Diplomarbeit

Bone marrow aspirations in Ewing sarcomas: are they still necessary?

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Zusammenfassung

Hintergrund: Derzeit ist das Vorhandensein von Metastasen bei Diagnosestellung einer der nützlichsten prognostischen Indikatoren bei Ewing Sarkomen (ES). Laut klinischen Leitlinien, wie den 2012 ESMO medizinischen Leitlinien und dem EURO Ewing 99 Studienprotokoll von 2006, ist, neben anderen bildgebenden Untersuchungen die Durchführung von Knochenmarksaspirationen und Biopsien im Staging Prozess bei ES vorgeschrieben. Allerdings ist der prognostische Wert dieser Ergebnisse kontrovers diskutiert. Daher war das primäre Ziel dieser retrospektiven Studie nachträglich Knochenmarksproben von Patienten mit ES zu analysieren. Als zweites Ziel**,** wurden die zu diesem Thema bereits veröffentlichten Studien gesucht. **Material und Methoden:** Diese retrospektive Studie umfasste 31 Patienten, bei denen zwischen 2000 und 2014 die Erstdiagnose eines ES gestellt wurde und die deshalb an der Abteilung für Orthopädie und orthopädische Chirurgie an der Universitätsklinik Graz behandelt wurden. Bei 5/31 Patienten waren zum Zeitpunkt der Diagnose bereits Metastasen vorhanden. Die Knochenmarkproben wurden aus dem Beckenkamm gewonnen und morphologisch sowie immunhistochemisch durch die Pathologen untersucht. Diese Befunde wurden auf das Vorhandensein oder Fehlen von Knochenmarksmetastasen durchsucht. Ferner waren in 15/31 Patienten die Proben noch vorhanden und wurden am Institut für Pathologie an der Universitätsklinik Graz mittels nested PCR erneut analysiert. Sekundär wurde in der PubMed-Datenbank nach relevanter Literatur gesucht. **Ergebnisse:** Morphologisch und immunhistochemisch gab es bei keinem der 31 Patienten Anzeichen für eine Knochenmarksinfiltration durch das bekannte ES, sogar alle Befunde der 5 metastatischen Patienten waren negativ. Die nested-PCR Ergebnisse waren ebenfalls in allen der 15 erneut getesteten Patienten negativ. 15 relevante Studien, mit unterschiedlichem Fazit, wurden in PubMed gefunden und zusammengefasst. **Fazit:** Unseren Ergebnissen zufolge und aufgrund der Erkenntnisse von Kopp et al. würden wir, wie Anderson und Valvi et al., das Weglassen von Knochenmarksaspirationen und Biopsien im Staging Prozess bei neu diagnostizierten Kindern und jungen Erwachsenen mit ES empfehlen.

Abstract

Background: Currently, one of the most useful prognostic indicators in Ewing sarcomas (ES) is the presence of metastatic disease at diagnosis. According to clinical guidelines, including the 2012 ESMO clinical practice guidelines and the EURO Ewing 99 study protocol from 2006, the assessment of bone marrow (BM) metastases, using light microscopically examination of bone marrow aspirates and biopsies (BMAB) is, besides other imaging investigations, mandatory. However, the prognostic value of BM positivity is discussed controversially. Therefore, the primary objective of this study was to retrospectively review BM samples from patients with ES and, as second objective, to review published literature concerning BM examination in ES. **Materials and Methods:** This retrospective study included 31 ES patients that were newly diagnosed between 2000 and 2014 and that have therefore been treated at the Department for Orthopaedics and Orthopaedic Surgery at the University Hospital of Graz. Metastases at diagnosis were present in 5/31 patients. BM samples were collected from the iliac crest and were morphologically and immunohistochemically examined by the pathologists. These findings were searched and screened for the presence or absence of BM metastases. Furthermore, in 15 of the 31 patients BM samples were still available and were reanalysed at the Department of Pathology at the University Hospital of Graz, using nested PCR. Secondary, PubMed database was searched for relevant literature. **Results:** Our study did not show BM involvement at diagnosis in any of our 31 ES patients, neither morphologically, nor immunohistochemically, not even in the 5 patients with metastatic disease at diagnosis. The nested PCR results were also negative in all of our 15 retested patients. 15 relevant studies, showing different results, were found and summarized. **Conclusion:** According to our results and due to the previous findings of Kopp et al. we would, like Anderson and Valvi et al., also suggest the elimination of BMAB in the initial staging process of newly diagnosed paediatric and young adult ES patients.

