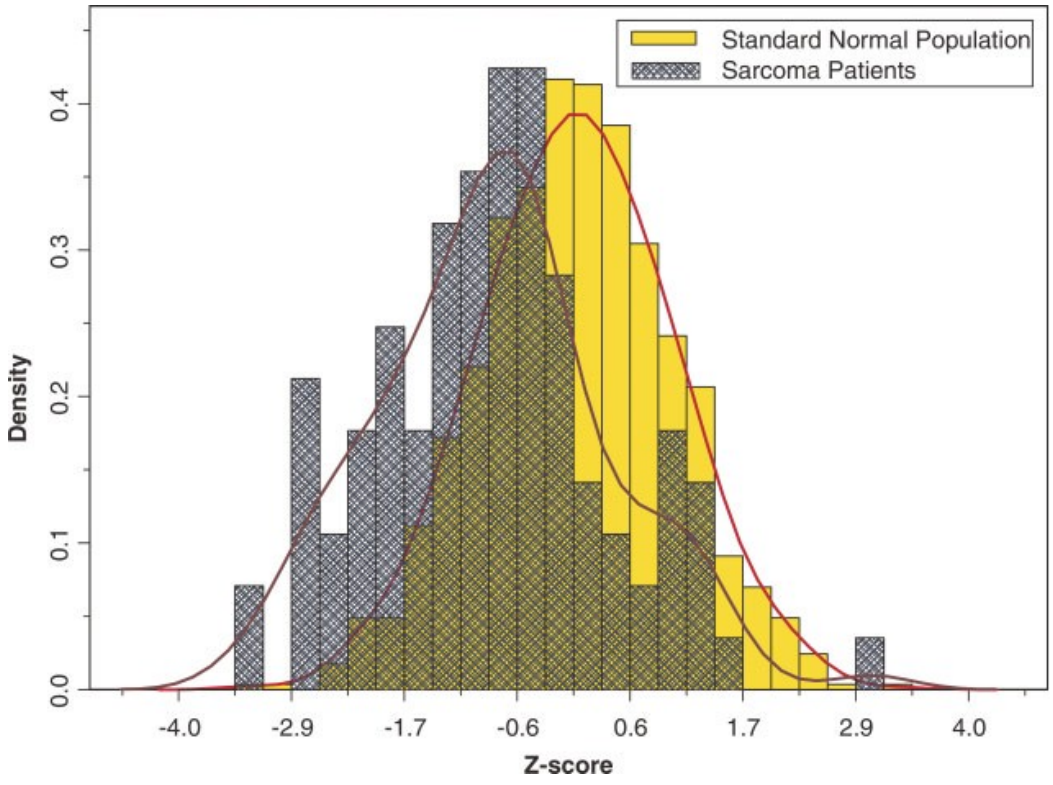
LEGEND OF TABLES

1 INTRODUCTION

1.1 Scientific background

The successful treatment of malignant bone tumours such as Ewing's sarcoma and osteosarcoma brought up a new generation of young childhood cancer survivors who are now facing long term effects of early cancer treatment. This cohort of patients is supposed to develop a lower peak bone mass with consecutive lower bone mineral density (BMD) in later life followed by premature osteopenia or osteoporosis and a higher risk of pathologic fractures (Figure 1). [(1), (2), (3), (4), (5)]

Figure 1. Histogram of QCT Z scores of 99 childhood sarcoma survivors (including 38% rhabdomyosarcoma, 25% osteosarcoma, 24% Ewing family tumours, 12% non-rhabdomyosarcoma soft-tissue sarcoma) compared to the normal population. (from Kaste et al.; Bone Mineral Density Deficits in Pediatric Patients Treated for Sarcoma; 2008)



In this context high-dose methothrexate (hd-MTX), a standard agent in the therapy protocols for osteosarcoma, has long been suspected to be one of the main triggers concerning the negative long-term effects on bone metabolism and bone mineral density. Cases of MTX-osteopathy and BMD-reduction have been reported in young patients treated for leukaemia or osteosarcoma in several studies declaring this agent next to glucocorticoids to one of the most suspicious chemotherapeutics causing premature osteoporosis. [6], [7], [8], [9], [10], [11] However, recent literature questioned this association as also the BMD of Ewing's sarcoma patients treated without methotrexate is known to be decreased. [12], [13]

Therefore we wanted to screen our patients who had been treated either for Ewing´s sarcoma or osteosarcoma at our institution for possible differences in bone loss. We invited all, since 1998 data base filed patients to a follow-up including densitometry and laboratory examination with the primary aim to quantify the effect of the various chemotherapeutic protocols on bone mineral density and bone metabolism. Bearing in mind that early intervention promises the best results in the therapy of osteoporosis we wanted to evaluate the need of an extended therapy including prophylactic or corrective medication to provide future patients at risk with a more efficient after care.

1.2 Bone sarcoma and treatment

1.2.1 PNET and Ewing´s sarcoma

Definition. PNET (=primitive neuroectodermal tumour) and Ewing´s sarcoma are both defined as round cell sarcomas, but whilst the first demonstrates neuroectodermal differentiation by light microscopy, immunohistochemistry and electron microscopy the latter does not. [14]

Epidemiology. PNET/Ewing´s sarcoma are the second most common sarcomatous tumours of bone and soft tissue in children with a peak age incidence in the second decade and a higher affection of males (♂ : ♀ = 1.4 : 1). The tumours are hardly ever found in patients aged above 30 years. [14]

Sites of involvement. Both, PNET and Ewing´s sarcoma, can affect the whole skeletal system, but the primary localisations are diaphysis and meta-diaphysis of the long bones (femur, tibia and humerus), ribs and pelvis (Figure 2). [14], [15], [16]

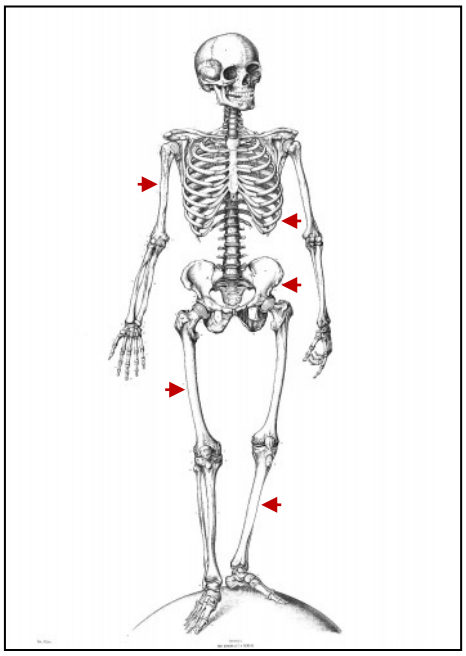


Figure 2. Most common primary localisation of PNET/Ewing´s sarcoma (arrows). (from www.schulbilder.org)

Macroscopy. The tumour mostly occurs tan-grey in bone and soft tissue with necrotic and haemorrhagic aspect and in some cases an association with large peripheral nerves in the soft tissue can be found (Figure 3). [14]

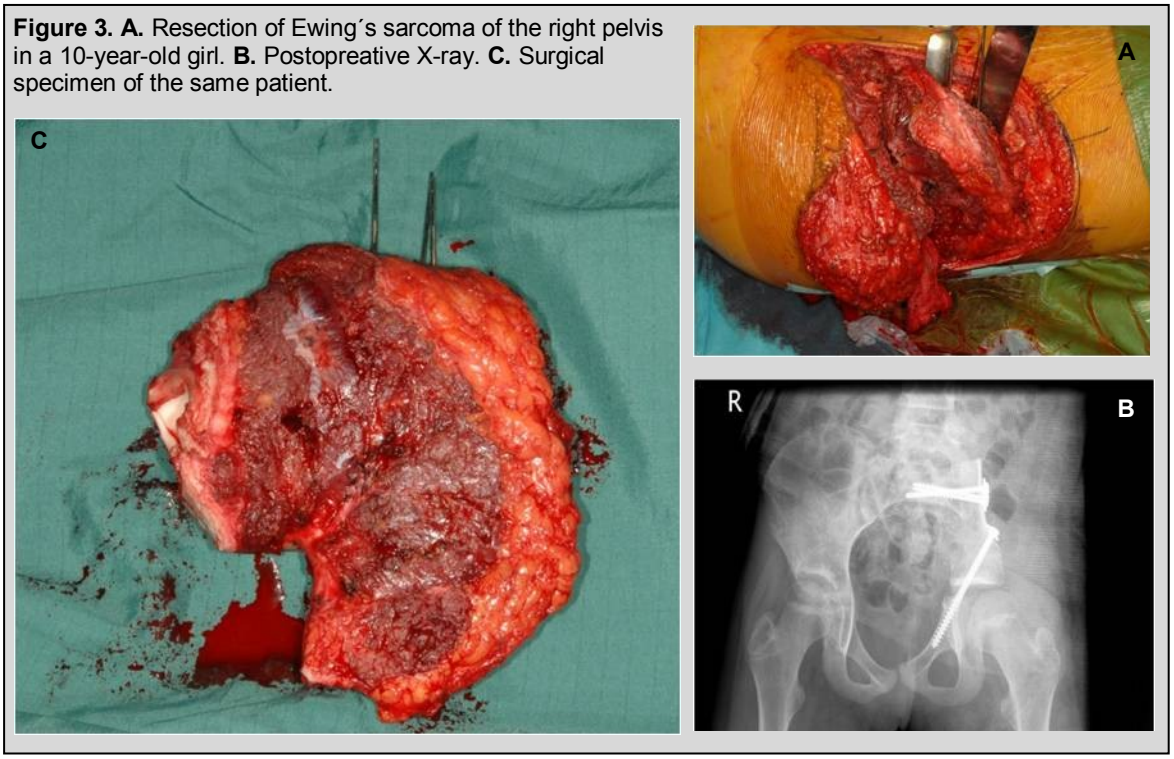


Figure 3. A. Resection of Ewing´s sarcoma of the right pelvis in a 10-year-old girl. **B.** Postopreative X-ray. **C.** Surgical specimen of the same patient.

Histopathology. Although the histological picture of the tumour shows high variability, frequent findings are multiple monomorphic cells with intracellular glycogen storing (PAS positive) and multifocal necrotic areas. [14], [15] Vital tumour zones around little vessels sometimes form a constellation known as Homer-Wright rosettes. [14], [15], [16]

Immunophenotype. PNET/Ewing´s sarcoma mostly express CD99 and frequently neural markers like neuron specific enolase (NSE). [14], [15]

Genetics. Characteristic for the whole Ewing family of tumours (EFT) is a recurrent t(11;22) (q24;q12) chromosomal translocation often accompanied by secondary chromosomal aberrations. [14], [15], [16]

Symptoms. The main symptoms are very similar to local inflammation like swelling, pain, localized warmth and limitation/loss of function. [16], [17] Additionally symptoms of displacement and signs of induration may be found.[16] Quite often therapy resistant pain occurs long before the first signs of local swelling.[15]

Diagnosis. In addition to an accurate clinical history and diagnostic imaging (e.g. x-ray with typical onionskin- and moth damage-like tumour growth) the biopsy is essential for diagnosis (Figure 4). [15],[16]

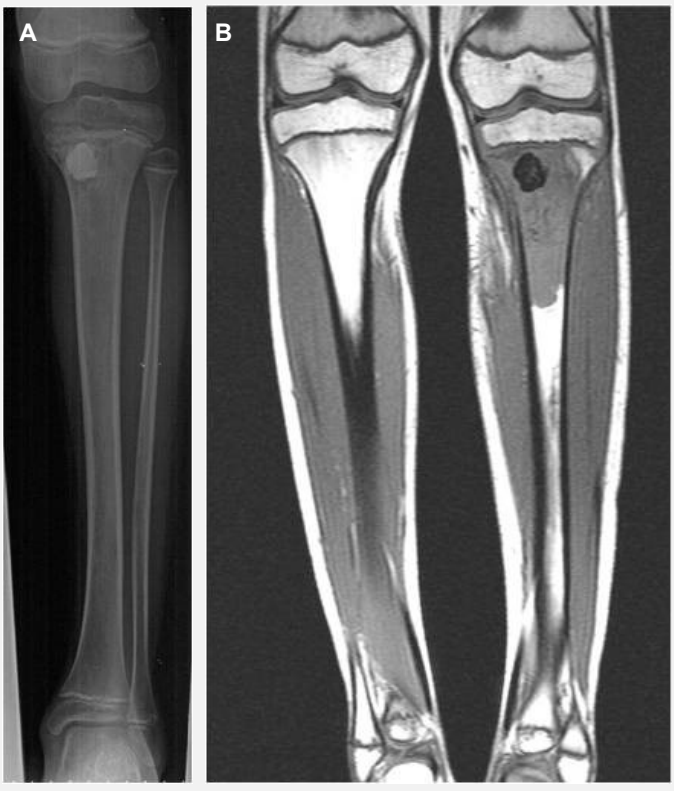


Figure 4. Diagnostic imaging: Ewing´s sarcoma located in the left proximal tibia in a 10-year-old boy. **A.** Anterior-posterior X-ray after biopsy. **B.** Frontal MRI. **C.** Axial MRI with contrast medium.

Differential diagnoses. Osteomyelitis, other types of bone sarcoma (e.g. osteosarcoma), neuroblastoma, leukaemia and skeletal metastases can present differential diagnoses of PNET and Ewing´s Sarcoma.[16], [17]

Metastases. At the time of first diagnosis 20-30% of cases show early haematogenous metastases in lungs, bone marrow and other locations of the skeletal system, but hardly ever lymph nodes are affected. [16], [17] Further 90% of patients with localised disease have already developed occult micro metastasis at the time of diagnosis, which requires a systemic treatment additionally to a local one. [16]

Therapy. The interdisciplinary treatment involves systemic chemotherapy (see 1.2.3), operation and/or radiotherapy depending on patient´s age, tumour size and localisation as well as the chemotherapeutic response/regression grade. [16], [15], [17]

Prognostic factors. Anatomic localisation, tumour stage and size present the major prognostic factors of survival. Patients with large tumours, pelvic affection and metastases tend to show a poorer outcome concerning the survival rate which in general improved during the last decades due to multimodal treatment (for further information see 1.2.3). [14], [15], [17]

1.2.2 Osteosarcoma

The term *osteosarcoma* is the hypernym for various subtypes of osteogenic tumours of which the conventional osteosarcoma is the most common one and will therefore be discussed in the following. Other subtypes are the teleangiectatic (<4%), small cell (~1.5%), low grade central (1-2%), parosteal (~4%), periosteal (<2%) and high grade surface osteosarcoma (<1%). Secondary osteosarcoma can develop on pre-existing abnormalities like alterations due to radiation (3.4-5.5%) or Mb. Paget, a bone disease leading to sarcomatous changes in estimated 0.7-0.95% of which osteosarcoma represent 50-60%. [14]

Definition and aetiology. Conventional osteosarcoma is defined as a primary intramedullary high grade tumour with still unclear precise aetiology, but associations between tumour development and trauma, Paget disease, various other bone tumours, non-neoplastic conditions like osteomyelitis or radiation

exposure are assumed. [14] Three groups of molecular alteration seem to play an important role in the pathogenesis of osteosarcoma. The inactivation of tumour suppressor genes like pRB110 (Rb) or p53 (both particularly associated with the hereditary osteosarcoma), the expression of the c-sis-proto-oncogene with a consecutive increase in the production of PDGF (=platelet-derived growth factor; mitogenious stimulation of mesenchymal cells) and the evidence of oncogenes (ras, raf, mos, myc, fos) or the oncornavirus. [15], [16]

Epidemiology. With an estimated incidence of 4-5 cases per million population osteosarcoma represents the most common, non-haemopoietic primary malignant bone tumour. [14], [15], [17] It develops most frequently in the second decade of life with approximately 60% of patients aged less than 25 years. It is diagnosed more often in male patients than in females (♂ : ♀ = 3 : 2). [14], [16]

Sites of involvement. In the majority of cases osteosarcoma affects the long bones (~ 91% metaphysis, <9% diaphysis) of the appendicular skeleton with preference of the distal femur and the proximal tibia (knee region~60%) as well as the proximal humerus. [14], [15], [16], [17] Epiphyseal involvement can be found, but occurs very seldom, as well as the primary affection of non-long bones like jaws, pelvis, spine and skull (Figure 5). [14], [16]

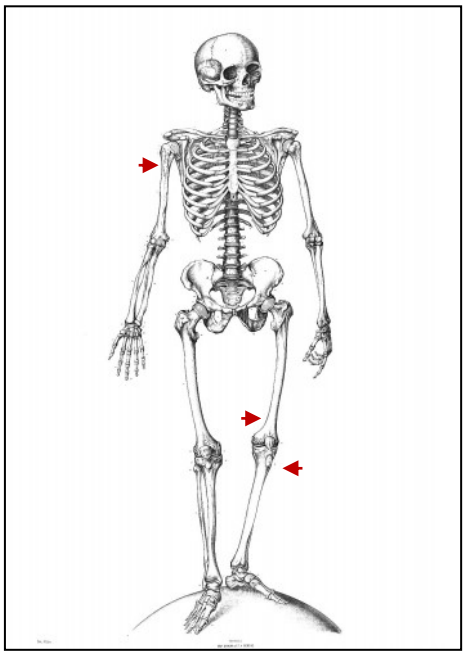


Figure 5. Most common primary localisation of osteosarcoma (arrows). (from www.schulbilder.org)

Macroscopy. Mostly conventional osteosarcoma is sized above 5 cm and develops metaphyseally centred with possible cartilaginary content, frequent transgression of the cortex and soft tissue association. [14] The macroscopic appearance reaches from grey-tan to yellow-white. [14], [15]

Histopathology. The highly anablastic and pleomorphic, osteoid producing tumour can be differentiated in dependence of the predominant matrix. Whilst in the past little prognostic significance has been given to this subtyping, recent data indicate a possible relation between subtype and survival rate. [14] The three major subtypes involve the osteoblastic osteosarcoma (50%) mainly consisting of bone and/or osteoid, the chondroblastic osteosarcoma (25%) with mostly hyaline cartilage mixed with non-chondroid elements, and the fibroblastic osteosarcoma (25%) with high histological similarity to fibrosarcoma or malignant fibrous histiocytoma (Figure 6). [14], [15]

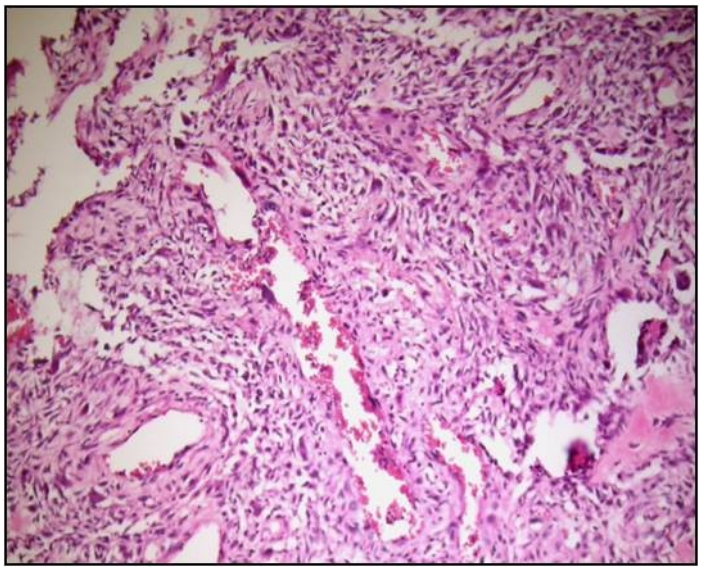


Figure 6. Conventional osteosarcoma consisting of mesenchymal cells which are producing osteoid and bone.

Immunophenotype. Adverse to the Ewing´s family of tumours the absence of reproducible specific findings makes immunohistochemistry or electron microscopy in osteosarcoma more important in order to exclude other tumours rather than to diagnose osteosarcoma. [14]

Genetics. Clonal chromosomal aberrations including numerical and structural alterations occur frequently in osteosarcoma, but similar to the immunophenotyping they are not very specific, although in the meantime certain frequently affected chromosomal regions have been detected. Furthermore patients suffering from hereditary retinoblastoma or Li-Fraumeni syndrome with a TP53 germline mutation are at higher risk to develop osteosarcoma. [14), 15)] (see also *definition and aetiology*)

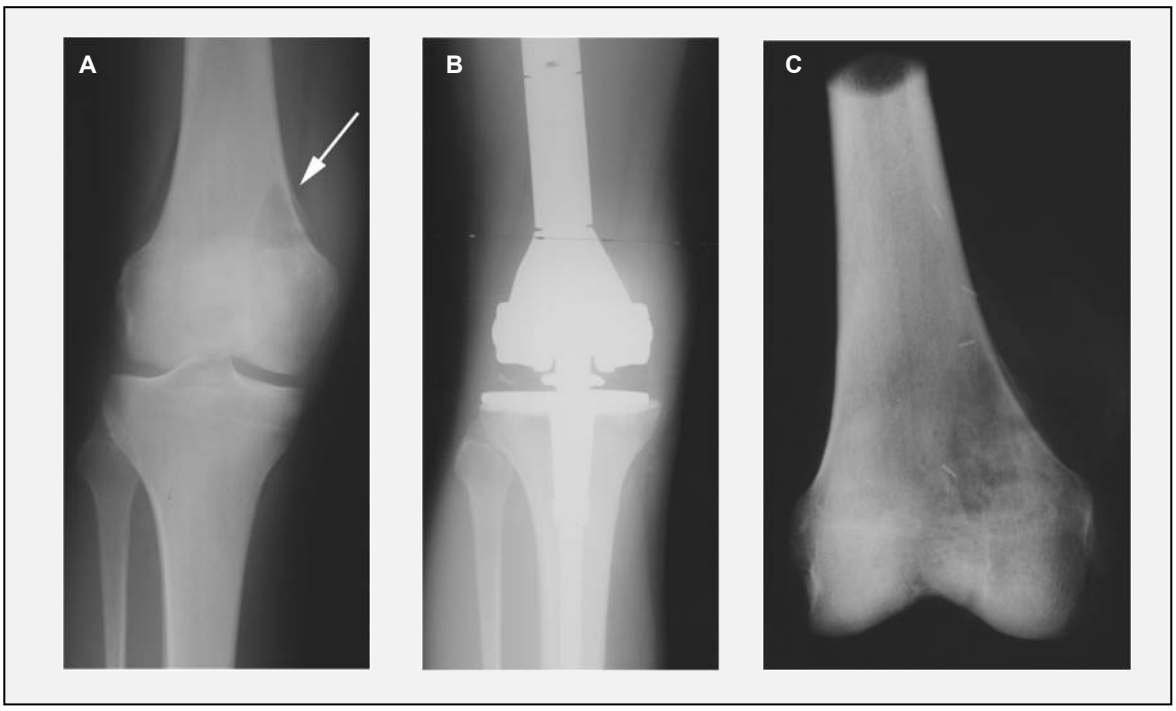
Symptoms. Patients with osteosarcoma tend to present as unspecific symptoms as those suffering from PNET/Ewing´s sarcoma (see 1.2.1). By physical examination a palpable, tender mass might be found which can be painless at the beginning. Deep, boring and severe pain which does not respond to symptomatical therapy mostly occurs if the tumour is growing rapidly with irritation/infiltration of the surrounding tissue. [14), 17)] In 10-15% of cases tumour diagnosis is due to a pathologic fracture. [17)]

Diagnosis. The clinical history should refer to the different risk factors and laboratory examinations may show an elevation in alkaline phosphatase and lactic acid dehydrogenase.[13), 14), 17)] The overall radiographic appearance of conventional osteosarcoma shows high variability (osteoblastic/osteolytic/mixed), but in most cases cortical destruction and soft tissue affection can be found. [14)] The process of periosteal elevation and reactive bone formation can lead to a configuration called Codman´s triangle in the X-ray (Figure 7). [14), 17)] Suspicion of osteosarcoma should always be clarified by magnetic resonance tomography (MRT) plus contrast medium and like in Ewing´s sarcoma biopsy is essential. [17)]

Differential diagnoses. Differential diagnoses of osteosarcoma are aneurysmatic bone cysts, osteoblastoma, reactive processes and myositis ossificans.[15)]

Metastases. At the time of first diagnosis 10-20% of cases show metastases basically in the lungs and the skeletal system, but like in Ewing´s sarcoma and PNET a high number of patients (80-90%) are supposed to have occult micro metastases. [16)]

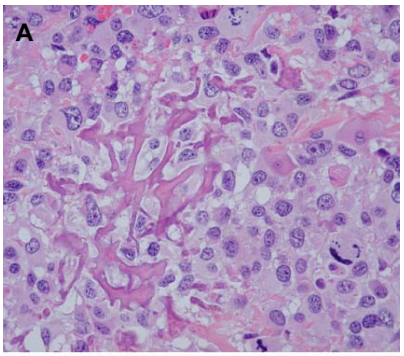
Figure 7. Osteosarcoma of the distal femur in a 21-year-old male patient. **A.** Typical Codman-triangle in the preoperative anterior-posterior X-ray (arrow). **B.** Postoperative X-ray with implanted tumour prostheses. **C.** Operation specimen.

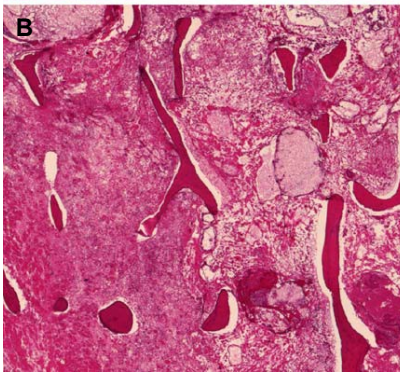


Therapy. The treatment of osteosarcoma is based on a combination of systemic chemotherapy (see 1.2.3) and operation, but different to Ewing´s sarcoma, osteosarcoma is usually resistant against radiotherapy which is therefore just used in exceptional cases. [16] Preoperative CT scan and MRI contribute to a more accurate delineation of the tumour extent and raise the chance for a limb-salvaging operative intervention.[14]

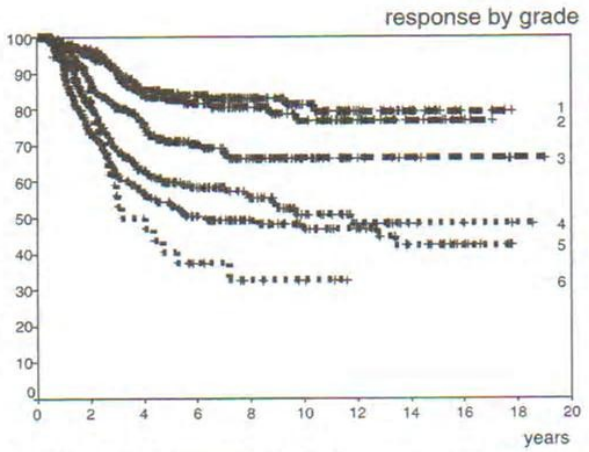
Prognostic factors. The most sensitive indicator of survival is the response to preoperative chemotherapy, sub-classified into six grades depending on tumour viability (Salzer-Kuntschik). [14], [18] Whilst the long-term surviving rate of *responders* (>90% tumour necrosis) according to the WHO ranges between 80-90%, *non-responders* (<90% tumour necrosis) show a survival of <15% if the postoperative therapeutic concept is not changed. [14] This association between remaining post-neoadjuvant amount of viable tumour cells and prognosis has also been documented by other investigators (Figure 8). [18]

Figure 8. A and B. Histology of osteosarcoma before (A) and after (B) chemotherapeutic treatment. **C.** Correlation of response to neoadjuvant chemotherapy (regression rate) and survival rate. **D.** Regression rate according to Salzer-Kuntschik. (A-C. from Carrle et al., Current strategies of chemotherapy in osteosarcoma, 2006; D. from Salzer-Kuntschik et al., 1983)





C



D

Grade I	no viable tumour cells
Grade II	scattered viable tumour cells
Grade III	viable tumour <10%
Grade IV	viable tumour 10-50%
Grade V	viable tumour >50%
Grade VI	no chemotherapeutic effect

1.2.3 Chemotherapeutic protocols

Because Ewing´s sarcoma/PNET and osteosarcoma are classified as high-malignant bone neoplasm, going along with occult metastases at the time of diagnosis in up to 90% of cases, their treatment follows a procedure of pre- and postoperative chemotherapy according to standardised study proved schemes in addition to a wide resection. The big benefit of this procedure is the significant increase in the patient´s survival. Whilst the 5-year survival rate of non-metastasised Ewing´s sarcoma patients lay below 10% in the past with resection only (± radiotherapy), it rose up to 50-75% with additional chemotherapeutic treatment. Similar results could be achieved in osteosarcoma patients where the 5-year survival increased from 15% up to 50-70%. Thus chemotherapy nowadays represents an essential and powerful part of successful sarcoma treatment. [16]

Ewing´s sarcoma (Figure 9). At the present Ewing´s sarcoma are treated according to the Euro-E.W.I.N.G 99 protocol (European Ewing Tumour Working Initiative of National Groups) which starts with six cycles of the neoadjuvant four-agent-combination VIDE including **v**incristine, **i**fosfamide, **d**oxorubicin (=adriamycin) and **e**topside. Depending on the chemotherapeutic response it is followed by at least one postoperative cycle VAI (**v**incristine, **a**ctinomycin-d and **i**fosfamide) which is further continued by either seven cycles VAC (**v**incristine, **a**ctinomycin-d and **c**yclophosphamide), VAI or one cycle Bu-Mel (**bu**sulfan and **mel**phalan). [16]

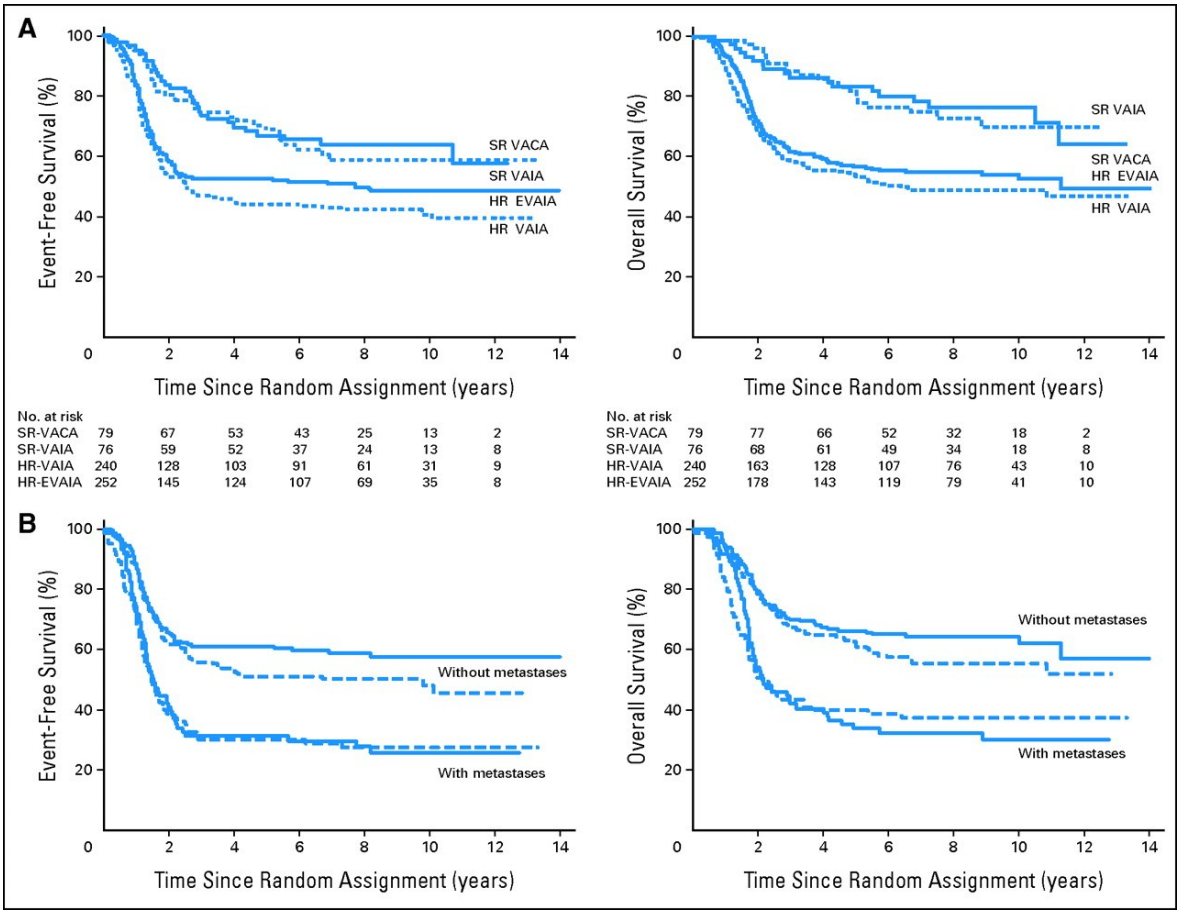
Figure 9. Chemotherapeutic treatment guidelines for Ewing´s sarcoma according to study proved protocols.

<table>
<tr><th colspan="5">Chemotherapeutic protocols - Ewing´s sarcoma</th></tr>
<tr><td rowspan="2">CESS (till 1992)</td><td colspan="2">VACA</td><td colspan="2">VAIA</td></tr>
<tr><td colspan="4">vincristine
actinomycin-d
cyclophosphamide | ifosfamide
adriamycin (=doxorubicin)</td></tr>
<tr><td>EICESS 92
(till 1999)</td><td colspan="4">CESS + etopside</td></tr>
<tr><td rowspan="2">Euro-Ewing 99</td><td>neoadjuvant</td><td colspan="3">adjuvant</td></tr>
<tr><td>VIDE
vincristine
ifosfamide
doxorubicin
etopside</td><td>VAI
vincristine
actinomycin-d
ifosfamide</td><td>VAC
cyclophosphamide</td><td>Bu-Mel
busulfan
melphalan</td></tr>
</table>

The average treatment duration is 10 to 12 months (around 3 weeks per chemotherapeutic cycle) in primary diagnosed Ewing´s sarcoma with localized disease. For metastatic or recurrent disease the treatment regimens are not as clearly defined and in general the outcome in both is much poorer. Whilst in patients with metastases intensified, time-compressed or high-dose chemotherapy has been assessed as beneficial, but with pending evidence from randomized trials, treatment possibilities of relapses are limited due to already achieved chemotherapeutic doses. In those patients the most reliable prognostic factor

seems to be the time to relapse which is related to a better outcome when it is longer than 2 years. [19]

Figure 10. Event-free and overall survival in EICESS-92. **A.** Outcome in standard risk (SR) and high risk (HR) patients related to the administered treatment scheme. **B.** Outcome of high risk patients with or without metastases treated according to VAIA (dashed line) or EVAIA (=VAIA + etopside; solid line). (from Paulussen et al., Results of the EICESS-92 Study, 2008)



Before the introduction of Euro-E.W.I.N.G. 99 Ewing´s sarcoma patients had been treated according to the CESS-protocols till 1992 using VACA (vincristine, actinomycin-d, cyclophosphamide, adriamycin) and VAIA (vincristine, actinomycin-d, ifosfamide, adriamycin) as the basic treatment regimes. [20] In 1992 the EICESS study, a collaborative approach of the German/Dutch/Austrian/Swiss Cooperative Ewing´s Sarcoma Studies (CESS) and the United Kingdom Children´s Cancer Study Group (UKCCSG), opened for recruitment by adding etopside for high risk patients (large localised tumours ≥100 mL or metastatic disease) and partially

replacing ifosfamide by the presumably less toxic cyclophosphamide in the standard-risk group (small localised tumours <100 mL). [21] Figure 10 presents the results of the EICESS-92 study concerning event-free and overall survival.

Osteosarcoma (Figure 11). Until in 2005 the European and American Osteosarcoma Study (EURAMOS 1) opened for recruitment osteosarcoma treatment in most European countries followed the guidelines of COSS (Cooperative Osteosarcoma Study) according to the protocol active at the time of enrolment. Basically the COSS protocol included a pre- and postoperative polychemotherapy (except the first COSS 77 trial with adjuvant chemotherapy only) consisting of **d**oxorubicin, **c**isplatin, high-dose **m**ethotrexate (with leukovorin rescue) and **i**fosfamide. All those agents still remain part of the EURAMOS 1 concept which was extended by additional interferon α maintenance therapy for good responders in a randomised trial and mainly targets young osteosarcoma patients less than 40 years of age. [16], [18], [22]

Figure 11. Chemotherapeutic treatment guidelines for Osteosarcoma according to study proved protocols.

<table>
<tr><th colspan="4">Chemotherapeutic protocols - osteosarcoma</th></tr>
<tr><td>COSS
(till 2005)</td><td colspan="3">doxorubicin
cisplatin
hd-MTX (+leukovorin rescue)
ifosfamide</td></tr>
<tr><td>EURAMOS 1
(below 40ys)</td><td colspan="3">COSS + interferon α</td></tr>
<tr><td rowspan="2">EURO-B.O.S.S.
(over 40ys)</td><td rowspan="2">neoadjuvant</td><td colspan="2">adjuvant</td></tr>
<tr><td>good / intermediate responders</td><td>bad responders</td></tr>
<tr><td>doxorubicin
cisplatin
ifosfamide</td><td>doxorubicin
cisplatin
ifosfamide</td><td>doxorubicin
cisplatin
ifosfamide
hd-MTX</td></tr>
</table>

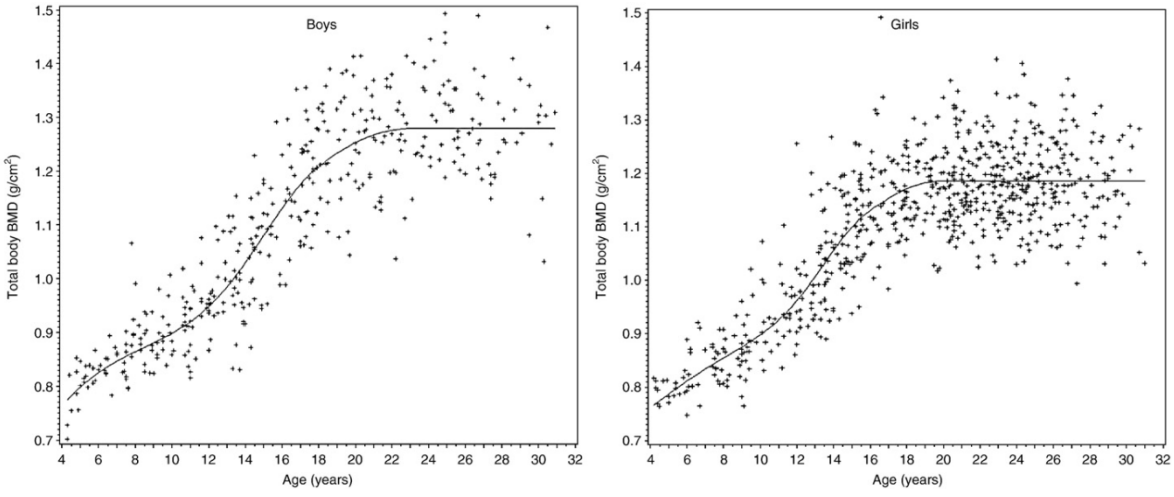
For patients of higher age the treatment scheme of EURO-B.O.S.S. (EUROpean Bone Over 40 Sarcoma Study) was introduced parallel to the EURAMOS 1 concept. EURO-B.O.S.S. differs between the option of solitary adjuvant chemotherapy including a combination of **d**oxorubicin, **c**isplatin and **i**fosfamide for primary operated patients on the one hand and a neoadjuvant-adjuvant concept with operation inbetween on the other hand. In the neoadjuvant line of the latter **d**oxorubicin, **c**isplatin and **i**fosfamide are used for all patients (same as in primary operated patients), whilst the adjuvant concept offers two possibilities depending on the chemotherapeutic response concerning the preoperative treatment. Good and intermediate responders (<50% viable tumour) continue postoperatively with the same chemotherapeutics as used for neoadjuvant treatment, whilst bad responders (≥50% viable tumour) get an extended treatment with additive hd-MTX. [16], [18]

The whole osteosarcoma treatment lasts for a period of 6-12 months and all patients are treated with a curative intent independently from metastatic status. Prognosis can be similar in metastatic and non-metastatic disease subject to the complete surgical removal of all known metastatic deposits, which highly influences the survival rate. Recurrences are primarily treated surgically as long as resection is possible and second-line chemotherapy frequently includes ifosfamide ± etopside ± carboplatin, but no generally accepted standard regime has been approved for those cases yet. Prognosis for patients with recurrent disease is very poor with a long-term post-relapse survival rate of <20%. [19]

1.3 Bone metabolism and chemotherapy

In general bone mineral density is influenced by genetic determinants as well as a number of endogenous and exogenous factors. [(1)] Thus, lower bone mineral density in childhood cancer survivors can be due to a multifactorial etiology, including the direct and indirect influence of the tumour itself through malignant infiltration as well as primary and secondary effects of sarcoma treatment like its influence on the bone metabolism and the interference with the endocrine system (e.g. hypopituitarism, hypogonadism). [(5)] Additionally, tumour and treatment can lead to suboptimal nutrition and decreased physical activity or immobilisation. All factors together may result in bone loss, diminished bone growth, reduced mineral accrual and the development of a suboptimal peak bone mass which is supposed to be reached in the early twenties in healthy young people (Figure 12). [(1), (4), (5), (23)]

Figure 12. Total body BMD [g/cm^2] of healthy young girls and boys plotted against age. (from Boot et al., Peak bone mineral density, lean body mass and fractures, 2009)



Although most of the sustaining chemotherapeutics used for bone sarcoma treatment showed an adverse influence on bone turnover in vitro the impact of each single agent is difficult to determine in vivo because of the multidrug regime (Figure 13). [(2), (12), (24), (25)] Therefore, the aim of our study was not the detection of the single mechanisms of chemotherapeutic agents. Much more we wanted to

reveal a weak spot in the follow-up of young cancer patients concerning the long-term effects of chemotherapy because at the moment strategies concerning early clinical diagnosis for patients at risk as well as therapeutic recommendations are sparse.