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Abbreviations

ACT: actinomycin-D

AJCC: American Joint Committee on Cancer

ARMS: pediatric alveolar rhabdomyosarcoma

BM: bone marrow

BMA: bone marrow aspiration

BMAB: bone marrow aspiration and biopsy

BMB: bone marrow biopsy

CD99: Cluster of differentiation antigen 99

CD57: Cluster of differentiation antigen 57

c-DNA: complementary deoxyribonucleic acid

CPA: cyclophosphamide

CT: chest computerized tomography

DFS: disease free survival

DNA: deoxyribonucleic acid

DSRCT: desmoplastic small round cell tumors

DXR: doxorubicin

EFS: event free survival

ES: Ewing sarcoma

ESFT: Ewing sarcoma family of tumors

FDG-PET: F18-fluorodeoxy-D-glucose-positron-emission tomography

FFPE: formalin-fixed paraffin embedded

HDCT: high-dose chemotherapy

HPRT: hypoxanthine-guanine phosphoribosyl transferase

IFM: ifosfamide

LDH: lactate dehydrogenase

MIC2: microneme protein 2

MPFC: multiparameter flow cytometry

MRI: magnetic resonance imaging

NSE: neuronal specific enolase

OS: overall survival

PAS: periodic acid-Schiff stain

PB: peripheral blood

PBSC: peripheral blood stem cell collections

PET scan: positron emission tomography

PFS: progression free survival

PNET: peripheral neuroectodermal tumor

RNA: ribonucleic acid

ROC: receiver operating characteristic

RSF: relapse-free survival

RT-PCR: reverse transcriptase-polymerase chain reaction

SCT: stem cell transplantation

VCR: vincristine

VIDE: vincristine, ifosfamide, doxorubicin and etoposide

VP16: etoposide

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General Part

1 Ewing sarcoma

1.1 Definition

Ewing sarcoma (ES) is the second most common primary malignant bone tumour in children and young adults and belongs to the Ewing sarcoma family of tumours (ESFT), a group of tumours that is also known as so called small round tumours of childhood.
This group includes ES, primitive neuroectodermal tumour (PNET) and Askin tumour. They are all characterized by common chromosomal translocations that result in the fusion of the EWS gene on chromosome 22q12 with different ETS related genes (1,2).
All Ewing sarcomas are high grade tumours (3).

1.2 History

In 1918, Arthur P. Stout reported a case of a tumour in the ulnar nerve of a 42 year old man which was composed of undifferentiated round cells forming rosettes. This may have been the first description of a primitive neuroectodermal tumour (PNET).
Ewing sarcoma is named after James Stephen Ewing, who was describing this tumour in 1921. He reported a case of a round cell tumour in the radius of a 14 year old girl as a “diffuse endothelioma of bone” (1)
James Ewing (1866-1943) was active in many fronts. He was an American pathologist and oncologist and helped to found, in 1907, the “American Association for Cancer Research” and in 1913, the “American Society for the Control of Cancer”, now known as “The American Cancer Society”. He was also responsible for the creation of present-day “Memorial Sloan Kettering Cancer Centre” in New York City, one of the most important multidisciplinary centres devoted to oncology in the world. In January 1931 he was on the cover of “Time magazine” titled as the “cancer man” (2).

Figure 1 James S. Ewing on the Time magazine.

1.3 Incidence and Epidemiology

Primary bone tumours are rare, accounting for 5% of all cancers in children and adolescents (4). Osteosarcoma is, with an incidence of 0.2-0.3/100 000/year the most frequent primary cancer of bone, with a higher incidence in adolescents at an age of 15-19 years (0.8-1.1/100 000/year) (4). Chondrosarcoma is the second most frequent bone tumour and, in the adulthood, the most frequent bone tumour with an incidence of 0.2/100 000/year (4).