Figure 13. Influences of the most suspicious chemotherapeutic agents on bone metabolism (arranged in alphabetical order).

Euro-E.W.I.N.G	**COSS EURAMOS 1 EURO-B.O.S.S**	**Effect on bone metabolism**
cyclophosphamide		↓ number of osteoclasts + osteoblasts on the bone surface of the mandibular condyles, effects the gonads (in neonatal and juvenile rats) [(2)]
doxorubicin (=adriamycin)		↓ mouse osteoblast proliferation and parameters of cell differentiation (in vitro) [(2)]
ifosfamide		↓ renal threshold for phosphate reabsorption (prox. tubule), ↓ serum osteocalcin [at doses ≥50g/cm^2 or in combination with cisplatin] [(2)]
	MTX (high-dose)	↑ bone resorption, ↓ bone formation (uncoupling of bone turnover) [(2)], interferes with DNA formation [(24)], inhibits strongly the proliferation of bone-derived human osteoblasts, but not significantly the differentiation [(25)]
vincristine		↓ the PICP (procollagen type I C-terminal peptide) [(24)]

2 PATIENTS AND METHODS

2.1 Patients

We started our investigation with a query of our data base, founded in 1998, and found 121 patients below 50 years of age with either Ewing´s sarcoma/PNET or osteosarcoma who had been treated for their bone malignancies at the Department of Orthopaedics and Orthopaedic Surgery (Medical University of Graz). Of those, 37 patients had already died of disease, two exceptionally did not receive any chemotherapeutic treatment and five underwent current chemotherapy at the time of our study. The remaining 77 patients, 38 Ewing´s sarcoma and 39 osteosarcoma, were invited to take part in our investigation either by telephone and/or letter, resulting in 49 patients who had bone densitometry performed (six in an external hospital and 43 at the Medical University of Graz). Fifteen patients were unfortunately lost to follow-up (five from other countries than Austria) and 13 decided not to take part.

Those 43 patients with densitometry at our institution, all of Caucasian origin, represent the final collective of our study including 18 Ewing´s sarcoma patients, ten male and eight female with a mean age of 26 years (± 8 SD), and 25 osteosarcoma patients, 16 male and nine female with a mean age of 27 years (± 10 SD). In all cases, with exception of one Ewing´s sarcoma with multiple lesions, the tumour affected a solitary localisation main-sited in the lower limbs in 34 patients, in the upper limbs in four and in other sites than the extremities in five cases. Metastases in the lungs had been found in three Ewing´s sarcoma and three osteosarcoma patients at the time of diagnosis. Further details about our patients are given in Table 1.

All 43 study participants received chemotherapy according to the treatment protocols active at the time of diagnosis in addition to a wide resection except one Ewing´s sarcoma and one osteosarcoma patient whose tumours had been determined as inoperable. Eight patients - six of them Ewing´s sarcoma and two osteosarcoma, additionally underwent local radiotherapy.

The reason for excluding all patients over 50 years of age from the very beginning was to minimize the possible influences of a menopausal or senile bone mineral density reduction which is assumed to arise earliest around the age of 50. [26]

Table 1. Patients´ details.

	Ewing´s sarcoma (n=18)	**Osteosarcoma (n=25)**	**Total (n=43)**
M:F	10:8	16:9	26:17
Mean [± SD] age at diagnosis (range) [yy]	18 ± 9.1 (3 to 38)	20 ± 11.3 (6 to 45)	19 ± 10.5 (3 to 45)
Mean [± SD] age at follow-up (range) [yy]	26 ± 7.6 (12 to 44)	27 ± 10.3 (7 to 49)	27 ± 9.3 (7 to 49)
Mean [± SD] time between diagnoses and follow-up [yy]	8 ± 4.2	7 ± 4.7	7 ± 4.6
Mean [± SD] heigth (range) [cm]	169 ± 12.3 (130 to 191)	172 ± 14.2 (123 to 195)	171 ± 13.5 (123 to 195)
Mean [± SD] weight (range) [kg]	61 ± 15.6 (25 to 95)	67 ± 19.1 (26 to 131)	64 ± 17.9 (25 to 131)
Mean [± SD] body mass index (range) [kg/m²]	20.9 ± 3.6 (14.8 to 29.4)	22.4 ± 5.5 (15.8 to 45.3)	21.8 ± 4.9 (14.8 to 45.3)

The study protocol was approved by the local Ethics committee and all patients gave their written informed consent.

2.2 Methods

2.2.1 Questionnaire

To gain information about our patient´s lifestyle, calcium intake, family history, other bone affecting diseases and the administration of osteoreductive medication we interviewed them according to a specially adapted questionnaire originally developed by the Division of Endocrinology and Metabolism (Department of Internal Medicine, Medical University of Graz).

In family history eight cases of osteoporosis and two cases of arthrosis were reported by Ewing´s sarcoma patients whilst in osteosarcoma the relation was 5:7; none of the relatives showed an increased incidence of fractures.

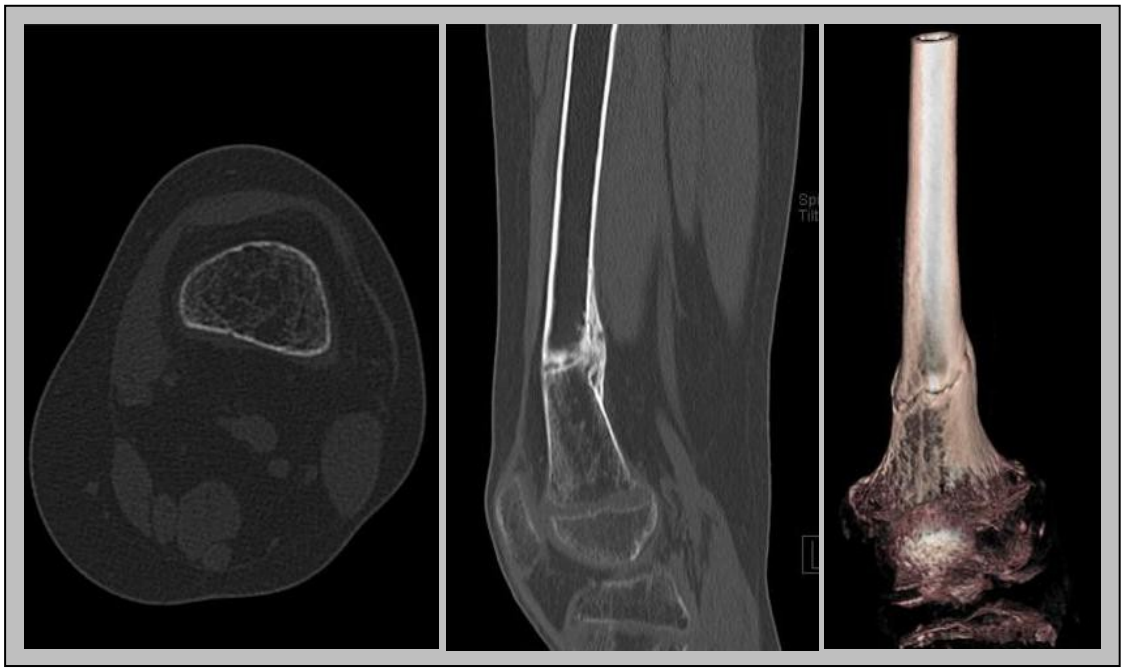
We also screened our patient´s files for fractures and in accordance to their own reports we found 17 non-vertebral fractures, 14 non-tumour-associated and three tumour-associated, in fifteen patients before chemotherapy (one patient of each tumour fractured twice at different times and localisations). Those fourteen non-tumour-associated fractures occurred after traumatic events affecting four times the radius in osteosarcoma patients and ten times other non-osteoporosis-associated skeletal parts. The three tumour-associated fractures all occurred in osteosarcoma patients.

After chemotherapeutic treatment two Ewing´s sarcoma (2/18) and five osteosarcoma patients (5/25) suffered from non-trauma and not directly tumour-associated fractures (Figure 14) localised in the proximal femur 30 and 72 months after tumour diagnosis, the distal femur after 29 and 72 months, the proximal tibia after 29, 32 and 192 months and digital after 36 months (one Ewing´s sarcoma patient suffered from fractures affecting both – the distal femur and the proximal tibia).

The majority of patients (ten Ewing´s sarcoma, 21 osteosarcoma) reported to be in a sedentary occupation and described their daily physical demand in job/school as

little or not physically stressfull. Nine patients admitted not to participate in any physical workout, whilst the remaining 34 patients performed recreational sport in different intensities (14 sparse, 18 regularly, two more than one hour a day).

Figure 14. Atraumatic fracture of the distal femur in an 11-year-old girl 27 months after chemotherapy (belonging to those six patients who did not participate in our study, but had densitometry performed in an external hospital).



Three patients were active smokers with a daily consumption between 2 and 20 cigarettes (0.5 – 7.5 pys) and one patient stopped smoking one year before our investigation (after around 1 py).

Fourteen patients commented not to drink any alcohol, 27 reported an occasional and two a regularly consummation of alcoholic drinks.

Prior to inclusion lactose intolerance was verified in one Ewing´s sarcoma patient by the H_2-respiratory test, but also four other patients reported that they did not tolerate milk well. Of all 43 probands 12 did not drink any milk, 23 consumed less and eight more than 0.25 litres per day. All patients ate cheese and/or other dairy products, but in different quantities - 17 sometimes and 26 at least once a day.

Sighting the sun exposure 28 patients exposed themselves rarely, 14 regularly and one even more often to the natural sun, but none of our patients used a solarium.

The question for other diseases with an indirect osteoreductive influence was negative except in one Ewing´s sarcoma patient with a toxic tubulopathy under current Renitec® and Reductio spezial® treatment and one osteosarcoma patient suffered from epilepsy medicated with Trileptal® and Seroquel® at the time of investigation. One Ewing´s sarcoma patient received Calcium and Vitamin D for the period of 3 years and in addition two times bisphosphonates i.v., and one patient with osteosarcoma had corticosteroids administered in course of his chemotherapeutic treatment. The intake of other possibly bone harming medication like anticoagulants or fluorine was negated by all patients.

The mean age at menarche of our 17 female study participants was 12.1 years (10 to 14) in Ewing´s sarcoma and 14.0 years (12 to 16) in osteosarcoma. One Ewing´s sarcoma patient aged 22 never had a menstruation and one girl with osteosarcoma aged 13 did not have it yet. Of the remaining 15 females nearly half (four Ewing´s sarcoma and three osteosarcoma patients) suffered from amenorrhoea during chemotherapy. At the time of investigation seven patients had a regular and three an irregular cycle, another three still had amenorrhoea and two ended up in an early menopause induced by the chemotherapeutic treatment. Four females received oestrogen-containing oral contraceptives, partly as hormonal replacement therapy, and one used a progesterone-based coil. Five women gave birth to eleven children in total (one per caesarean) with a mean breast feeding time of 4.4 months in each child. No incidence of miscarriage was reported. Two Ewing´s sarcoma patients had gynaecological operations - one ovariectomy and one uterus cystectomy.

2.2.2 Denstitometry

To quantify bone mineral density in clinical practice the dual-energy x-ray absorptiometry (DEXA) currently is the most commonly used technique and was therefore our method of choice. Other methods to measure body composition are the quantitative computed tomography (QCT-peripheral and central), quantitative ultrasound (QUS) and MRI. But neither of those is accomplishable with DEXA concerning costs, availability and radiation exposure. [5], [27]

In general the BMD can be quantified either as T score according to the definition of the World Health Organisation (WHO) or as Z score. [3]

- T score ($\rightarrow BMD_{max}$)
  - Osteopenia (-1.0 SD) – (-2.5 SD)
  - Osteoporosis < -2.5 SD

- Z score ($\rightarrow BMD_{age\ and\ gender\ related}$)
  - Osteopenia (-1.0 SD) – (-2.0 SD)
  - Osteoporosis < -2.0 SD

The difference between those two scores is the control group to which the DEXA result is related to. The T score compares the BMD of the patient with the maximum physiological bone content of a young healthy adult, which is normally reached in the twenties, whilst the Z score relates to age- and usually gender-matched controls.

In our study we ascertained the T-Score for patients over 20 years of age mainly to gain international comparable data according to the WHO and the Z-Score for all patients independently from the age for statistical evaluation. This decision was due to several recommendations which are suggesting the Z score as the more suitable method to judge the BMD of children, adolescents and young adults, because it connects bone mineral density and chronologic age. [2], [3], [5]

Furthermore, premature bone loss in those young patients would be better described as *low bone density for chronologic age* than with the terms *osteopenia* and *osteoporosis.* Nevertheless, for reasons of simplicity and comparability, we will still stick to these assignations. [28]

2.2.3 Laboratory

We took blood samples of approximately 25 ml in the morning hours with the Vacutainer system Vacuette® (Greiner bio-one GmbH) to gain the fasting values of the following parameters:

- BONE METABOLISM (serum/*EDTA)
  - Osteoporosis profile:
    - PTH (parathyroid hormone)*
    - 25(OH) vitamin D_3
    - Beta-crosslaps*
    - OC (osteocalcin)*
    - TSH (thyroid stimulating hormone)
    - fT3 (trijodthyronin)
    - fT4 (thyroxin)
  - PINP (N-terminal telopeptide procollagen)
  - bALP (bone specific alkaline phosphatase)
  - TRAP (tartrate resistant acidic phosphatase)
  - OPG (osteoprotegerin)
  - RANKL (NF α B-receptor ligand)

- HYPOPHYSE AND GONADS (Serum)
  - HGH (growth hormone)
  - IGF-1 (insulin-like growth factor 1)
  - SHBG (sexual hormone binding globulin)
  - Testosterone total (♀ and ♂)
  - Testosterone free (♀ and ♂)

- LH (luteotrope hormone)
- FSH (follicle stimulating hormone)

- OSTEOPOROSIS - GENETIC ANALYSIS for later analysis (EDTA)
  - SNPs (single nucleotide polymorphism) in candidate genes for osteoporosis (COLIA - Collagen Type I, LRP5)

- ADDITIONAL SERUM- PROFILE
  - Creatinine
  - BUN (blood urea nitrogen)
  - AST (aspartate aminotransferase)
  - ALT (alanine aminotransferase)
  - GGT (gamma glutamyl transferase)
  - Phosphate
  - Total calcium
  - GE (total protein)
  - Alb (albumin)

- ADDITIONAL SERUM SAMPLES for later analysis
  - 2 ml serum
  - 2 ml EDTA plasma

The lab analyses were performed in three laboratories at the Medical University of Graz according to their specialization (general laboratory, endocrinological laboratory, gynaecological laboratory).

2.3 Evaluation and statistical analysis

For calculations R 2.12.0 (www.r-project.org) was used and p-values below 0.05 were considered to be statistically significant, Bonferroni-corrected when necessary because of multiple testing. Correlations between measurements were tested with Spearman's rank correlation coefficient. The exact Wilcoxon rank sum test was used for comparisons of two groups, and in order achieve more power compared to Bonferroni corrections a global test according to Goeman et al. (2004), which had been developed for comparison of two groups with respect to many measurements, was applied. In diagrams group´s measurements were displayed by box-and-whiskers plots, in which the box marked the first and the third quartile and the median was indicated by an asterisk inside the box. The whiskers were drawn from the box to the minimum and maximum of those measurements that were within 1.5 times the interquartile range from the end of the box. Measurements outside this range were outlined by circles.

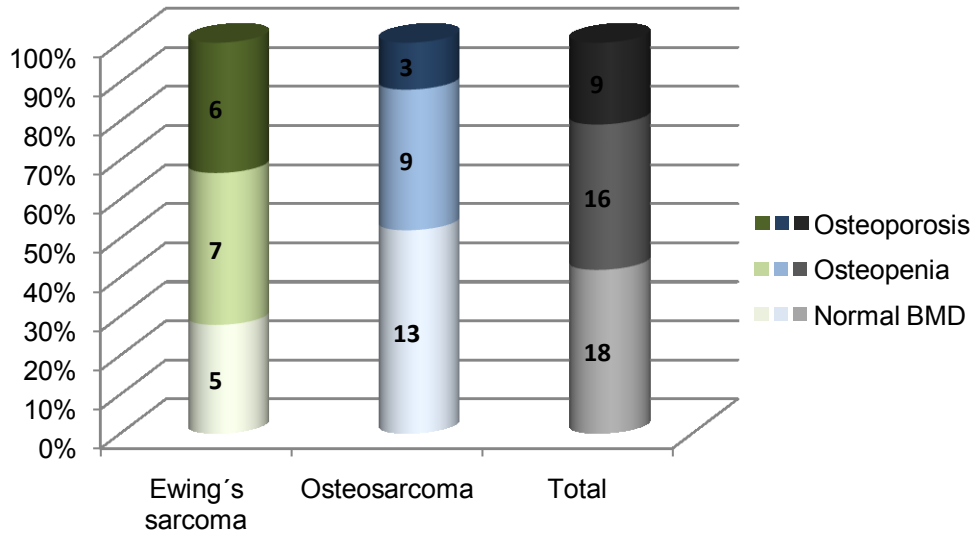
3 RESULTS

3.1 Densitometry

3.1.1 General results

The following densitometry results are all referring to the age and gender matched Z score and display the BMD of the lumbar spine (L1 to L4), the femoral neck and the total femur, both of the tumour unaffected side. The results of the lumbar spine include the primary collective of 43 patients, but concerning the femoral evaluation we had to exclude six patients in which densitometry of the tumour unaffected side could not be performed for different reasons.

Figure 15. Distribution of osteopenia ((-1 SD) – (-2 SD)) and osteoporosis (< -2 SD) according to the Z score in 43 patients (grey) including 18 Ewing´s sarcoma (green) and 25 osteosarcoma (blue).



Taking the worst measured BMD independently from the localisation we found beyond our Ewing´s sarcoma patients six cases (33%) of osteoporosis which correlates with Z scores below -2 SD, seven cases (39%) of osteopenia (Z score between -1 and -2 SD) and just five patients (28%) reached a bone mineral density regarded as normal. Beyond our osteosarcoma patients bone assessment displayed three cases (12%) of osteoporosis, nine (36%) of osteopenia and 13 patients (52%) with a normal BMD. Ewing´s sarcoma and osteosarcoma together presented nine times (21%) osteoporosis, 16 times (37%) osteopenia and in less than half of patients (18/43; 42%) we found a normal BMD (Figure 15).

3.1.2 BMD and measured localisation

Our next step was the separate evaluation of the measured localisations (lumbar spine/femur) due to the reason that the BMD levels of our individual patients differed depending on the measured spots. This implies that for example a decreased BMD in the lumbar spine did not stringently correlate with the same BMD reduction in the femur (Figure 16).