Ewing sarcoma is the third most common bone tumour, but the second most common occurring in the childhood and in adolescents and is accounting for approximately 3% of all paediatric malignancies and 10% of all primary malignant bone tumours (3). ES seldom occur in adults, but if so, especially the rare extra skeletal variety is seen. ES is diagnosed in white Caucasians at an incidence of 0.3/100 000/year, but is very uncommon in the African and Asian population (3).

ES may be diagnosed at a wide range of ages from infants to the elderly, but the median age at diagnosis is 15 years (1). The peak incidence is during the second decade of life. Patient age has diagnostic importance. In patients older than 30 years tumours like small cell carcinoma of the lung and large cell lymphoma need to be ruled out. In patients under 5 years metastatic leukaemia, rhabdomyosarcoma, medulloblastoma and neuroblastoma must be considered. ES is slightly more common in males than in females, with a ratio of 1.5:1 (1,4,5).

Patient Group	Incidence Rate (per 10^6 persons)	
	Ewing Sarcoma	PNET
All races		
Boys	4.0	0.5
Girls	2.8	0.6
White race		
Boys	4.6	0.6
Girls	3.2	0.7
Other races		
Boys	3.1	~
Girls	~	~

*From U.S. Cancer Statistics Working Group. *United States Cancer Statistics: 1999–2001 Incidence and Mortality Web-based Report Version.* Atlanta (GA): Department of Health and Human Services, Centers for Disease Control and Prevention, and National Cancer Institute, 2004.

~ Numbers are suppressed due to low incidence.

PNET, primitive neuroectodermal tumor.

Figure 2 Incidence Rates in Ewing sarcomas (Khoury et al. 2005).

1.4 Signs and Symptoms

The most common presenting and earliest symptom for ES is locoregional pain, which can be intermittent and show off in variable intensity. Often the pain does not disappear during the night. It can also be mistaken for "bone growth" as most of the ES patients are diagnosed in their adolescents (6).

Paraspinal tumours are often associated with back pain and neurologic symptoms may be present. Besides pain, large pelvic tumours may lead to signs of bowel or bladder disturbances (1). Following this, tumour growth may get visible as swelling or palpable mass in about 1/3 of patients. This swelling is rapidly increasing and occurs as a tense, elastic, hard, tender mass with local heat. If the tumour bulk is localized in the pelvis or chest wall it may be indiscernible for a longer period of time (6).

About 1/3 of patients are presenting with systemic signs and symptoms, including fever, loss of appetite and weight, anaemia and nonspecific signs of inflammation, such as increasing

sedimentation rate, moderate leucocytosis and an increase in serum lactate dehydrogenase (LDH) (7). In advanced and/or metastatic cases, those symptoms are frequent. Differential diagnosis include osteomyelitis, which can be distinguished by the missing palpable mass, the occurrence of systemic illness and of course by biopsy evaluation of all suspected sites (1,8).

A delay between the duration of symptoms prior to diagnosis is common. Delays between weeks and months have been reported, with a median of 4 to 6 months (7). Especially in pelvic tumours a longer delay is common, because the mass is not visible until it becomes rather large. The initial misdiagnosis varies along different age groups. Older patients are most frequently misdiagnosed with tendinitis (21%) and sciatica (11%), whereas in young patients it is coxitis simplex (9%) and osteomyelitis (6%) (7,8).