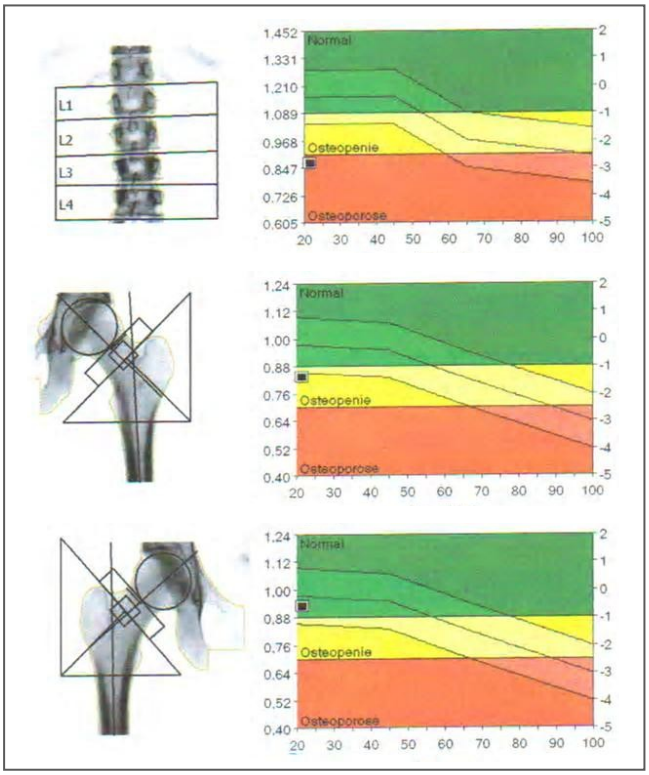


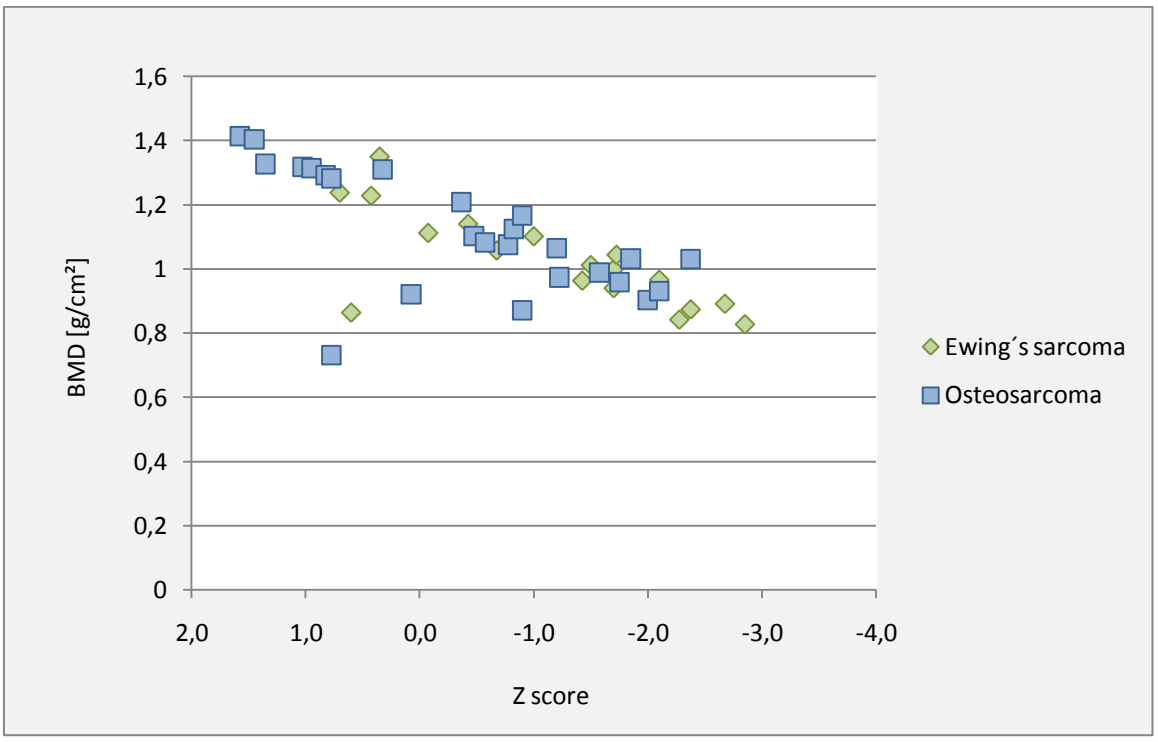
Figure 16. Densitometry in a 22-year old female 185 months after diagnosis of Ewing´s sarcoma located in the left pelvis. The patient is presenting all three categories of bone assessment (top down): osteoporosis (lumbar spine), osteopenia (left femur), normal BMD (right femur)

Lumbar spine. Our total study group of 18 Ewing´s sarcoma and 25 osteosarcoma patients presented a mean bone mineral density of 1.076 g/cm² ± 0.172 SD in the lumbar spine and a mean Z score of -0.702 ± 1.230 SD. Better mean BMD values and Z scores were found in the osteosarcoma group.
For more details see Table 2 and Figure 17.

Table 2. Mean BMD [g/cm²] und Z scores evaluated in the lumbar spine (L1-L4) in 18 Ewing´s sarcoma and 25 osteosarcoma patients.

	Lumbar spine L1-L4	
	BMD (mean ± SD)	Z score (mean ± SD)
Ewing´s Sarcoma (n=18)	1.025 ± 0.144	-1.135 ± 1.134
Osteosarcoma (n=25)	1.113 ± 0.180	-0.391 ± 1.201
Total (n=43)	1.076 ± 0.172	-0.702 ± 1.230

Figure 17. General distribution of bone mineral densities [g/cm²] and Z scores evaluated in the lumbar spine (L1-L4) in 18 Ewing´s sarcoma (green) and 25 osteosarcoma (blue) patients.



Searching for cases of reduced bone mineral density we found seven (7/43; 16%) patients with osteoporotic and eleven (11/43; 26%) with osteopenic BMD-levels, which results in 18 patients (18/43; 42%) who presented lower bone mineral

densities than regarded as healthy. In the remaining 25 patients (25/43; 58%) normal BMD-levels could be measured. Details concerning mean bone mineral densities and Z scores are listed in Table 3 and the accurate distribution of osteopenia and osteoporosis between Ewing´s sarcoma and osteosarcoma group is illustrated in Figure 18.

Figure 18. Distribution of osteopenia ((-1 SD) – (-2 SD)) and osteoporosis (< -2 SD) in the lumbar spine (L1-L4) according to the Z score in 43 patients (grey) including 18 Ewing´s sarcoma (green) and 25 osteosarcoma (blue).

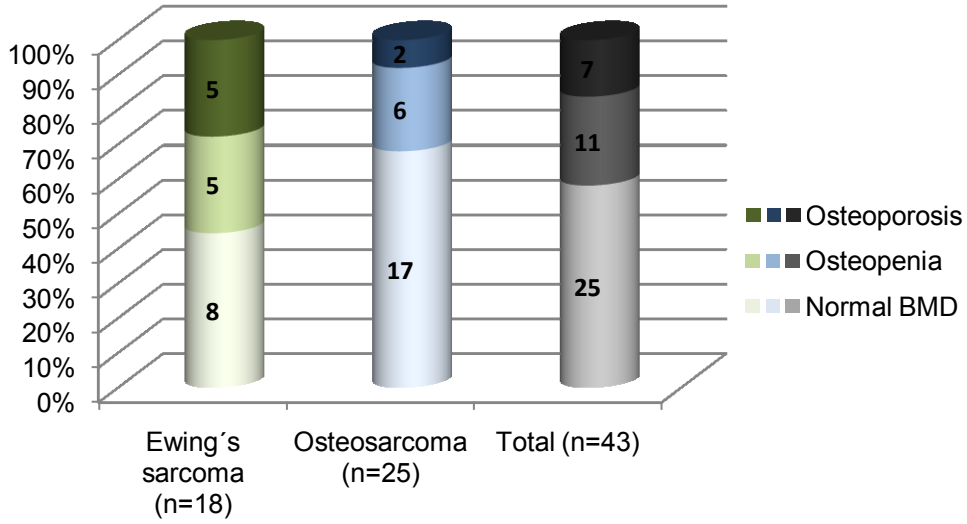


Table 3. Mean bone mineral densities [g/cm²] of the lumbar spine (L1-L4) in 43 patients including 18 Ewing´s sarcoma and 25 osteosarcoma.

		Lumbar spine L1-L4 (n=43)	
	n	BMD (mean ± SD)	Z score (mean ± SD)
Ewing´s sarcoma (n=18)			
Normal BMD	8	1.136 ± 0.135	-0.013 ± 0.591
Osteopenia	5	0.991 ± 0.036	-1.610 ± 0.123
Osteoporosis	5	0.880 ± 0.049	-2.455 ± 0.272
Osteosarcoma (n=25)			
Normal BMD	17	1.173 ± 0.187	0.253 ± 0.869
Osteopenia	6	0.987 ± 0.052	-1.600 ± 0.302
Osteoporosis	2	0.981 ± 0.050	-2.238 ± 0.137
Total (n=43)			
Normal BMD	25	1.161 ± 0.173	0.168 ± 0.801
Osteopenia	11	0.989 ± 0.045	-1.605 ± 0.238
Osteoporosis	7	0.909 ± 0.067	-2.393 ± 0.260

Femur. In the femur we were measuring bone mineral densities in the femoral neck as well as the total femur of both sides (left and right) and found, given that the tumour affected one of the weight bearing lower limbs, a significant lower BMD level in the limb where the tumour was primary located (Wilcoxon signed ranks test: $p_{femoral\ neck} = 0.0005$, $p_{total\ femur} = 0.0019$).

Assuming that this might be partly influenced by a lower load on the affected leg, due to the patients´ attitude to go easy on the operated limb, we decided to concentrate further statistical evaluations on the tumour unaffected side which was supposed to be the one with the higher BMD level and less impact of immobilisation.

As already mentioned we had to reduce our patients´ collective to 37 probands concerning the evaluation of the femur because in six patients densitometry data were only available from the affected side. In probands in which the tumour did not affect the lower limbs the results of left-sided measurement were taken for evaluation as this is the standard side at our institution.

In Table 4 mean bone mineral densities and Z scores of both measured localisations in the femur (neck/total) are listed for the total of 37 patients and separately for the two tumour groups. Again, like in the lumbar spine, it seems that Ewing´s sarcoma patients were presenting worse results in the femoral neck as well as the total femur than patients treated for osteosarcoma.

Table 4. Mean BMD [g/cm²] and Z scores evaluated in the femur in 17 Ewing´s sarcoma and 20 osteosarcoma patients.

	Femoral neck		**Total femur**	
	BMD (mean ± SD)	Z score (mean ± SD)	BMD (mean ± SD)	Z score (mean ± SD)
Ewing´s Sarcoma (n=17)	0,888 ± 0.122	-0.994 ± 1.051	0.921 ± 0.140	-0.876 ± 1.011
Osteosarcoma (n=20)	0,953 ± 0.173	-0.430 ± 1.126	0.963 ± 0.183	-0.500 ± 1.182
Total (n=37)	0.923 ± 0.155	-0.689 ± 1.128	0.943 ± 0.166	-0.673 ± 1.122

In Figure 19 and 20 the general distribution of bone mineral densities among the two different tumour groups is displayed. The quantification of bone loss in the two femoral localisations, neck and total femur, will be discussed separately in the following.

Figure 19. General distribution of bone mineral densities [g/cm²] and Z scores evaluated in the femoral neck in 17 Ewing´s sarcoma (green) and 20 osteosarcoma (blue) patients.

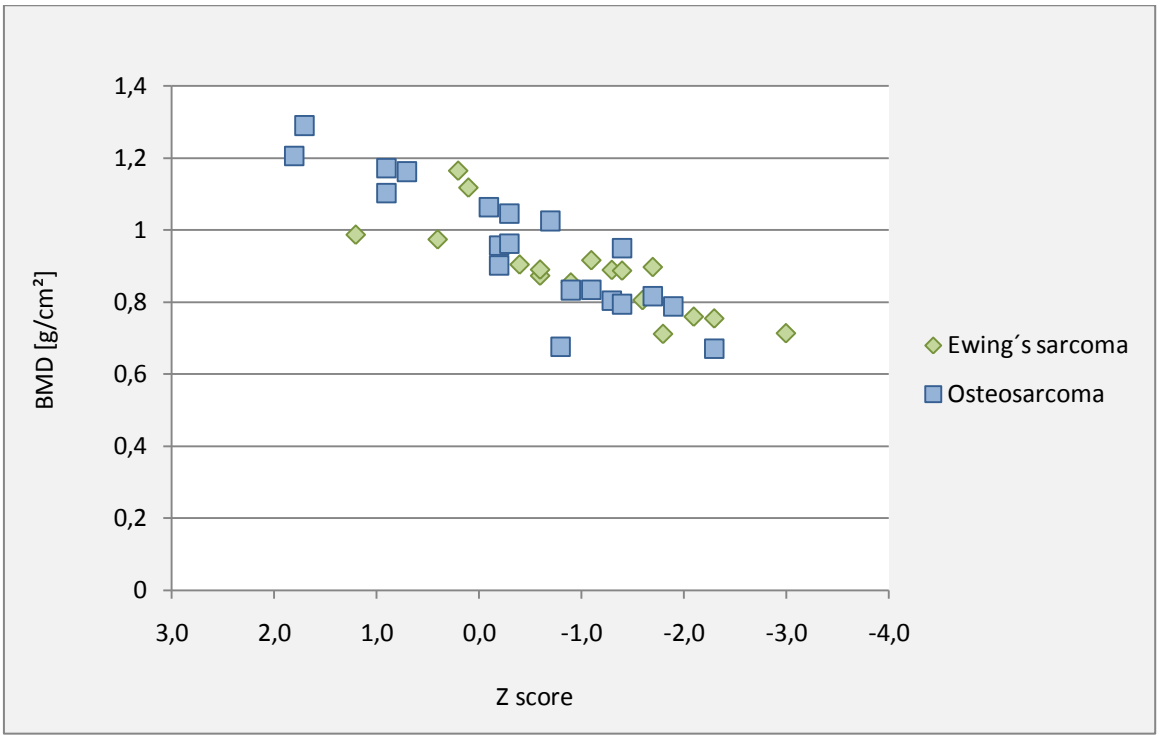
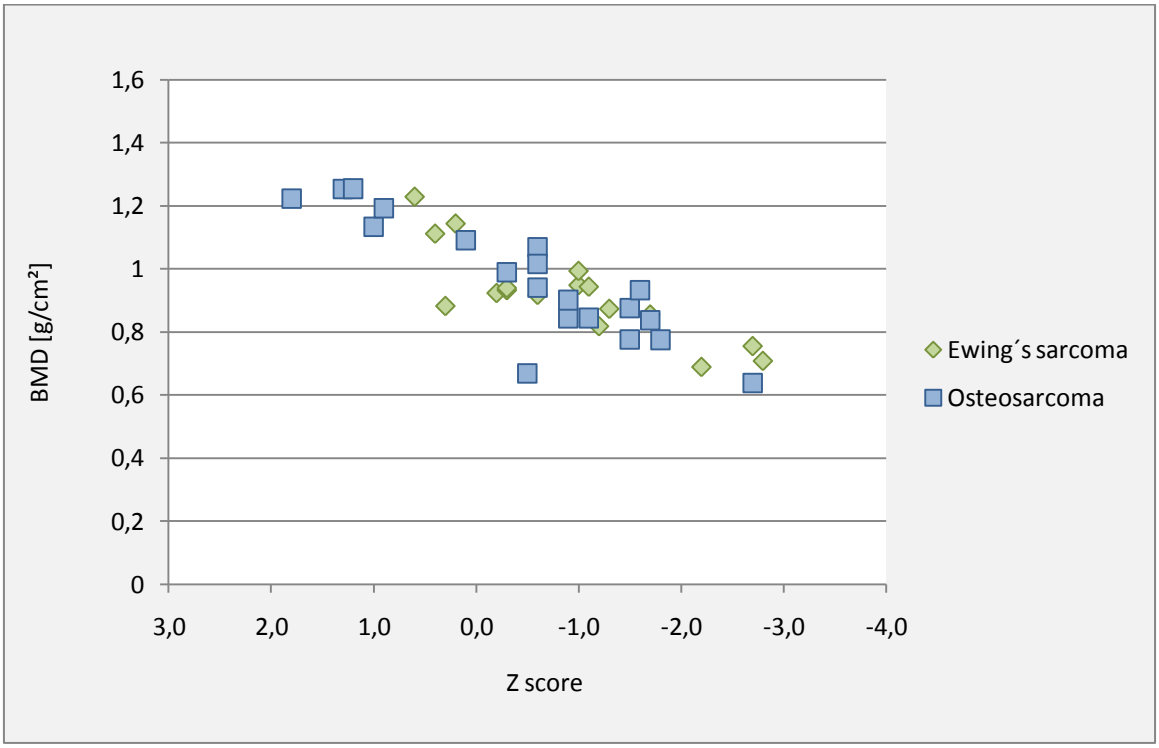


Figure 20. General distribution of bone mineral densities [g/cm²] and Z scores evaluated in the total femur in 17 Ewing´s sarcoma (green) and 20 osteosarcoma (blue) patients.



Femoral neck (tumour unaffected). In the femoral neck osteoporosis occurred in four (11%) and osteopenia in twelve (32%) cases whilst the BMD of 21 patients (57%) was judged as normal. Table 5 and Figure 21 are presenting the detailed results of measurement in the femoral neck in dependence of the tumour type.

Figure 21. Distribution of osteopenia ((-1 SD) – (-2 SD)) and osteoporosis (< -2 SD) in the femoral neck according to the Z score in 37 patients (grey) including 17 Ewing´s sarcoma (green) and 20 osteosarcoma (blue).

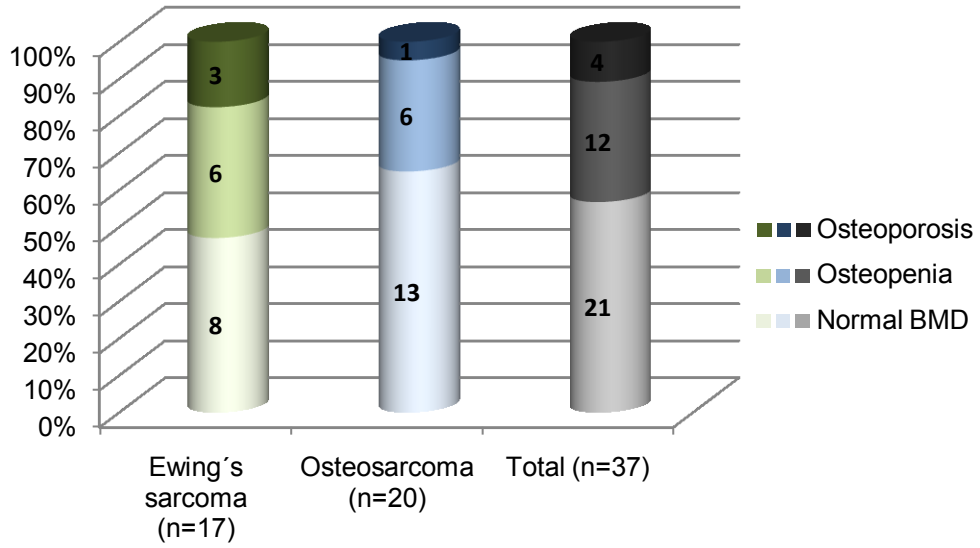


Table 5. Mean bone mineral densities [g/cm²] of the femoral neck in 37 patients including 17 Ewing´s sarcoma and 20 osteosarcoma.

	Femoral neck (tumour unaffected side): n=37		
	n	BMD (mean ± SD)	Z score (mean ± SD)
Ewing´s sarcoma (n=17):			
Normal BMD	8	0.971 ± 0.108	-0.075 ± 0.642
Osteopenia	6	0.851 ± 0.072	-1.483 ± 0.241
Osteoporosis	3	0.742 ± 0.021	-2.467 ± 0.386
Osteosarcoma (n=20)			
Normal BMD	13	1.030 ± 0.160	0.192 ± 0.876
Osteopenia	6	0,831 ± 0,055	-1.476 ± 0.262
Osteoporosis	1	0.670 ± 0.000	-2.300 ± 0.000
Total (n=37)			
Normal BMD	21	1.008 ± 0.146	0.090 ± 0.805
Osteopenia	12	0.841 ± 0.065	-1.475 ± 0.252
Osteoporosis	4	0.724 ± 0.036	-2.425 ± 0.342

Total femur (tumour unaffected). Bone mineral assessment of the total femur revealed four cases (11%) of osteoporosis, ten (27%) of osteopenia and 23 (62%) with normal BMDs. Figure 22 displays the exact proportional distribution of osteopenia and osteoporosis related to tumour histology and Table 6 gives information about the correlating mean bone mineral densities and Z scores.

Figure 22. Distribution of osteopenia ((-1 SD) – (-2 SD)) and osteoporosis (< -2 SD) in the total femur according to the Z score in 37 patients (grey) including 17 Ewing´s sarcoma (green) and 20 osteosarcoma (blue).

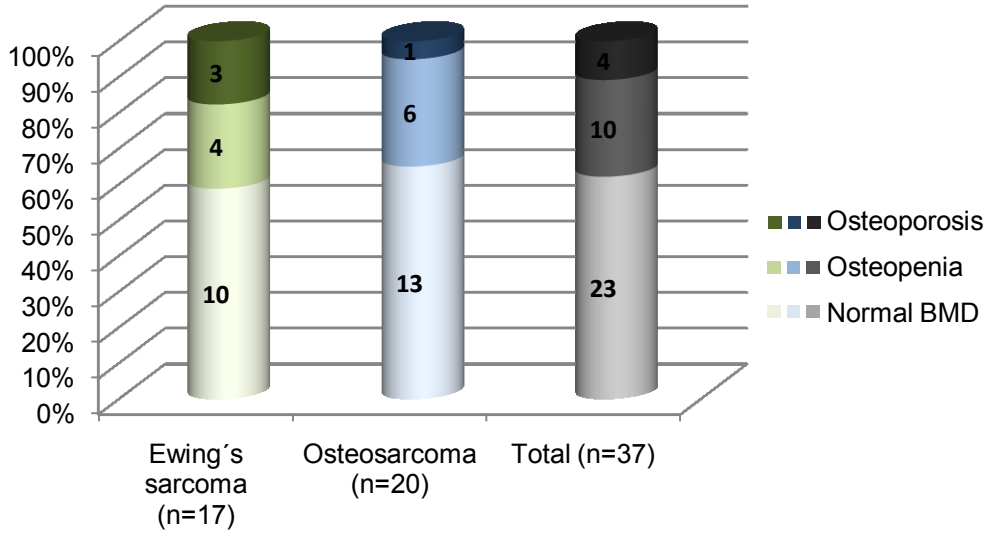


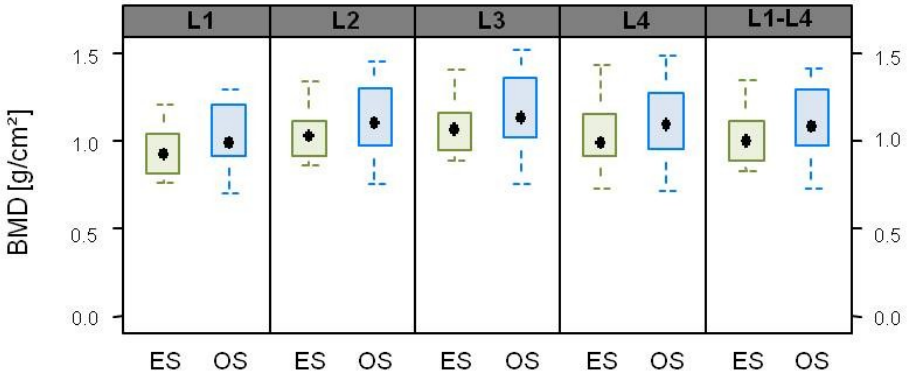
Table 6. Mean bone mineral densities [g/cm²] of the total femur in 37 patients including 17 Ewing´s sarcoma and 20 osteosarcoma.

	Total femur (tumour unaffected side): n=37		
	n	BMD (mean ± SD)	Z score (mean ± SD)
Ewing´s sarcoma (n=17):			
Normal BMD	10	1.002 ± 0.111	-0.190 ± 0.536
Osteopenia	4	0.872 ± 0.045	-1.325 ± 0.228
Osteoporosis	3	0.717 ± 0.028	-2.567 ± 0.262
Osteosarcoma (n=20)			
Normal BMD	13	1.044 ± 0.168	0.146 ± 0.918
Osteopenia	6	0.840 ± 0.055	-1.533 ± 0.221
Osteoporosis	1	0.638 ± 0.000	-2.700 ± 0.000
Total (n=37)			
Normal BMD	23	1.026 ± 0.148	0.000 ± 0.793
Osteopenia	10	0.853 ± 0.054	-1.450 ± 0.246
Osteoporosis	4	0.698 ± 0.042	-2.600 ± 0.235

3.1.3 BMD and tumour type

Shortly summarized the evaluation of our patients´ densitometry results with the BMD of healthy age and gender matched pairs shows a lower bone mineral density than regarded as normal (including osteopenia and osteoporosis) in 43% in the femoral neck, followed by 42% in the lumbar spine (L1 to L4) and 38% in the total femur. In this context the question raises if there exists a significant difference in BMD reduction between the two tumour types treated with various chemotherapeutic agents with the main difference in the use of hd-MTX for osteosarcoma patients. The following figures graphically oppose the median BMD (Figure 23) and Z-scores (Figure 24) of our patients in dependence of tumour type and measured localisation.

Figure 23. Comparison of bone mineral densities (BMD) [g/cm²] measured in the lumbar spine in 18 Ewing´s sarcoma (ES; green) and 25 osteosarcoma (OS; blue) patients, and in the tumour unaffected total femur (F tot)/femoral neck (F neck) in 17 Ewing´s sarcoma (ES; green) and 20 osteosarcoma (OS; blue) patients (* median).



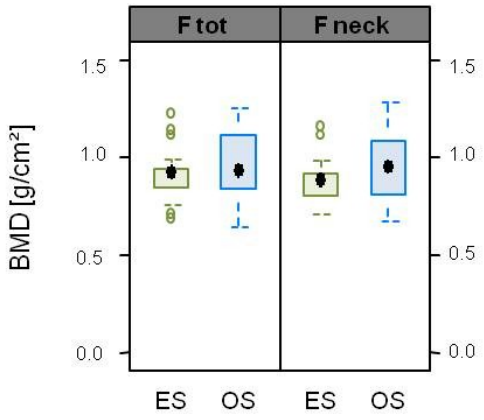
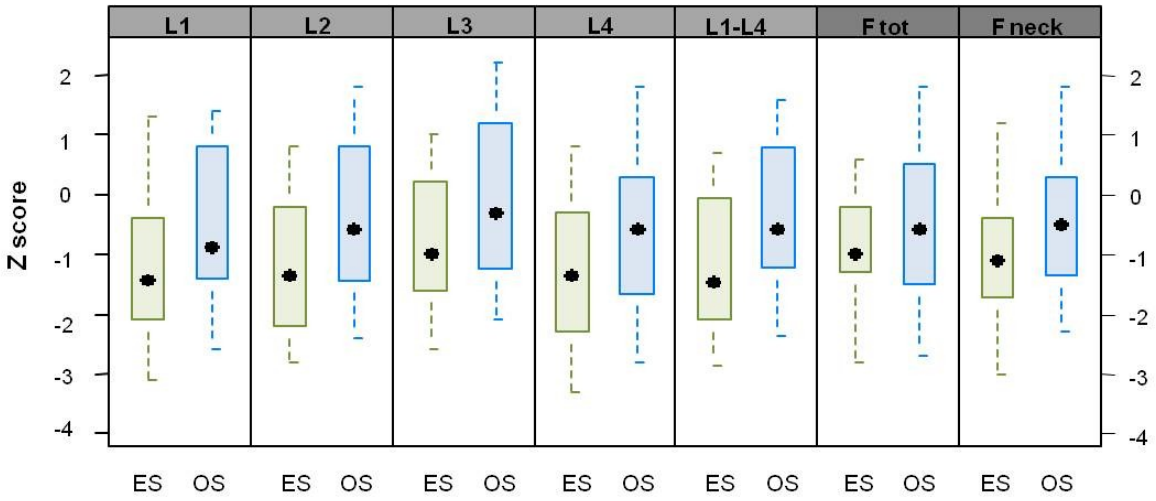


Figure 24. Comparison of the evaluated Z scores concerning the lumbar spine (L1-L4) in 18 Ewing´s sarcoma (ES; green) and 25 osteosarcoma (OS; blue) patients ,and the tumour unaffected total femur (F tot)/femoral neck (F neck) in 17 Ewing´s sarcoma (ES; green) and 20 osteosarcoma (OS; blue) patients (* median).



We used the Wilcoxon test for getting the exact p-values for the different localisations of densitometry and the Global test for evaluating all measured localisations together (lumbar spine and femur) with a non significant result neither concerning the BMD (p_{global} = 0.1271) nor the Z score (p_{global} = 0.0612). In general, however, a slight tendency of a lower bone mineral density in Ewing´s sarcoma patients was outlined against the values of osteosarcoma patients especially in the lumbar spine, but the p-values did not reach significant levels.

3.1.4 BMD and gender / age at diagnosis / passed time since CTX

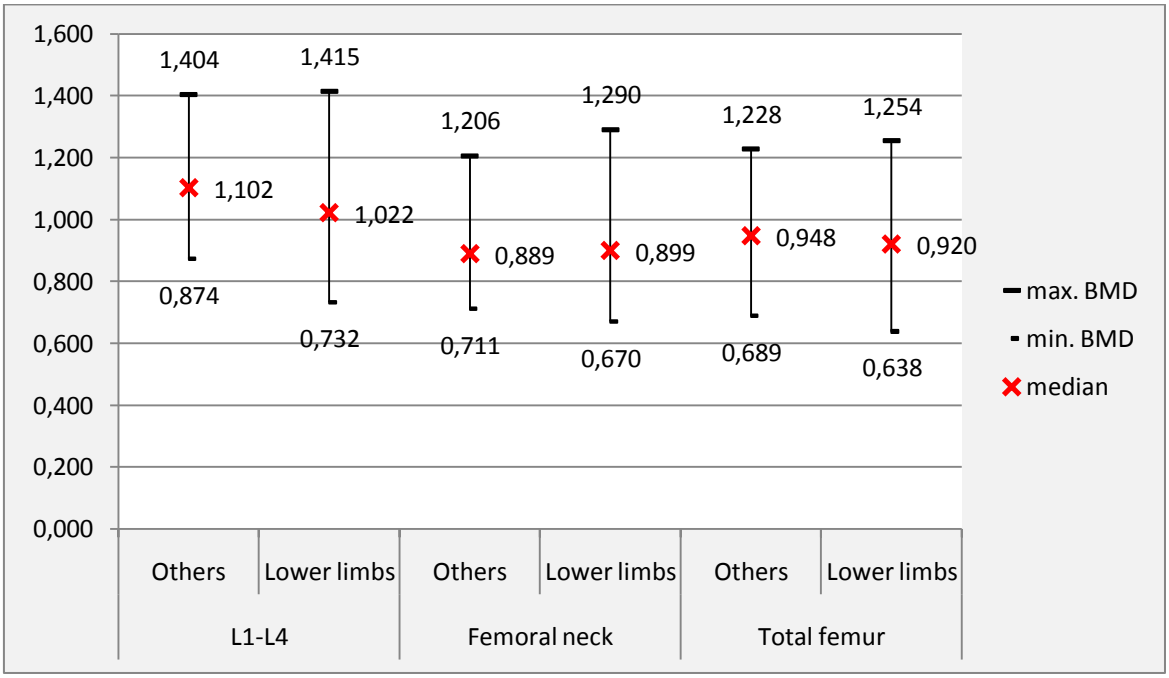
The BMD of our male participants was tested against those of the females (Wilcoxon test) as well as the influence of age at chemotherapy and the time interval passed between chemotherapeutic application (CTX) and our investigation (Spearman rank correlation). After testing all measured localisations separately and correcting for multiple testing according to Bonferroni neither gender (p = 0.2874), nor age at diagnosis (p = 0,5236), nor the time passed since chemotherapy (p = 0,2050) had significantly influenced our results.

3.1.5 BMD and primary tumour localisation

As already mentioned in the introduction the majority of our patients (34/43; 79%), presented a primary tumour localisation in one of the weight bearing lower limbs. Consequently the statistical results of densitometry mainly reflect the proportional distribution of osteopenia and osteoporosis in those patients and may overshadow the results of patients whose tumour did not affect the lower extremities. In this context it might be possible that patients with tumour-unaffected legs did not underlie the same amount of immobilisation and therefore might show higher BMD levels.

In concrete terms we are talking about nine patients (9/43; 21%) who presented a primary tumour affection of the upper limbs in four, and of any other part of the skeleton (except the lower limbs) in five cases. Figure 25 compares the median measured bone mineral densities of those nine probands (=others) with the median BMD of patients with lower limb affection (=lower limb). Slightly better median BMD values could be recognised in the lumbar spine as well as in the total femur in the group with tumour unaffected legs.

Figure 25. Comparison of median bone mineral densities [g/cm²] and its range in relation to the primary tumour localisation.



3.1.6 BMD and radiation

In total eight patients (8/43; 19%) received radiotherapy - five Ewing´s sarcoma and one osteosarcoma additionally to surgery, and one patient of each tumour type instead of it. Beyond them we found four (4/8) with normal BMD levels in all measured localisations and the other half showed a reduction of bone mineral density in different amounts and localisations, but just in two cases (*) the decrease might have been additionally influenced by pelvic radiation. Details about bone mineral densities and Z scores are displayed in Table 7.

Table 7. Bone mineral densities [g/cm²] and Z scores of six Ewing´s sarcoma (ES) and two osteosarcoma (OS) patients who underwent radiotherapy either additionally or instead of surgery.

Localisation of tumour / radiaton	Histol. Diag.	L1-L4		F neck (tumour unaffected)		F tot (tumour unaffected)	
		BMD	Z	BMD	Z	BMD	Z
Os pubis dext.	ES	1,238	0,7	0,904	-0,4	0,939	-0,3
Costa VIII dext.	ES	1,102	-1,0	0,889	-1,3	0,948	-1,0
Os ileum sin.*	ES	0,940	-1,7	0,890	-0,6	0,890	-0,6
M. erector spinae sin.	ES	1,057	-0,7	0,711	-1,8	0,689	-2,2
Cervical vertebra (C5)	ES	1,350	0,4	1,165	0,2	1,228	0,6
Calcaneus dext.	ES	0,864	0,6	0,987	1,2	0,882	0,3
Sacroiliacal dext.	OS	1,124	-0,8	0,957	-0,2	0,941	-0,6
Femur prox. dext.*	OS	0,931	-2,1	0,816	-1,7	0,837	-1,7

* possible correlation of local radiotherapy and bone mineral density decrease because of coherence of application and measuring localisation

3.1.7 BMD and fractures

Seven of our study participants (7/43; 16%) suffered from non-trauma and not directly tumour-associated fractures after chemotherapeutic treatment, all affecting the lower limbs, except in one case with affection of the finger (see 2.2.1). Table 8 gives the densitometry results of those patients in dependence of the measured localisation.

Table 8. BMDs [g/cm²] and Z scores of two Ewing´s sarcoma (ES) and five osteosarcoma (OS) patients who suffered from at least one non-traumatic fracture after chemotherapeutic treatment (CTX)

Localisation of fracture	Time [mo] after CTX	Type of tumour	L1-L4		F neck (tumour unaffected)		F tot (tumour unaffected)	
			BMD	Z	BMD	Z	BMD	Z
Prox. Femur	36	OS	0,974	-1,2	0,670	-2,3	0,638	-2,7
Prox. Femur	72	ES	0,842	-2,3	0,619*	-2,7	0,582*	-3,1
Dist. Femur	192	OS	1,065	-1,2	0,821*	-1,8	0,689*	-2,9
Dist. Femur, prox. Tibia	29	ES	0,891	-2,7	0,759	-2,1	1,111	0,4
Prox. Tibia	32	OS	1,102	-0,5	0,962	-0,3	0,989	-0,3
Prox. Tibia	30	OS	0,921	0,1	0,563*	-3,2	0,548*	-3,1
Digitum	72	OS	1,415	1,6	1,102	0,9	1,133	1,0

* tumour affected side (because the tumour-unaffected side could not be measured)

Just two patients with fractures showed normal BMD levels in all measuring points, the remaining five presented decreased values in the femur as well as in the lumbar spine, except in one osteosarcoma patient with osteoporosis in the femur (neck and total) and normal bone mineral content in the lumbar spine.
In detail, in the lumbar spine two patients (2/7) showed osteoporotic and another two (2/7) osteopenic BMD levels. In the femur the result was even worse with four (4/7) times osteoporosis in the femoral neck and the total femur, and one (1/7) time osteopenia in the femoral neck.

A limitation of these results are the missing bone mineral densities of the tumour unaffected femur in three patients in which we had to take the results of the tumour affected side for this fracture associated evaluation.

3.1.8 BMD and height / weight / body mass index

Height, weight and body mass index (BMI) turned out to be strongly influencing factors of bone mineral density in our patients. All three showed a directly positive correlation with the measured density values in all localisations by Spearman rank correlation and after correcting for multiple testing according to Bonferroni (p_{height} = 0.0267, p_{weight} = 0.0003, p_{BMI} = 0.0003).

3.1.9 BMD and menarche

Assuming that advanced pubertal status at chemotherapeutic application might have positively influenced the outcome, we tested how far age at menarche ($p = 0.390$) as well as the passed time between menarche and chemotherapeutic application ($p = 1.000$) correlated with the measured BMD in our female patients by using the Spearman rank correlation and Bonferroni correction again, but finding no significant association.

3.2 Laboratory

3.2.1 General laboratory

The basic diagnostics of our patients´ bone metabolism presented unexpectedly normal *calcium* values, with just one osteosarcoma patient below the reference value. *Phosphate* was decreased in six Ewing´s sarcoma and four osteosarcoma patients (23%) with the mean and median phosphate levels ranging in the lower third of the reference value among the total collective of patients (Table 9). Related to this we also found a general upward tendency in *parathyroid hormone*, but still within physiological range except in one Ewing´s sarcoma and two osteosarcoma patients (7%) with an increase above the upper reference limit (Table 10).

Table 9. General laboratory values of 18 Ewing´s sarcoma and 25 osteosarcoma patients (median values (range)) and statistical comparison of the two tumour types (p values).

GENERAL LABORATORY	Ewing´s sarcoma (n=18)	Osteosarcoma (n=25)	Total (n=43)	p values
Electrolytes				
Total calcium [mmol/l]	2.4 (2.3 to 2.6)	2.4 (2.2 to 2.6)	2.4 (2.2 to 2.6)	0.460
Phosphate [mg/dl]	2.7 (1.9 to 4.1)	3.3 (2.2 to 4.4)	3.1 (1.9 to 4.4)	0.096
Kidney				
Creatinine [mg/dl]	1.1 (0.8 to 1.8)	0.9 (0.4 to 1.3)	1.0 (0.4 to 1.8)	0.043
Urea [mg/dl]	30.5 (16.0 to 57.0)	30.0 (19.0 to 55.0)	30.0 (16.0 to 57.0)	0.719
Blood urea nitrogen [mg/dl]	14.2 (7.5 to 26.6)	14.0 (8.9 to 25.7)	14.0 (7.5 to 26.6)	0.719
Liver				
Gamma-glutamyl transferase [U/l]	15.5 (10.0 to 511.0)	17.0 (7.0 to 115.0)	17.0 (7.0 to 511.0)	0.578
Aspartate aminotransferase [U/l]	29.0 (17.0 to 93.0)	25.0 (17.0 to 56.0)	27.0 (17.0 to 93.0)	0.490
Alanine aminotransferase [U/l]	21.5 (11.0 to 186.0)	22.0 (10.0 to 119.0)	22.0 (10.0 to 186.0)	0.898
Serum proteins				
Total globulin [g/dl]	7.9 (6.5 to 9.1)	7.7 (6.8 to 9.0)	7.7 (6.5 to 9.1)	0.336
Albumin [g/dl]	5.2 (4.2 to 5.9)	5.0 (4.2 to 5.5)	5.1 (4.2 to 5.9)	0.044

With a mean of 15.8 ng/ml in Ewing´s sarcoma and 16.6 ng/ml in osteosarcoma 88% of our patients (15 Ewing´s sarcoma and 23 osteosarcoma) presented a deficiency in vitamin D which plays a key role in bone metabolism (Table 10).

Liver and kidneys, both involved in the endocrine vitamin D production, also showed some disorders. *Creatinine* was marginal high in both tumour types with levels above the reference limit of 1 mg/dl in 12 Ewing´s sarcoma and ten osteosarcoma patients (51%), but on the other hand the levels of *urea* were physiological midranged with an increase just in two Ewing´s sarcoma and three osteosarcoma patients (12%). *Total protein* was above the reference range in seven Ewing´s and four osteosarcoma patients (26%) and in albumin the relation was 5:3 (19%). The hepatic transaminases, the *gamma-glutamyl transferase* (γGT / GGT) as well as the *alanine aminotransferase* (ALT), were slightly increased in 16% and the *aspartate aminotransferase* (AST) in 28%. One Ewing´s sarcoma patient showed an extraordinary high γGT-level of 511 U/l (Table 9).

By the exact Wilcoxon test a statistically significant difference between the two tumour types could just be evaluated in creatinine ($p = 0.043$) and albumin ($p = 0.044$), and in both osteosarcoma patients presented tendentially lower levels. All other general laboratory findings did not differ significantly (for exact p values see Table 9).

3.2.2 Specific laboratory

Bone specific alkaline phosphatase (BAP) was elevated in 86% (16 Ewing´s sarcoma, 21 osteosarcoma), *N-terminal telopeptide procollagen* (PINP) in 60% (eight Ewing´s sarcoma, 18 osteosarcoma) and *osteocalcin* (OC) in 47% (eight Ewing´s sarcoma, 12 osteosarcoma) of our study probands. *Beta-crosslaps* were above reference range in 12 Ewing´s sarcoma and 16 osteosarcoma patients (65%) with a general tendency towards the upper limit. *The tartrate resistant alkaline phosphatase* in all patients, except in two Ewing´s sarcoma with a decrease below reference range, gained levels close to the upper reference limit and in 23% (four Ewing´s sarcoma and six osteosarcoma patients) this limit was even exceeded. *Osteoprotegerin* generally ranged close to the upper borderline, with exception of one Ewing´s sarcoma, and 26% of our probands (three Ewing´s

sarcoma and eight osteosarcoma) even reached values above. The serum levels of *rankl* presented themselves inverse to osteoprotegerin with a decrease below the reference limit in 51%, including eight Ewing´s sarcoma and 14 osteosarcoma patients (Table 10).

Table 10. Endocrinological and bone specific laboratory values of 18 Ewing´s sarcoma and 25 osteosarcoma patients (median values (range)) and statistical comparison of the two tumour types (p values).