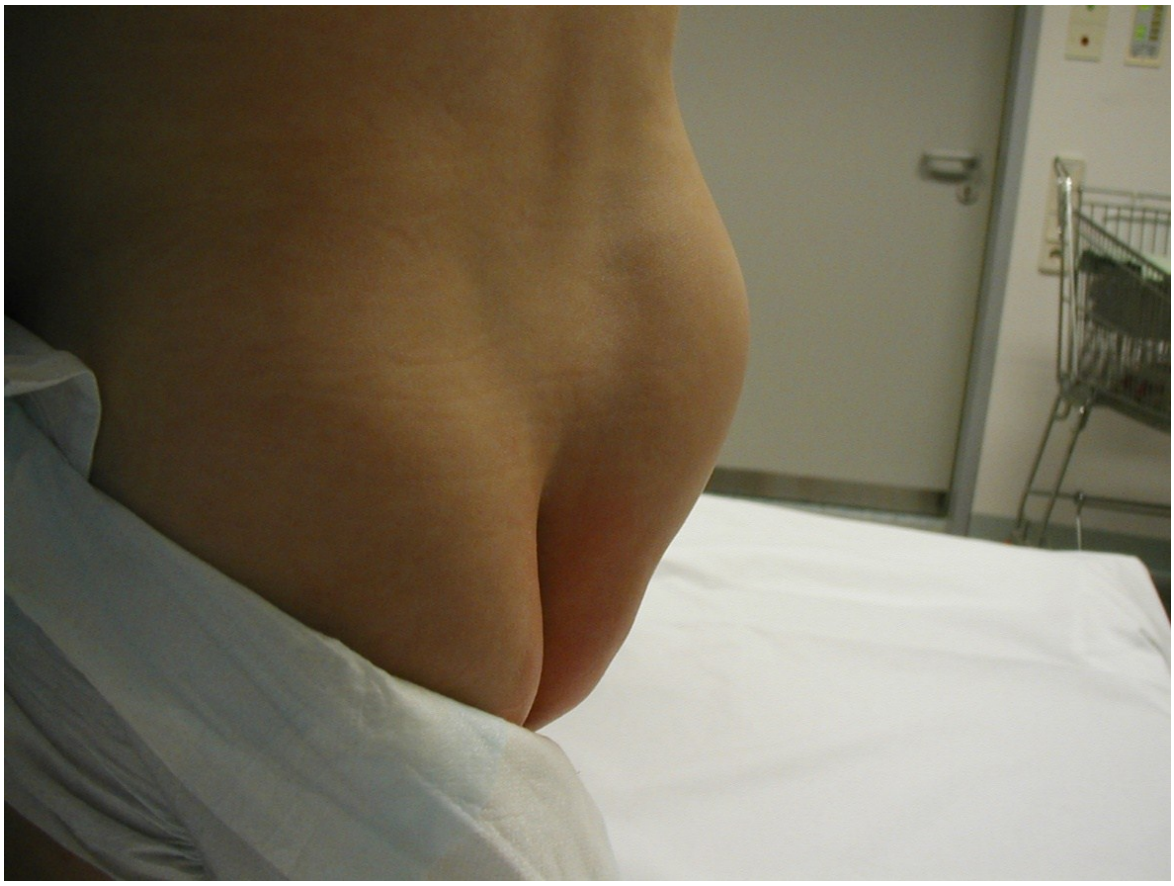

Figure 3 Visible swelling of an ES at the right pelvic site of a 2-year old boy.

1.5 Localization

ES mainly occurs in bones and almost any bone can be affected (1).
Soft tissue involvement is rare, with about 10% of all cases, but it is more likely seen in adults. An analysis of patients with <40 years of age reported a frequency of extra skeletal soft tissue ES in about 31%. The most common extra skeletal sites are the chest wall, para-vertebral muscles, extremities, buttocks and the retro-peritoneal space (7,9).
ES shows a predilection for the trunk and long bones. The axial skeleton is affected in about 50%, with about 25% arising in the pelvis alone, followed by the scapula, vertebral column, ribs and clavicle (7,8). 46% of the tumours occur in long bones, where they typically arise from the diaphysis. About 20% of them affect the femur, followed by the humerus, tibia and bones of the forearm (7,8).