ENDOCRINOLOGICAL LABORATORY	Ewing´s sarcoma (n=18)	Osteosarcoma (n=25)	Total (n=43)	p values
Thyroid gland				
Thyroid stimulating hormone [µU/ml]	1.5 (1.1 to 3.3)	1.6 (0.4 to 2.7)	1.6 (0.4 to 3.3)	0.530
Trijodthyronin [pmol/l]	5.3 (4.1 to 6.4)	5.2 (4.0 to 6.8)	5.2 (4.0 to 6.8)	0.253
Thyroxin [pmol/l]	15.9 (12.1 to 22.9)	14.4 (11.2 to 24.2)	14.8 (11.2 to 24.2)	0.976
Parathyroid gland/bone metabolism				
Parathyroid hormone [pg/ml]	38.1 (18.7 to 65.6)	45.7 (21.8 to 77.8)	43.3 (18.7 to 77.8)	0.453
25 (OH) vitamin D3 [ng/ml]	15.8 (5.0 to 50.2)	16.6 (7.6 to 104.6)	16.4 (5.0 to 104.6)	0.468
Bone specific alkaline phosphatase [µg/l]	31.4 (16.6 to 75.5)	29.4 (15.8 to 97.8)	31.1 (15.8 to 97.8)	0.579
Osteocalcin [ng/ml]	30.7 (1.5 to 255.7)	34.4 (15.0 to 168.2)	31.9 (1.5 to 255.7)	0.793
N-terminal telopeptide procollagen [ng/ml]	62.4 (29.7 to 417.3)	81.1 (31.5 to 722.4)	78.8 (29.7 to 722.4)	0.518
Osteoprotegerin [pmol/l]	3.2 (0.1 to 7.0)	3.6 (1.3 to 7.7)	3.5 (0.1 to 7.7)	0.325
NF α B-rezeptor ligand [pmol/l]	0.04 (0.0 to 0.4)	0.02 (0.0 to 1.1)	0.03 (0.0 to 1.1)	0.556
Tartratresistant alkaline phosphatase [U/l]	3.1 (1.7 to 5.6)	3.4 (2.4 to 11.5)	3.3 (1.7 to 11.5)	0.205
Beta-crosslaps [ng/ml]	0.7 (0.2 to 3.7)	0.6 (0.3 to 2.8)	0.6 (0.2 to 3.7)	0.692
Gonads				
Testosterone total (♂) [ng/ml]	4.7 (0.2 to 8.8)	4.8 (0.1 to 8.8)	4.7 (0.1 to 8.8)	0.698
Testosterone free (♂) [pg/ml]	18.2 (2.4 to 34.3)	15.7 (0.2 to 28.1)	16.4 (0.2 to 34.3)	0.391
Sexual hormone binding protein (♂) [nmol/l]	37.1 (22.3 to 111.1)	37.0 (23.3 to 200.0)	37.1 (22.3 to 200.0)	0.776
Testosterone total (♀) [ng/ml]	0.3 (0.2 to 0.6)	0.4 (0.1 to 0.6)	0.4 (0.1 to 0.6)	0.763
Testosterone free (♀) [pg/ml]	1.5 (1.0 to 2.9)	1.7 (0.8 to 3.0)	1.6 (0.8 to 3.0)	0.888
Sexual hormone binding globulin (♀) [nmol/l]	118.6 (35.1 to 173.8)	47.10 (22.8 to 133.6)	88.5 (22.8 to 173.8)	0.074
Neuroendocrinology				
Growth hormone [ng/ml]	2.8 (0.2 to 11.8)	0.6 (0.1 to 15.7)	1.3 (0.1 to 15.7)	0.325
Insulin-like growth factor 1 [ng/ml]	230.0 (112.0 to 489.0)	242.0 (72.6 to 418.0)	238.0 (72.6 to 489.0)	0.588

Apart from the direct bone specific parameters an indirect bone reductive influence caused by thyroid imbalances could be excluded. But other hormones affecting bone mineral density showed disturbed levels. An increase in *growth hormone* was seen in 23% of patients and in 28% in its regulator, the *insulin-like growth factor 1* (Table 10).

The median levels of *free (=bioactive) testosterone* in patients aged above 17 years (n=37) were 16.6 pg/ml in men and 1.5 pg/ml in women, both tending more towards the lower reference limit (in Table 10 the values of the whole study group, n=43, are given). Related to this the *follicle stimulating hormone* (FSH) showed an increase above reference range in 35% of patients, presenting an equal percental distribution in males and females. A non-physiological raise was also found in *luteotrope hormone* (LH) in 19% of patients, but here an increase was seen more in men than in women (♂:♀=9:1). For more details see Table 11.

Table 11. Gynaecological/Gonadal laboratory values of 18 Ewing´s sarcoma and 25 osteosarcoma patients (median values (range)) and statistical comparison of the two tumour types (p values).

GYNAECOLOGICAL / GONADAL LABORATORY	Ewing´s sarcoma (n=18)	Osteosarcoma (n=25)	Total (n=43)	p values
Follicle stimulating hormone (♂) [mIE/ml]	6.3 (1.0 to 31.9)	8.9 (0.9 to 33.7)	6.8 (0.9 to 33.7)	0.683
Follicle stimulating hormone (♀) [mIE/ml]	40.4 (0.3 to 115.4)	7.3 (2.4 to 183.7)	13.3 (0.3 to 183.7)	0.277
Luteotrope Hormone (♂) [mIE/ml]	6.3 (0.4 to 13.8)	4.5 (0.3 to 16.0)	5.6 (0.3 to 16.0)	0.201
Luteotrope Hormone (♀) [mIE/ml]	18.2 (0.3 to 48.4)	5.6 (1.7 to 71.8)	13.8 (0.3 to 71.8)	0.815

Table 10 and 11 also display the exact p values of the statistical comparison of the two tumour types (Ewing´s sarcoma versus osteosarcoma) concerning our specific laboratory findings by the exact Wilcoxon test. Out of all tested laboratory results just the sexual hormone binding globulin (SHBG) reached nearly significant p levels in women with higher ranges in Ewing´s sarcoma patients, but as SHBG is menstrual cycle dependant and strongly influenced by oral contraceptives this should not be overestimated.

3.2.3 Screening for lactose intolerance

A genetic determination for lactose intolerance was found in 44% of Ewing´s sarcoma and 31% of osteosarcoma patients, which results in 37% of the total collective. The remaining probands were split into 40% with a slight tendency to develop lactose intolerance and 23% who did not display any genetic predisposition to develop problems with lactose digestion (Table 12).

Table 12. Relation of genetic determination of lactose intolerance and bone mineral density (mean values ± SD) in Ewing´s sarcoma and osteosarcoma patients.

LACTOSE INTOLERANCE	n=43	Lumbar spine (mean BMD ± SD)	n=37	Femoral neck (mean BMD ± SD)	Total femur (mean BMD ± SD)
TT (no genetic determination)	10	1.086 ± 0.183	9	0.948 ± 0.188	0.952 ± 0.209
TC (little to no genetic determination)	17	1.052 ± 0.092	14	0.919 ± 0.112	0.934 ± 0.125
CC (genetic determination)	16	1.096 ± 0.220	14	0.910 ± 0.166	0.948 ± 0.169

Using the exact Wilcoxon test again no significant difference could be found between Ewing´s sarcoma and osteosarcoma patients ($p = 0.363$). Interestingly, the predisposition for lactose intolerance itself did not significantly correlate with the measured BMD ($p = 1.000$) when tested by Spearman rank correlation and after Bonferroni correction.

4 DISCUSSION

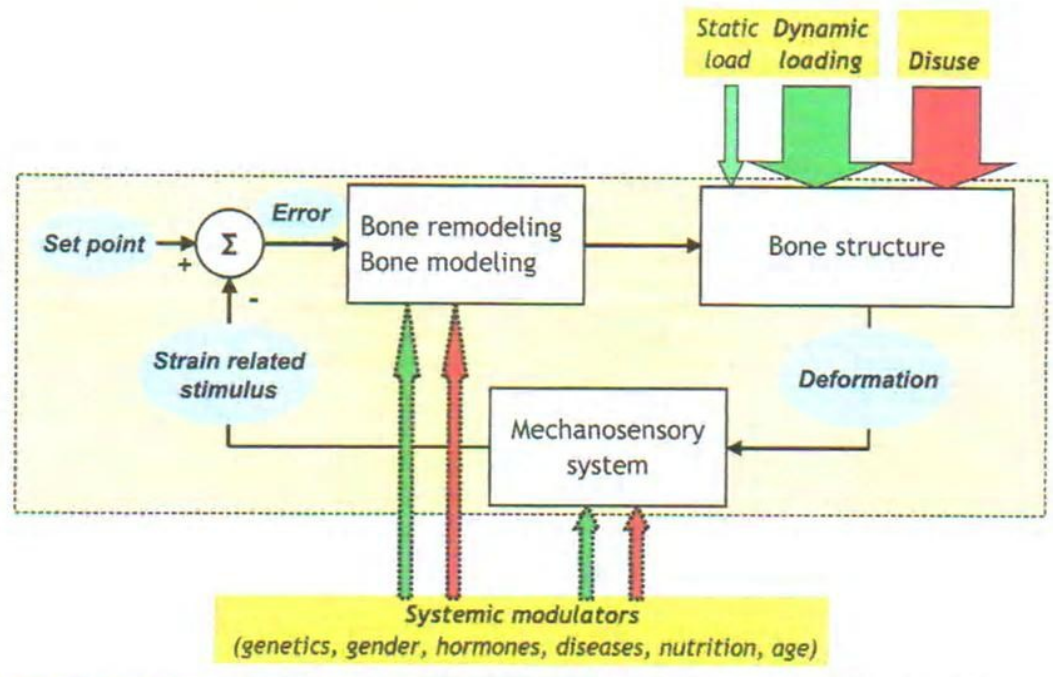
The invention of chemotherapeutic treatment presented a big benefit for patients suffering from bone sarcoma in childhood and adolescence because it significantly increased the chance of survival compared with just operative intervention. However, this increase in the survival rate also confronts us nowadays with many yet unknown longterm effects of childhood cancer and its treatment. One of those is the suspected bone reductive effect of the different chemotherapeutic agents used in a multidrug treatment scheme which was already discussed in several studies in context of leukaemia and osteosarcoma, mainly focusing on the application of high-dose methotrexate. [6], [7], [8], [9], [10], [11]

4.1 Densitometry

Ewing´s sarcoma versus osteosarcoma. In our study we were measuring bone mineral density after chemotherapeutic treatment in two different groups of patients with bone tumours occurring majorly in the first two decades of life and found a reduction in BMD in 58% (37% osteopenia, 21% osteoporosis) of our study participants in at least one of the measured localisations either in the lumbar spine or in the femur with no significant alteration between the two tumour groups. This result indicates that not only osteosarcoma patients treated with hd-MTX are threatened by premature bone loss, but also Ewing´s sarcoma treatment implicates a harmful potential on bone mineral density. This is underlined by the fact that in our study even more Ewing´s sarcoma (10/18; 56%) than osteosarcoma (8/25; 32%) patients showed a reduction of BMD in the lumbar spine, which is suspected to be more sensitive to therapeutic modalities because of the trabecular bone as the main component. [9], [29] Additionally the longer average treatment duration of 10-12 months in Ewing´s sarcoma compared to periods of 6-12 months in osteosarcoma might have contributed to this outcome. [19]

Immobilisation. On the other hand we also found a femoral bone mineral density reduction in both types of tumour which might additionally be the consequence of physical inactivity caused by the illness itself as immobilisation is supposed to show a higher impact on weight bearing bones than on the lumbar spine. [29], [30]
A three-dimensional network of osteocytes nestling in mineralized bone matrix builds the basis of a mechanosensory system which enables our skeleton to permanently adapt to varying static and dynamic loading (Figure 26).

Figure 26. Schematic representation of the homeostatic negative feedback control system of bone mechanical competence: depending on loading or disuse the mechanosensory system regulates bone remodelling in relation to an apparent evolutionary set point of bone tissue, all under influence of a variety of systemic excitatory (green arrows at the bottom) and inhibitory modulators (red arrows at the bottom) not yet fully understood. (from Sievanen et al., Immobilization and bone structure in humans, 2010)



If immobilisation disturbs the balance between bone formation and resorption cortical thinning and trabecular bone loss are the consequences, affecting the epiphysis more than the diaphysis. [31], [32] In general bone loss seems to be higher in the first weeks of disuse due to diminished bone formation consequently to the sudden removal of mechanical loading. Thus approximately 70% of initial bone reduction are due to a decrease in bone formation and just

30% result from bone resorption. After the first half year of immobilisation bone loss is supposed to reach a steady state and although individuals present high variability, depending on the initial skeletal status, the duration of disuse shows the greatest impact on the amount of BMD reduction. [31] Here again the longer treatment periods of our Ewing´s sarcoma patients might have fundamentally influenced densitometry results. For example, bone recovery after a three-monthly period of immobilisation can earliest be expected after around one year given a full functional rehabilitation, but it is still unanswered if phenotype and strength of bone are akin to the state prior to disuse. [31]

Radiation. In childhood cancer treatment additionally to immobilisation and chemotherapeutic application also radiotherapy might contribute to a bone mineral density reduction. Especially Ewing´s sarcoma treatment involves radiation if complete surgery is impossible, or postoperative either in case of inadequate surgical margins or poor responding to neoadjuvant chemotherapy (i.e. >10% viable tumour cells in the surgical specimen), whilst in osteosarcoma radiotherapy is administered just in highly selected cases or for palliation. [19] Investigations revealed spontaneous fractures of the femoral neck accompanied by trabecular bone atrophy (i.e. osteoporosis) after high-dose local radiotherapy for gynaecological malignancies in adults, whilst in children radiation delivered to the epiphyseal plate has impacted bone growth in diverse studies. Nevertheless the quantification of possible effects of local radiotherapy on bone mineral density still seems to be very difficult due to the complexity of tumour treatment. [33]

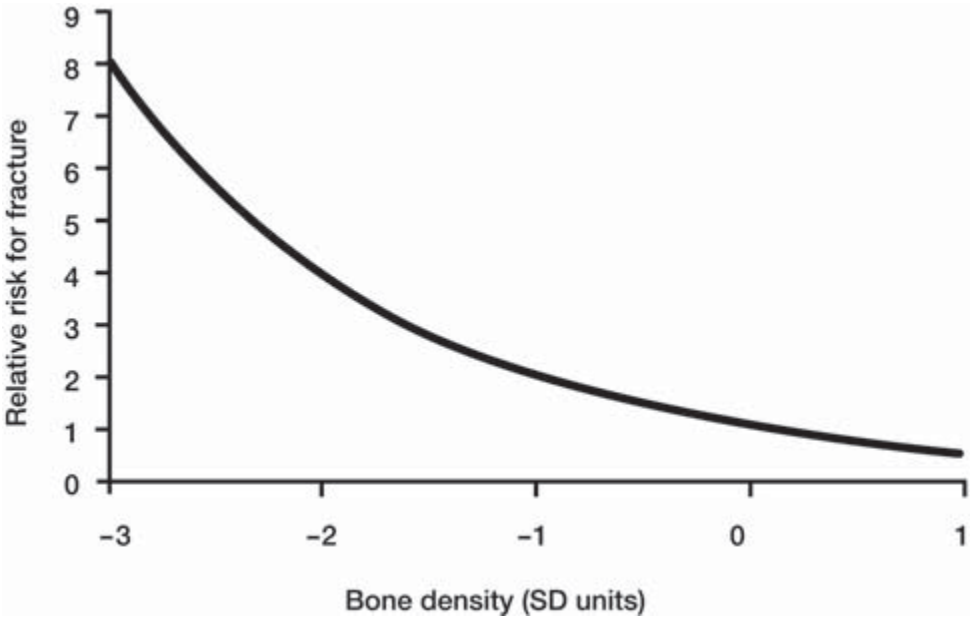
In our study this question also remains unanswered, but the influence of local radiotherapy, administered to eight of our study participants (six Ewing´s sarcoma and two osteosarcoma), should not have biased the general results. Just in two probands the measured reduction of bone mineral density might have been additionally deteriorated by pelvic radiation.

Skeletal/cancer related events. Bone loss is a silent disease [34] and is often first diagnosed by skeletal related events (SREs) like bone pain or pathologic fractures. This happened to seven of our study participants who suffered from non-traumatic

fractures after chemotherapeutic treatment. Densitometry revealed a central BMD reduction in five of them in different extents, which is quite alarming because studies indicate that osteoporotic fractures in patients´ history are associated with a higher risk for subsequent events. [35] Furthermore the absence of fractures does not automatically imply an age-related healthy bone status, because according to Kaste S.C. children often do not suffer from fractures even if very low Z score levels are reached. [36]

In general the estimated lifetime-fracture risk due to postmenopausal or senile osteoporosis in patients above 50 years ranges at 40% in women and 13% in men. It is exponentially related to bone mineral density, which implies that a small decrease in BMD correlates with a much higher increase in fracture risk (Figure 27). [35]

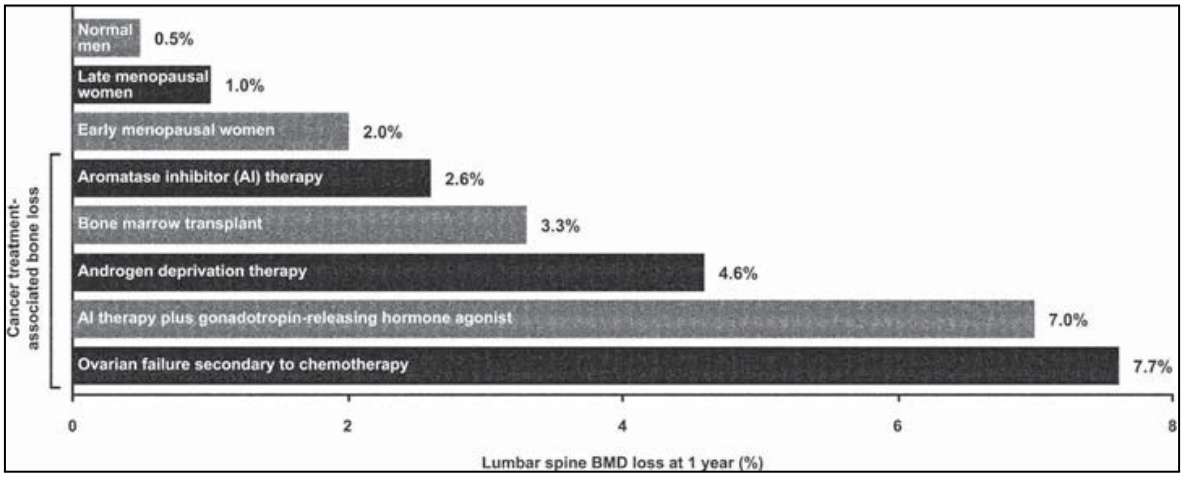
Figure 27. Exponential relationship between bone mineral density (BMD) and fracture risk (SD = T score standard deviation used in adult bone assessment). (from Guise TA, Bone Loss and Fracture Risk Associated with Cancer Therapy, 2006)



Bone loss in people without cancer history starts in midlife and counts 0.5-1.0% per year in men and 2% for the first 5-10 postmenopausal years with a decline afterwards in women. Related to tumour-treatment bone mineral density decrease is assessed to be up to tenfold higher, especially in association with

cancer related hypogonadism (Figure 28). [35] Therefore bone mineral density reduction in childhood cancer survivors has to be taken at least as seriously as midlife or senile osteoporosis.