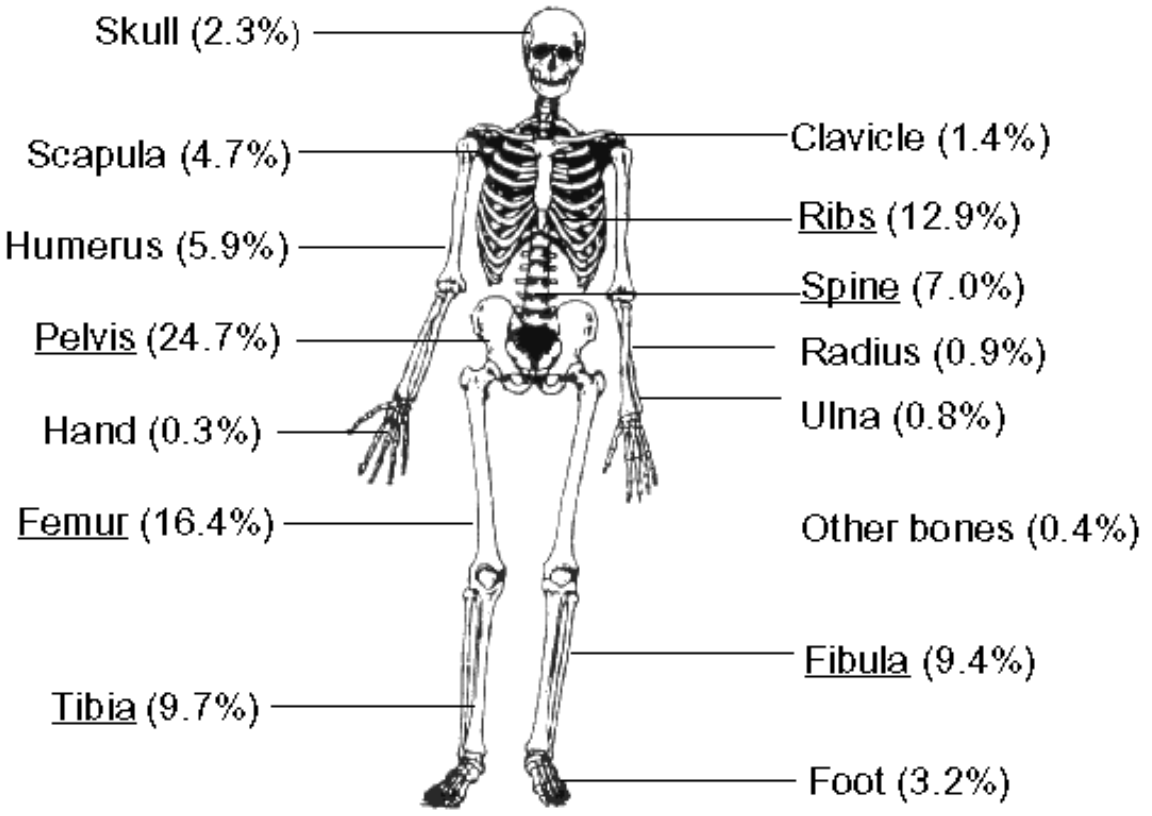

Figure 4 Localization of skeletal ES (Cotteril et al. 2015).

1.6 Diagnosis

1.6.1 Macroscopy

Ewing sarcomas present as grey- white tumours and often show a variable amount of necrosis, haemorrhages or cyst formation (10).

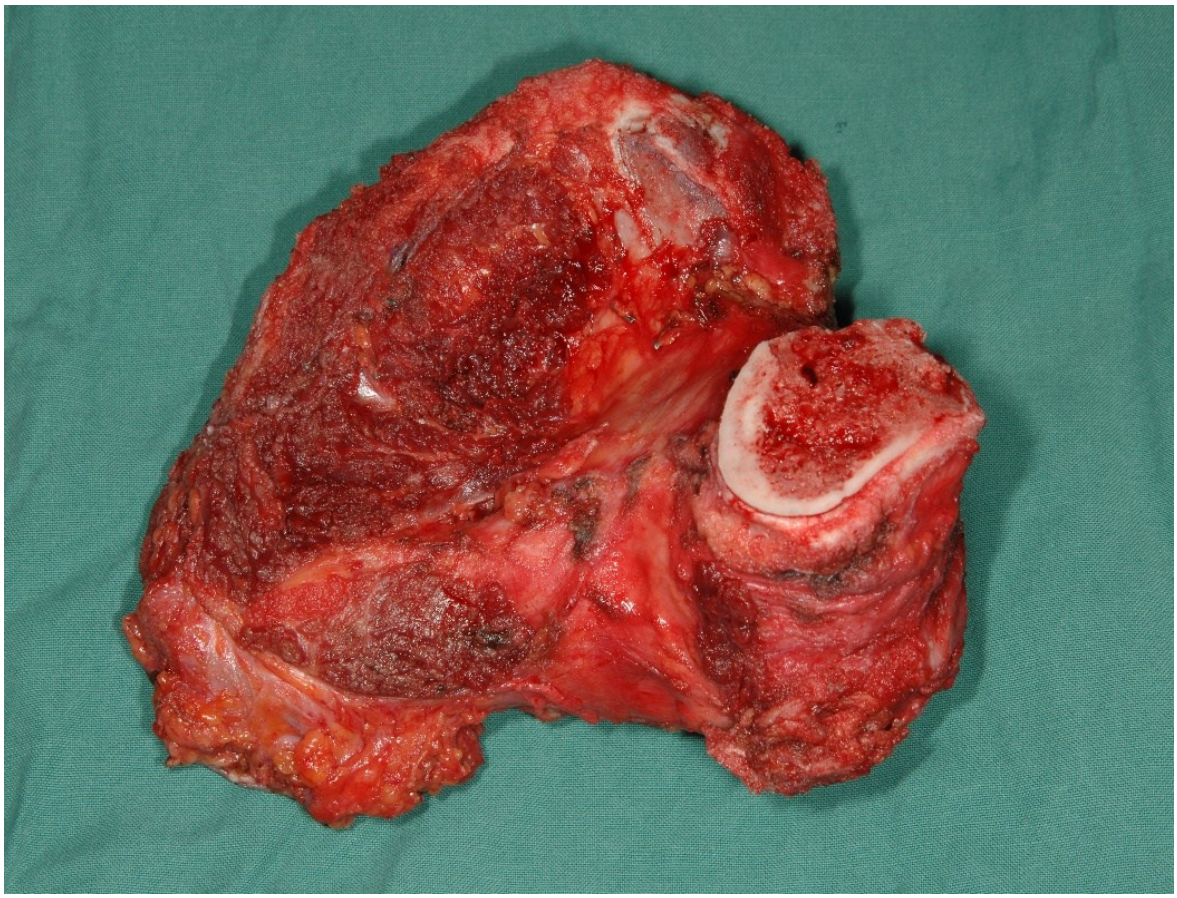

Figure 5 Resected pubic ES.

1.6.2 Histopathology

The morphology of ES is variable. Most cases are composed of sheets of uniform small round tumour cells with a high nuclear-to cytoplasmic ratio. The cells typically show round nuclei containing fine chromatin and little mitotic activity. The cytoplasm is scanty clear or weakly eosinophilic with indistinct cytoplasmic membranes. Usually, the cytoplasm

contains glycogen, making the cells positive on periodic acid-Schiff stain (<90% PAS positive). Necrosis is common and in some cases (<20%) Homer-Wright rosettes are present. Tumour cells in the soft tissue variety rarely have a spindle cell morphology (1,10).

Three larger categories appear, according to their predominant morphological criteria: classical/typical ES, PNET with neuroectodermal features and atypical ES, compromising subtypes distinct from the other two types (9).

As Ewing sarcomas are classified into the group of small blue cell tumours, that includes neuroblastoma, alveolar rhabdomyosarcoma and lymphoplastic lymphoma, the differentiation between them based on histomorphological features may be difficult (11).