Figure 28. Rates of bone loss displaying substantially higher rates in tumour-treatment related BMD reduction (from Guise TA, Bone Loss and Fracture risk Associated with Cancer Therapy, 2006)



In adults bisphosphonates are generally well accepted for antiresorptive therapy of benign as well as malignant bone conditions. In osteoporosis and cancer treatment, especially in tumours associated with hormonal dysbalances, consecutive hypogonadism and cases of metastatic disease, bisphosphonates build the mainstay of effective fracture-risk management by slowing down bone loss. [35], [37], [38]

Latest therapeutic strategies in bone tumours even consider the involvement of bisphosphonates in primary tumour treatment focusing on limiting the osteolytic component of the tumour itself as well as protection from metastatic settlement in lungs and bone. [39], [40] Zoledronic acid (ZOC), a nitrogen-containing high-potential bisphosphonate of the third generation, represents promising results in Ewing´s sarcoma mice-model by inhibiting tumour development in bone and showing a synergistic effect with ifosfamide in the soft tissue. Clinicians now sense a chance to improve overall survival in Ewing´s sarcoma patients with metastases which is still below 40% (15% for bone metastases!), as well as to reduce chemotherapeutic doses. Furthermore bisphosphonates may provide a promising

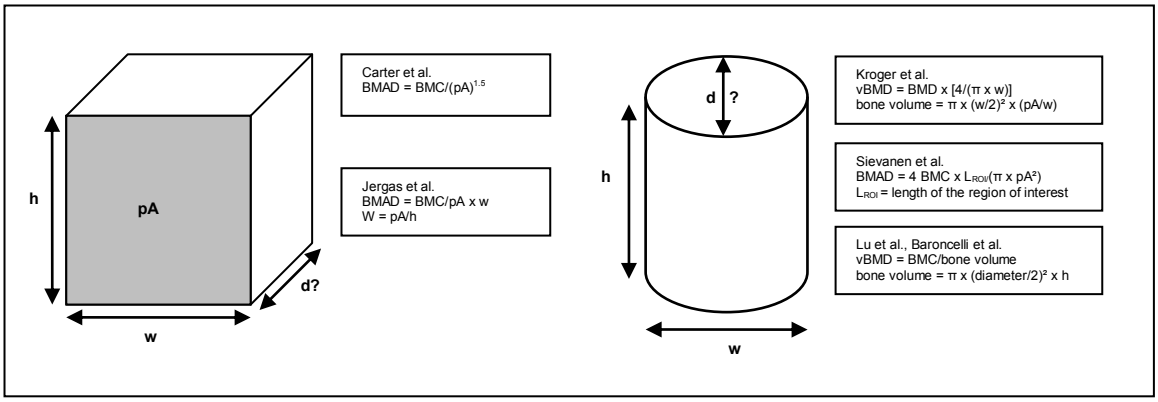
future strategy especially for patients with tumour relapse (30%!) or chemotherapeutic non-responders, most of them still with poor prognosis under current Euro-E.W.I.N.G. 99 therapy. [39], [41], [42]

In osteosarcoma rat model zoledronic acid was able to limit tumour induced cortical bone destruction, but failed in pulmonary metastases known to cause death in over 30% of patients. [40] In mice ZOC showed the same corticoprotective effect in dependence of application frequency (single/weekly), but primary tumour growth seemed to be unaffected. [43]

Using bisphosphonates in basic tumour treatment may positively influence the outcome of childhood cancer survivors for two different reasons. First, chemotherapeutic dose reduction might result in a lower direct and indirect bone toxicity, and second, bisphosphonates themselves may contribute to a higher resistance against chemotherapeutic bone loss as well as metastatic settlement in bone. This seems to be a welcome future therapy objective, but does not retroactively adjust our current patients´ deficits in bone mineral density. Therefore we want to return to our initial topic of diagnosing BMD-deficits in bone sarcoma survivors treated by chemotherapeutic protocols of the last years.

Limitation of densitometry. Taking bone mineral density measurement alone for diagnosing osteopenia or osteoporosis in children and adolescents may accidentally lead to wrong results and overtreatment because its adequate interpretation presents a generally well known problem due to the continuing process of growth and all its multiple influencing factors. [44], [45], [46] Furthermore dual-energy X-ray absorptiometry (DEXA), which is in common use because of its rapid and accurate measurement in addition to a relatively low radiation exposure, presents some further pitfalls. Whilst real bone mass depends on the factors density and size and is a volumetric unit (g/cm^3), densitometry just enables the investigator to measure a two-dimensional area (g/cm^2). [46], [46], [47], [48] No matter if we use one of the mathematical models (Figure 29) to calculate vBMD (=volumetric bone mineral density) or the Z score system, as we did in our study, real BMD and its clinical assessment will still remain partly estimated.

Figure 29. Mathemathical models proposed to calculate vBMD (=volumetric bone mineral density) by assuming the vertebral body to have the shape of a cube (A) or cylinder (B). Abbreviations: BMAD = vBMD, pA = projected area, h = vertebral height, w = vertebral width, d = vertebral depth. (from Baroncelli et al., Critical Ages and Stages of Puberty in the Accumulation of Spinal and Femoral Bone Mass: The validity of Bone Mass Measurements, 2000)



Furthermore densitometry does not differ between cortical (= 80% component of the total body) and trabecular (= main element of the lumbar spine) bone and does not provide any information about important quality criteria like matrix mineralisation and bone turnover.[47], [45]

All this seems to be the prize for lower radiation because it is not arguable to use the more accurate quantitative computed tomography, which is measuring true volumetric BMD, in children and adolescents in routine follow-up because of the high radiation exposure.

A novel approach on this sector is high-resolution MRI (μMRI) which performs a “virtual bone biopsy” giving a three-dimensional quantitative and qualitative imaging of bone.[35] This method could become a powerful diagnostic tool in the future to assess bone loss and the consecutive risk of fractures, but unfortunately this method is not in standard use yet. Therefore we decided to base our study on densitometry and endorse it with laboratory investigations.

For evaluation of bone mineral density we determined the Z score system as more appropriate than the T score which is usually used for diagnosing postmenopausal or senile osteoporosis. The Z score appears to be more suitable to evaluate the BMD in a child or young adult because it better compensates possible gender and age related influences. Taking the T score as reference seems meaningless in children because it is comparing the patient´s BMD with the

maximum bone mass (= peak bone mass) of an adult which is supposed to be reached one or two years after completion of growth, mostly in the early twenties. [3]. [44], [48], [23]

In this context it has to be considered that the process of gaining proper peak bone mass is closely related to an undisturbed onset of puberty under growth- and sex-hormonal control. Approximately up to one half of total skeletal bone mass is gained during this sensitive period of human development when growth in length is often associated with a delayed growth in width and consolidation, which alone entails a higher risk of fractures. [44], [49]

Therefore results in densitometry should be regarded with caution and also the scoring system itself can lead to artificial misinterpretation because of the possible mismatch concerning height and pubertal status of probands compared with the reference group taken for defining the Z score limits. For example a measured lower bone mineral content in study participants could have also been due to a smaller skeleton with an underestimated BMD as well as tall patients with large bone sizes could have been overestimated. [46], [44] This correlates with our findings of increasing values of bone mineral density with height, weight and body mass index.

Nevertheless, densitometry seems to be a suitable diagnostic tool, but as the evaluation faces the investigator with lots of pitfalls a combination with laboratory examinations appeared to us to be the more appropriate way to assess deficiencies in bone mineral density because it additionally provides important clinical information about the possible causes of BMD reduction.

4.2 Laboratory

Bone metabolism. Bone underlies a steady more or less balanced coupling of formation and resorption, a process which may be classified more accurately by special parameters. *Bone specific alkaline phosphatase* (BAP), *osteocalcin* (OC) and the *N-terminal telopeptide procollagen* (PINP) represent the activity of osteoblasts and bone formation. All those parameters showed an increase above the physiological reference range in our study group. Consequently also the parameters of osteoclast activity, b*eta-crosslaps and tartrate resistant alkaline phosphatase (TRAP),* showed higher serum levels. The rankl-inhibitor *osteoprotegerin*, produced by activated osteoblasts, generally ranged close to the upper borderline or even above with consecutive inverse levels of rankl.

All those markers reflect the simultaneously ongoing process of skeletal modelling and remodelling (= bone turnover) which is supposed to be higher during childhood and adolescence as a consequence of growth and the net-gain of bone. [50] In the therapy of osteoporosis they seem to be more associated with the risk of fractures and the success of antiresorptive treatment than the BMD itself, as well as reduced rates of skeletal remodelling contradictorily seem to correlate with reaching a higher bone mass during puberty. [51], [50]

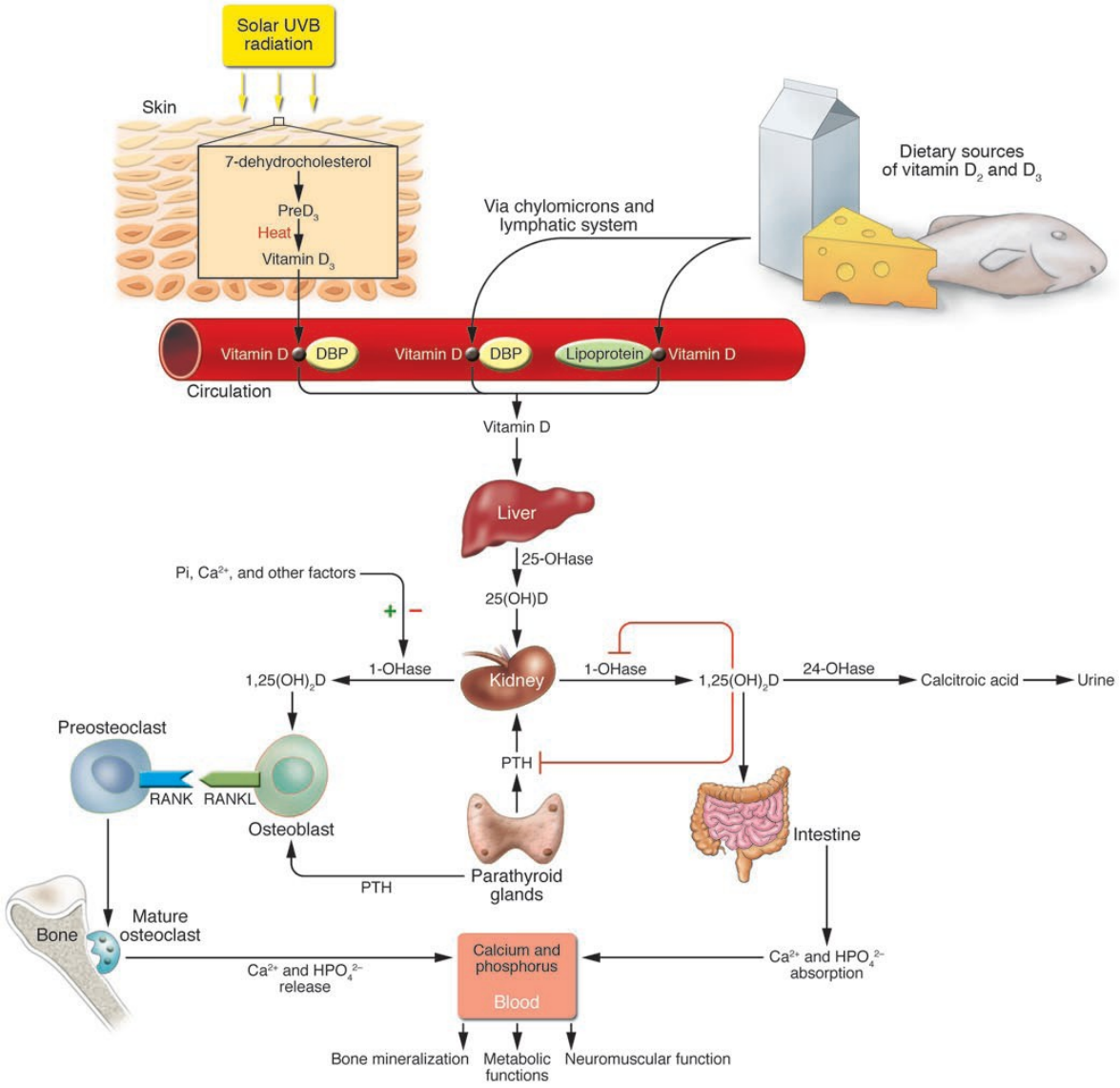
Among our juvenile study participants we anticipated higher levels in those bone specific markers compared to the older ones as it would reflect the growing organism, but we still expected levels within reference range. What we found instead was an increase above the physiological reference limit in more than two third of our patients, concerning children as well as adults, which displays more or less what our densitometry measurements already suggested.

Vitamin D. Searching for possible reasons for this high bone-turnover and under consideration of the compiled data of our laboratory examinations organic diseases with direct or indirect influences on the skeletal system or bone metabolism did not seem to be the main cause. Rather unexpected high deficiency in vitamin D (88%), combined with PTH-levels close to the upper reference limit,

and an unusual high percentage of probands with genetic determination for lactose intolerance (37%) seemed to be the origin of this dysbalances.

Inadequate vitamin D levels are, next to the extreme of developing rickets, closely related to children's inability of attaining their genetically programmed peak bone mass. In adults vitamin D deficiency exacerbates osteoporosis or results in osteomalacia. [52], [53], [54]

Figure 30. Photoproduction and metabolism of Vitamin D in relation to calcium, phosphate and bone metabolism. (from Holick MF, Resurrection of vitamin D deficiency and rickets, 2006)



Sources of vitamin D are on the one hand nourishment like fish and fish oils, egg yolks and special vitamin D fortified products (e.g. margarines, cereals, milk), and on the other hand the endogenous, UVB-dependant (wavelengths of 290-315 nm) production in the skin which shows seasonal variation in latitudes above 37°N and below 37°S (Figure 30). [52]

Due to a wide spread rare nutritional vitamin D supply solar irradiation remains the main source of gaining sufficient vitamin D levels. This presents a problem during the winter season when sunlight is insufficient to induce cutaneous vitamin D_3 synthesis in our latitudes and our body has to resort to its vitamin D stores gained during the summer months, which leads to a seasonal alteration in serum vitamin D in adults as well as in children. [52], [55] Our study was performed in springtime and therefore seasonal vitamin D insufficiency could have contributed to lower results in bone mineral density.

Several investigations indicate a relation between vitamin D deficiencies and decreased duodenal calcium absorption as well as an increase in serum PTH (secondary hyperparathyroidism) with consecutive renal loss of phosphate. This results in a raise in bone-turnover, impaired mineralisation and loss of bone mineral density, going along with higher incidences of fractures.[56], [57], [58], [59] All those seem to correlate with our laboratory and densitometry findings and offer a plausible explanation why bone loss occurred unaffected by gender, age at chemotherapeutic application and the time passed since chemotherapy.

Lactose intolerance. Another discussable result of our study is the high percentage of patients with genetic determination of lactose intolerance (37%) compared to the middle European average of 10-15%, which additionally raises the chance of gaining lower bone mineral density. In this context study findings have not been uniform, but patients´ habit to avoid intolerance symptoms by reducing the consumption of calcium-rich dairy products seem to be more likely to cause lower calcium intake in this population than the lactose intolerance itself. [60], [61], [62], [63]

Our study questionnaire displayed possible imbalances in the amount of calcium regularly consumed by our probands. Although all study participants drank

milk and/or ate other dairy products with supposed high calcium ratio, the consumed calcium amounts showed high variation. Therefore lactose intolerance might have contributed to lower nutritional calcium supplement in some cases triggered by intestinal malabsorption due to insufficient vitamin D levels. [64] Furthermore, although the serum calcium levels of our patients were found within reference range, this is no proof of sufficient nutritional calcium supply. Physiological serum calcium values could also be due to an increased bone mobilisation as a consequence of low calcium intake. This again would correlate with the raise in serum PTH and the increased bone turnover found in about two third of our patients. [64]

GH/IGF-1. One further laboratory finding which correlates with higher bone metabolism was the increase in growth hormone (23%) and insulin-like growth factor 1 (28%). Both play an important role in bone growth regulation including activation of osteoblast differentiation, stimulation of chondrocyte proliferation, modulation of tubular phosphate retention and renal 25-hydroxyvitamin D_3 hydroxylation. Furthermore in adults growth hormone and insulin-like growth factor 1 contribute to the maintenance of bone mass by interfering with the remodelling process. [65], [66], [67]

Excessive increase in GH/IGF-1 and its effects on bone turnover and bone mineral density can be best observed in patients with acromegaly, a disease which serves as a model of hypersomatotropism. [68] It is supposed that in those patients a raise in growth hormone and its liver derived modulator IGF-1 leads to an increase in bone turnover with promotion of bone formation more than bone resorption. [68], [69] In healthy people the secretion of growth hormone reaches its peak level during puberty accompanied by very high circulating IGF-1 levels, both gradually declining during adulthood. [66]

Approximately one third of our study participants presented levels of growth hormone and/or insulin-like growth factor 1 which were slightly increased above reference range, findings which correlate with the diagnosed high bone turnover, but which can also be due to hypoglycaemia due to the overnight fast and possible influences of stress. [70]

Hypogonadism. Last but not least we would like to focus on very important secondary effects of chemotherapeutic treatment which indirectly affect bone mineral density - gonadal dysfunction and fertility problems.

Many chemotherapeutic agents are claimed to have adverse influences on the hypothalamic-pituitary-gonadal axis ending up in hypogonadism and all its further consequences on human metabolism. Especially alkylating drugs as cyclophosphamide, busulfan and melphalan (used for Ewing´s sarcoma treatment) as well as ifosfamide (part of Ewing´s sarcoma and osteosarcoma protocols) are determined as high risk agents concerning their gonadal harmful potential. The platinum analogue Cisplatin (part of osteosarcoma treatment) and doxorubicin (used for both tumour types) rank somewhere in the middle, followed by the low risk plant derivate vincristine, the antibiotic actinomycin-d (both used for Ewing´s sarcoma treatment) and the antimetabolite methotrexate (used for osteosarcoma therapy). In the end not just the type of drug, but also the cumulative dose as well as the combinations used in the multidrug treatment schemes, the patient´s age and pretreatment gonadal status seem to impact the harming effect of chemotherapy on gonadal function. [71]

In females, next to infertility, chemotherapy causes premature ovarian failure which is associated with oestrogen deficiency and a higher risk of osteoporosis. In male patients the chemotherapeutic effect on germ cells seems to be higher than on Leydig cells and therefore the risk of infertility is assumed to be the more aggravating problem than hypogonadism. [71] Nevertheless, the whole collective of patients presented free (=active) testosterone values ranging close to the lower reference limit in correlation with an increase in the pituitary regulating hormones FSH and LH.

Several studies discussed osteoporosis due to perimenopausal hormonal changes in women, but also in men sex hormones, androgens as well as estrogens, show an important influence on bone turnover. The former is supposed to impact more the bone formation and both independently influence bone resorption. [72] Next to alcoholic excess and glucocorticoid treatment hypogonadism presents one of the most common causes of secondary osteoporosis in aging men. [73] Therefore hypogonadism in male cancer survivors

should not be underestimated concerning its effect on bone mineral density, although according to Boot et al. the major determinant of BMD in childhood in boys seems to be weight, whilst in girls pubertal development shows the major impact. [45]

The timing of pubertal changes is strongly dependant on the function of the hypothalamic-pituitary-ovarian system in girls where the onset of estrogenization first leads to a reduction in bone mass, deceleration of growth and GH-decrease, and is then followed by weight gain, growth spurt including a raise in GH, IGF-1 and estradiol and a decline in SHBG (=sexual hormone binding globulin). [74] The latter in general functions as a transport protein of sex hormones and its level is the major inverse determinant of free (active) estrogen and testosterone. [59], [75] Aging leads to a reduction in androgens and hence to a raise in the sexual hormone binding globulin, which showed a significant inverse correlation with bone mineral density (especially in trabecular bone) in elderly women in several studies. [59], [76], [77], [78] SHBG even seems to be the better predictor of bone loss than endogenous estrogen levels in elderly as well as young women. [59], [79], [80]

Interestingly in our study group the average levels of SHBG were well midranged, although the average level of free testosterone was relatively low. An increase was just seen in six women, in which the higher SHBG levels were possibly due to the intake of contraceptives. [70] Of three we definitely know it and the others possibly did not report it in the questionnaire. Three male patients also presented SHBG-levels above reference range, but two of those were prepubertal and one was under antiepileptic treatment, a medication known to side-effect SHBG raise. [70]

4.3 Treatment strategies

In childhood and young adolescence many factors besides chemotherapy are affecting bone mineral density and therefore it is hard to assess the true effect of chemotherapeutic treatment on the skeletal system as the evaluation of our data reasserted. But there are some parameters which are assumed to be important for appropriate bone development also in healthy people, like sufficient vitamin D and calcium supply in combination with physical activity and sensitive sun exposure. [45], [52], [55]

Young patients undergoing cancer treatment experience long time of hospitalisation, possible lack in nourishment and contrary to healthy people of the same age are partially forced to inactivity in a life period of high importance for gaining their genetically programmed peak bone mass. When all this is cumulating with seasonal or permanent vitamin D insufficiency/deficiency in combination with calcium malnutrition plus a possible genetic determination for lactose intolerance, as in 37% of our patients, it might potentiate the direct and indirect bone harming effects of cancer therapy.

Therefore supporting our study participants with sufficient vitamin D and calcium can provide an easy, efficient and cost-saving first step to prevent later osteoporosis which is probably to occur earlier in this cohort of patients than in the average population and might additionally be accompanied by further skeletal related events like pathologic fractures.

Whilst in primary tumour treatment benefits of bisphosphonates (e.g. zoledronic acid) may overweigh possible adverse events like renal dysfunction and jaw-necrosis, especially in comparison to other chemotherapeutic agents, [81] we do not welcome bisphosphonates as first line therapy in childhood bone deficiencies as long as less toxic strategies are applicable.

4.4 Limitations of the study

The small number of participating patients as well as the different time intervals between chemotherapeutic application and investigation represent the major limitations of our study. Furthermore densitometry measurement of the tumour unaffected femoral side was not possible in six probands who therefore could not be involved in part of our evaluation. Additionally our study was set in springtime when seasonal vitamin D insufficiency may have worsened the results and overshadowed the influence of chemotherapy itself, but due to the multiple treatment modalities this seems to be a desperate intention anyway.

4.5 Conclusion

Our study results indicate that a reduction in bone mineral density in young childhood cancer survivors presents a possible threat not only in osteosarcoma patients treated with high-dose methotrexate, but also in patients with Ewing´s sarcoma. Therefore preventive after-care treatment should be part of a sensitive patients´ management.