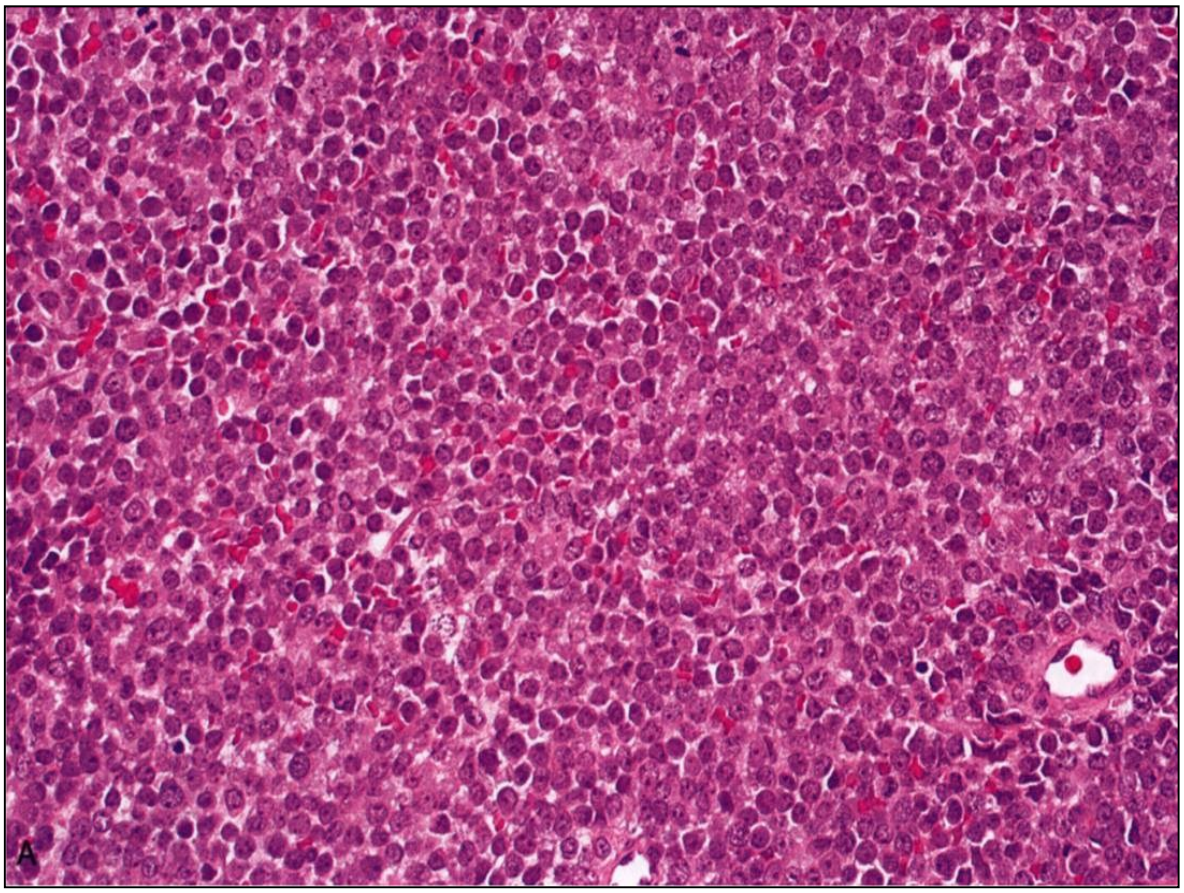

Figure 6 Histology of a typical ES.

1.6.3 Immunohistochemistry

Immunohistochemical features could be helpful to differentiate ES from other neoplasms, but they should interpreted alongside other findings, like molecular genetics to be definitive (1).

More than 90% of ES cells express the adhesion receptor CD99, also known as MIC2 or single-chain type-1 glycoprotein. Although CD99 is characteristic for ES it is not a specific marker (11). CD99 is a 32-kDa transmembrane protein that is encoded by the MIC2, located in the pseudo autosomal region at the end of the short arms of the X and Y chromosomes (12).

According to the degree of neuroectodermal differentiation, ES cells can also express neural cell markers, like neuronal specific enolase (NSE), S-100 protein, CD57, as well as neurofilaments and synaptophysin (11).

Vimentin stains most ES tumour cells and they are, in about 20% of all cases, reactive with anti-cytoceratin bodies (10).