Due to the results of our investigation consultation and adequate treatment for their bone deficits were offered to our study participants by the Division of Endocrinology and Metabolism (Department of Internal Medicine, Medical University of Graz) and we hope to sensitise further clinicians for the possible threat of premature bone loss in young survivors of bone sarcoma!

5 REFERENCES

1. **Kaste SC, Chesney RW, Hudson MM, Lustig RH, Rose SR, Carbone LD.** Bone mineral status during and after therapy of childhood cancer: an increasing population with multiple risk factors for impaired bone health. *J Bone Miner Res 1999 Dec; 14(12):2002-2009.*

2. **Pfeilschifter J, Diel IJ.** Osteoporosis due to cancer treatment: pathogenesis and management. *J Clin Oncol 2000 Apr; 18(7):1570-1593. Review.*

3. **Sala A, Barr RD.** Osteopenia and cancer in children and adolescents: the fragility of success. *Cancer 2007; 109(7):1420-1431.*

4. **van der Sluis IM, van den Heuvel-Eibrink MM.** Osteoporosis in children with cancer. *Pediatr Blood Cancer 2008; 50:474-478.*

5. **Wasilewski-Masker K, Kaste SC, Hudson MM, Esiashvili N, Mattano LA, Meacham LR.** Bone mineral density deficits in survivors of childhood cancer: long-term follow-up guidelines and review of the literature. *Pediatrics 2008 Mar; 121(3):e705-e713.*

6. **Ragab AH, Frech RS, Vietti TJ.** Osteoporotic fractures secondary to methotrexate therapy of acute leukemia in remission. *Cancer 1970 Mar; 25(3):580-585.*

7. **O'Regan S, Melhorn DK, Newman AJ.** Methotrexate-induced bone pain in childhood leukemia. *Am J Dis Child 1973 Oct; 126(4):489-490.*

8. **Stanisavljevic S, Babcock AL.** Fractures in children treated with methotrexate for leukemia. *Clin Orthop Relat Res 1977 Jun; (125):139-144.*

9. **Gnudi S, Butturini L, Ripamonti C, Avella M, Bacci G.** The effects of methotrexate (MTX) on bone. A densitometric study conducted on 59 patients with MTX administered at different doses. *Ital J Orthop Traumatol 1988 Jun; 14(2):227-231.*

10. **Ecklund K, Laor T, Goorin A M, Connolly L P, Jaramillo D.** Methothrexate osteopathy in patients with osteosarcoma. *Radiology 1997; 202:543-547.*

11. **Holzer G, Krepler P, Koschat MA, Grampp S, Dominkus M, Kotz R.** Bone mineral density in long-term survivors of highly malignant osteosarcoma. *J Bone Joint Surg (Br) 2003; 85-B:231-237.*

12. **Ruza E, Sierrasesúmaga L, Azcona C, Patiño-Garcia A.** Bone mineral density and bone metabolism in children treated for bone sarcomas. *Pediatr Res 2006 Jun; 59(6):866-871. Epub 2006 Apr 26.*

13. **Müller C, Winter CC, Rosenbaum D, Boos J, Gosheger G, Hardes J, Vieth V.** Early decrements in bone density after completion of neoadjuvant chemotherapy in pediatric bone sarcoma patients. *BMC Musculoskelet Disord 2010 Dec; 11(1):287.*

14. **IARC, International Agency for Research on Cancer.** *World Health Organization Classification of Tumours; IARC Press Lyon 2002.*

15. **Böcker W, Denk H, Heitz PU.** Pathologie. *Elsevier GmbH München 2004.*

16. **Berger DP, Engelbrecht R, Mertelsmann R.** Das Rote Buch - Hämatologie und Internistische Onkologie. *Verlagsgruppe Hüthig Jehle Rehm GmbH 2006.*

17. **Wülker N.** Orthopädie und Unfallchirurgie. *Georg Thieme Verlag 2005.*

18. **Carrle D, Bielack SS.** Current strategies of chemotherapy in osteosarcoma. *International Orthopaedics (SICOT) 2006; 30:445-451. Review.*

19. **ESMO/EUROBONET Working Group, Athanasou N, Bielack S, De Alava E, Tos AP, Ferrari S, Gelderblom H, Grimer R, Hall KS, Hassan B, Hogendoorn PC, Jurgens H, Paulussen M, Rozeman L, Taminiau AH, Whelan J, Vanel D.** Bone sarcomas: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol 2010 May; 21 Suppl 5:204-213.*

20. **Dunst J, Ahrens S, Paulussen M, Rübe C, Winkelmann W, Zoubek A, Harms D, Jürgens H.** Second malignancies after treatment for Ewing's sarcoma: a report of the CESS-studies. *Int J Radiat Oncol Biol Phys 1998 Sep 1; 42(2):379-384.*

21. **Paulussen M, Craft AW, Lewis I, Hackshaw A, Douglas C, Dunst J, Schuck A, Winkelmann W, Köhler G, Poremba C, Zoubek A, Ladenstein R, van den Berg H, Hunold A, Cassoni A, Spooner D, Grimer R, Whelan J, McTiernan A, Jürgens H und EICOSS-92.** Results of the EICESS-92 Study: two randomized trials of Ewing's sarcoma treatment--cyclophosphamide compared with ifosfamide in standard-risk patients and assessment of benefit of etoposide added to standard treatment in high-risk patients. *J Clin Oncol 2008 Sep 20; 26(27):4385-4393.*

22. **Kager L, Zoubek A, Dominkus M, Lang S, Bodmer N, Jungt G, Klingebiel T, Jürgens H, Gadner H, Bielack S.** Osteosarcoma in very young children. *Cancer 2010 (accepted January 20, 2010); 000:000-000. Published online in WileyInterScience (wileyonlinelibrary.com).*

23. **Boot AM, de Ridder MA, van der Sluis IM, van Slobbe I, Krenning EP, Keizer-Schrama SM.** Peak bone mineral density, lean body mass and fractures. *Bone 2010 Feb; 46(2):336-341. Epub 2009 Oct 13.*

24. **Davies JH, Evans BA, Jenney ME, Gregory JW.** Effects of chemotherapeutic agents on the function of primary human osteoblast-like cells derived from children. *J Clin Endocrinol Metab 2003 Dec; 88(12):6088-6097.*

25. **van der Veen MJ, Scheven BA, van Roy JL, Damen CA, Lafeber FP, Bijlsma JW.** In vitro effects of methotrexate on human articular cartilage and bone-derived osteoblasts. *Br J Rheumatol 1996 Apr; 35(4):342-349.*

26. **Herold G, et al.** Innere Medizin. *Köln 2008.*

27. **Gilsanz V.** Bone density in children: a review of the available techniques and indications. *Eur J Radiol 1998 Jan; 26(2):177-182.*

28. **Baroncelli GI, Bertelloni S, Sodini F, Saggese G.** Osteoporosis in children and adolescents: etiology and management. *Paediatr Drugs 2005; 7(5):295-323.*

29. **Arikoski P, Komulainen J, Riikonen P, Voutilainen R, Knip M, Kröger H.** Alterations in bone turnover and impaired development of bone mineral density in newly diagnosed children with cancer: a 1-year prospective study. *J Clin Endocrinol Metab 1999 Sep; 84(9):3174-3181.*

30. **Minaire P.** Immobilization osteoporosis: a review. *Clin Rheumatol 1989 Jun; 8 Suppl 2:95-103.*

31. **Sievänen H.** Immobilization and bone structure in humans. *Arch Biochem Biophys 2010 Nov 1; 503(1):146-152. Epub 2010 Jul 14. Review.*

32. **Rittweger J, Simunic B, Bilancio G, De Santo NG, Cirillo M, Biolo G, Pisot R, Eiken O, Mekjavic IB, Narici M.** Bone loss in the lower leg during 35 days of bed rest is predominantly from the cortical compartment. *Bone 2009 Apr; 44(4):612-618. Epub 2009 Jan 9.*

33. **Hopewell JW.** Radiation-therapy effects on bone density. *Med Pediatr Oncol 2003 Sep; 41(3):208-211. Review.*

34. **Kaste SC.** Bone-mineral density deficits from childhood cancer and its therapy. *Pediatr Radiol 2004; 34:373-378. Review.*
35. **Guise TA.** Bone loss and fracture risk associated with cancer therapy. *Oncologist 2006 Nov-Dec; 11(10):1121-1131. Review.*
36. **Kaste SC.** Skeletal Toxicities of Treatment in Children with Cancer. *Pediatr Blood Cancer 2008; 50:469-473.*
37. **Brown SA, Guise TA.** Cancer treatment-related bone disease. *Crit Rev Eukaryot Gene Expr 2009; 19(1):47-60. Review.*
38. **Lipton A.** Implications of bone metastases and the benefits of bone-targeted therapy. *Semin Oncol 2010 Oct; 37 Suppl 2:S15-S29.*
39. **Odri GA, Dumoucel S, Picarda G, Battaglia S, Lamoureux F, Corradini N, Rousseau J, Tirode F, Laud K, Delattre O, Gouin F, Heymann D, Redini F.** Zoledronic acid as a new adjuvant therapeutic strategy for Ewing's sarcoma patients. *Cancer Res 2010 Oct 1; 70(19):7610-7619. Epub 2010 Sep 14.*
40. **Labrinidis A, Hay S, Liapis V, Findlay DM, Evdokiou A.** Zoledronic acid protects against osteosarcoma-induced bone destruction but lacks efficacy against pulmonary metastases in a syngeneic rat model. *Int J Cancer. 2010 Jul 15; 127(2):345-54.*
41. **Dirksen U, Jürgens H.** Approaching Ewing sarcoma. *Future Oncol 2010 Jul; 6(7):1155-1162. Review.*
42. **Zhou Z, Guan H, Duan X, Kleinerman ES.** Zoledronic acid inhibits primary bone tumor growth in Ewing sarcoma. *Cancer 2005 Oct 15; 104(8):1713-1720.*
43. **Labrinidis A, Hay S, Liapis V, Ponomarev V, Findlay DM, Evdokiou A.** Zoledronic acid inhibits both the osteolytic and osteoblastic components of osteosarcoma lesions in a mouse model. *Clin Cancer Res 2009 May 15; 15(10):3451-3461. Epub 2009 Apr 28.*
44. **Baroncelli GI, Saggese G.** Critical ages and stages of puberty in the accumulation of spinal and femoral bone mass: the validity of bone mass measurements. *Horm Res 2000; 54 Suppl 1:2-8.*
45. **Boot AM, de Ridder MA, Pols HA, Krenning EP, de Muinck Keizer-Schrama SM.** Bone mineral density in children and adolescents: relation to puberty, calcium intake, and physical activity. *J Clin Endocrinol Metab 1997 Jan; 82(1):57-62.*

46. **Mølgaard C, Thomsen BL, Michaelsen KF.** Influence of weight, age and puberty on bone size and bone mineral content in healthy children and adolescents. *Acta Paediatr 1998 May; 87(5):494-499.*

47. **Bacchetta J, Boutroy S, Vilayphiou N, Fouque-Aubert A, Delmas PD, Lespessailles E, Fouque D, Chapurlat R.** Assessment of Bone Microarchitecture in Chronic Kidney Disease: A Comparison of 2D Bone Texture Analysis and High-Resolution Peripheral Quantitative Computed Tomography at the Radius and Tibia. *Calcif Tissue Int 2010 Aug 15.*

48. **Lu PW, Briody JN, Ogle GD, Morley K, Humphries IR, Allen J, Howman-Giles R, Sillence D, Cowell CT.** Bone mineral density of total body, spine, and femoral neck in children and young adults: a cross-sectional and longitudinal study. *J Bone Miner Res 1994 Sep; 9(9):1451-1458.*

49. **Parfitt AM.** The two faces of growth: benefits and risks to bone integrity. *Osteoporos Int 1994 Nov; 4(6):382-398.*

50. **Slemenda CW, Peacock M, Hui S, Zhou L, Johnston CC.** Reduced rates of skeletal remodeling are associated with increased bone mineral density during the development of peak skeletal mass. *J Bone Miner Res 1997 Apr; 12(4):676-682.*

51. **Garnero P.** Biomarkers for osteoporosis management: utility in diagnosis, fracture risk prediction and therapy monitoring. *Mol Diagn Ther 2008; 12(3):157-170. Review.*

52. **Holick MF.** High prevalence of vitamin D inadequacy and implications for health. *Mayo Clin Proc 2006 Mar; 81(3):353-573. Review.*

53. **Unuvar T, Buyukgebiz A.** Nutritional rickets and vitamin D deficiency in infants, children and adolescents. *Pediatr Endocrinol Rev 2010 Mar-Apr; 7(3):283-291.*

54. **Outila TA, Kärkkäinen MU, Lamberg-Allardt CJ.** *Vitamin D status affects serum parathyroid hormone concentrations during winter in female adolescents: associations with forearm bone mineral density.* Am J Clin Nutr 2001 Aug; 74(2):206-210.

55. **Docio S, Riancho JA, Pérez A, Olmos JM, Amado JA, González-Macías J.** Seasonal deficiency of vitamin D in children: a potential target for osteoporosis-preventing strategies? *J Bone Miner Res 1998 Apr; 13(4):544-548.*

56. **Pasco JA, Henry MJ, Kotowicz MA, Sanders KM, Seeman E, Pasco JR, Schneider HG, Nicholson GC.** Seasonal periodicity of serum vitamin D and parathyroid hormone, bone resorption, and fractures: the Geelong Osteoporosis Study. *J Bone Miner Res 2004 May; 19(5):752-758. Epub 2004 Jan 19.*

57. **Dawson-Hughes B, Dallal GE, Krall EA, Harris S, Sokoll LJ, Falconer G.** Effect of vitamin D supplementation on wintertime and overall bone loss in healthy postmenopausal women. *Ann Intern Med 1991 Oct 1; 115(7):505-512.*

58. **Krall EA, Sahyoun N, Tannenbaum S, Dallal GE, Dawson-Hughes B.** Effect of vitamin D intake on seasonal variations in parathyroid hormone secretion in postmenopausal women. *N Engl J Med 1989 Dec 28; 321(26):1777-1783.*

59. **Ooms ME, Lips P, Roos JC, van der Vijgh WJ, Popp-Snijders C, Bezemer PD, Bouter LM.** Vitamin D status and sex hormone binding globulin: determinants of bone turnover and bone mineral density in elderly women. *J Bone Miner Res 1995 Aug; 10(8):1177-1184.*

60. **Jackson KA, Savaiano DA.** Lactose maldigestion, calcium intake and osteoporosis in African-, Asian-, and Hispanic-Americans. *J Am Coll Nutr 2001 Apr; 20(2 Suppl):198S-207S. Review.*

61. **Kull M, Kallikorm R, Lember M.** Impact of molecularly defined hypolactasia, self-perceived milk intolerance and milk consumption on bone mineral density in a population sample in Northern Europe. *Scand J Gastroenterol 2009; 44(4):415-421.*

62. **Honkanen R, Pulkkinen P, Järvinen R, Kröger H, Lindstedt K, Tuppurainen M, Uusitupa M.** Does lactose intolerance predispose to low bone density? A population-based study of perimenopausal Finnish women. *Bone 1996 Jul; 19(1):23-28.*

63. **Bob A, Bob K et al.** Innere Medizin. *Thieme Verlag Stuttgart 2009.*

64. **Segal E, Dvorkin L, Lavy A, Rozen GS, Yaniv I, Raz B, Tamir A, Ish-Shalom S.** *Bone density in axial and appendicular skeleton in patients with lactose intolerance: influence of calcium intake and vitamin D status.* J Am Coll Nutr 2003 Jun; 22(3):201-207.

65. **Giustina A, Mazziotti G, Canalis E.** Growth hormone, insulin-like growth factors, and the skeleton. *Endocr Rev 2008 Aug; 29(5):535-559. Epub 2008 Apr 24.*

66. **Perrini S, Laviola L, Carreira MC, Cignarelli A, Natalicchio A, Giorgino F.** The GH/IGF1 axis and signaling pathways in the muscle and bone: mechanisms underlying age-related skeletal muscle wasting and osteoporosis. *J Endocrinol 2010 Jun; 205(3):201-210. Epub 2010 Mar 2. Review.*
67. **Ueland T.** Bone metabolism in relation to alterations in systemic growth hormone. *Growth Horm IGF Res 2004 Dec; 14(6):404-417.*
68. **Ueland T, Fougner SL, Godang K, Schreiner T, Bollerslev J.** Serum GH and IGF-I are significant determinants of bone turnover but not bone mineral density in active acromegaly: a prospective study of more than 70 consecutive patients. *Eur J Endocrinol 2006 Nov; 155(5):709-715.*
69. **Bolanowski M, Daroszewski J, Medraś M, Zadrozna-Sliwka B.** Bone mineral density and turnover in patients with acromegaly in relation to sex, disease activity, and gonadal function. *J Bone Miner Metab 2006; 24(1):72-78.*
70. **Neumeister B, Besenthal I, Böhm BO.** Klinikleitfaden Labordiagnostik. *Urban & Fischer 2009.*
71. **Brydøy M, Fosså SD, Dahl O, Bjøro T.** Gonadal dysfunction and fertility problems in cancer survivors. *Acta Oncol 2007; 46(4):480-489. Review.*
72. **Leder BZ, LeBlanc KM, Schoenfeld DA, Eastell R, Finkelstein JS.** Differential effects of androgens and estrogens on bone turnover in normal men. *J Clin Endocrinol Metab 2003 Jan; 88(1):204-210.*
73. **Kamel HK.** Male osteoporosis: new trends in diagnosis and therapy. *Drugs Aging 2005; 22(9):741-748. Review.*
74. **Blogowska A, Rzepka-Górska I, Krzyzanowska-Swiniarska B.** Growth hormone, IGF-1, insulin, SHBG, and estradiol levels in girls before menarche. *Arch Gynecol Obstet 2003 Oct; 268(4):293-296. Epub 2002 Oct 2.*
75. **Selby C.** Sex hormone binding globulin: origin, function and clinical significance. *Ann Clin Biochem 1990 Nov; 27(Pt 6):532-541. Review.*
76. **Wild RA, Buchanan JR, Myers C, Lloyd T, Demers LM.** Adrenal androgens, sex-hormone binding globulin and bone density in osteoporotic menopausal women: is there a relationship? *Maturitas 1987 Apr; 9(1):55-61.*
77. **Brody S, Carlström K, Lagrelius A, Lunell NO, Möllerström G, Pousette A.** Serum sex hormone binding globulin (SHBG), testosterone/SHBG index,

endometrial pathology and bone mineral density in postmenopausal women. *Acta Obstet Gynecol Scand 1987; 66(4):357-360.*

78. **Rapuri PB, Gallagher JC, Haynatzki G.** Endogenous levels of serum estradiol and sex hormone binding globulin determine bone mineral density, bone remodeling, the rate of bone loss, and response to treatment with estrogen in elderly women. *J Clin Endocrinol Metab 2004 Oct; 89(10):4954-4962.*

79. **van Hemert AM, Birkenhäger JC, De Jong FH, Vandenbroucke JP, Valkenburg HA.** Sex hormone binding globulin in postmenopausal women: a predictor of osteoporosis superior to endogenous oestrogens. *Clin Endocrinol (Oxf) 1989 Oct; 31(4):499-509.*

80. **Daniel M, Martin AD, Drinkwater DT.** Cigarette smoking, steroid hormones, and bone mineral density in young women. *Calcif Tissue Int 1992 Apr; 50(4):300-305.*

81. **Coleman R, Burkinshaw R, Winter M, Neville-Webbe H, Lester J, Woodward E, Brown J.** Zoledronic acid. *Expert Opin Drug Saf 2010 Nov 29.*

APPENDIX – Abstracts, Posters, Presentations

Osteoporosis – a late effect after chemotherapy for bone sarcomas. Pirker-Frühauf U, Leithner A, Windhager R. Abstract book of the 22nd EMSOS meeting, 172:4.P.25; May 13-16, 2009, Stuttgart, GERMANY. [Poster]

Reduction of bone mineral density after chemotherapy for bone sarcomas. Pirker-Frühauf U, Obermayer-Pietsch B, Windhager R, Leithner A. Abstract book of the 23rd EMSOS meeting, 70:7.7; May 5-7, 2010, Birmingham, UK. [Oral Presentation]

Osteoporose als Langzeitfolge der Chemotherapie bei Patienten mit malignen Knochentumoren. Pirker-Frühauf U, Leithner A, Maurer-Ertl W, Windhager R. Abstract CD des DGOU 2010; PO20-1188, Berlin, GERMANY [Poster and Short Oral Presentation]

Premature bone loss in Ewing´s sarcoma and osteosarcoma patients after chemotherapeutic treatment. Pirker-Frühauf UM, Obermayer-Pietsch B, Leithner A. Abstract accepted for the AAOS meeting 2011, San Diego, US. [Poster]