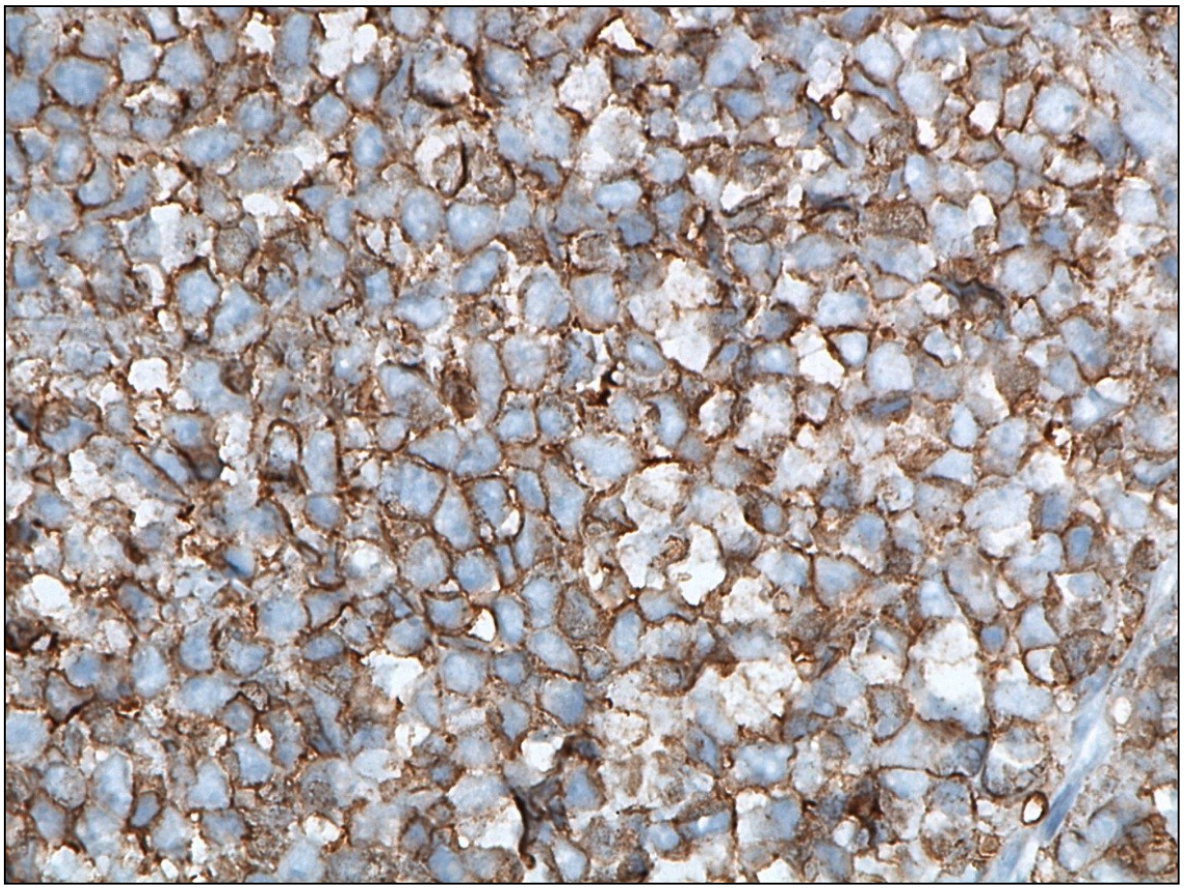

Figure 7 Membranous CD99 immunostaining.

1.6.4 Genetics

There are two classes of sarcomas, subdivided based on the genetic mutations associated with their development. One class summarizes tumours that bear complex karyotypic abnormalities without particular pattern. The second class includes Ewing sarcomas and defines tumours that are associated with unique chromosomal translocations that cause to specific fusion genes (11).

Molecular genetic studies using fluorescence in situ hybridisation (FISH) and/or reverse transcriptase chain reaction (PCR) are, along with histopathology and immunochemistry, indispensable essential in diagnosing Ewing sarcomas (6).

ES is characterised by a specific karyotype with only a few structural and numeric aberrations (6).

The translocation t(11;22)(q24;q12), which leads to the EWS-FL1 fusion gene is pathognomonic for the disease and is found in about 85% of cases. This rearrangement results in the translocation of the 3´ portion of the friend leukaemia virus integration site 1 (fli1) gene from chromosome 11 to the 5´ portion of the ES gene EWS on chromosome 22. In the other rare variant translocations, EWS is fused to the genes erg, e1af/etv4/pea3, etv1/er81, or fev, which are closely related to fli1 (6).

However, it is important to know that, although the occurrence is rare, other tumours (e.g. rhabdomyosarcoma) can also carry EWS-FL1 transcripts (1). In about 10-15% of all cases the translocation t(21;12)(22;12) causes the EWS-ERG fusion. The remaining 1-5% of cases carry one of several possible translocations, each of them resulting in a fusion gene containing a portion of the EWS gene and a member of the ETS family of transcription factors (6,11).

In about 20% additional structural changes affect chromosome 1 and 16. This mostly leads to a gain of 1q and a loss of 16q. Furthermore, trisomy 8 and/or trisomy 12 numeric chromosome changes are observed in half and one third of cases of ES (6).

The incidence of p53 mutations in ES is about 12% (13).

Thus, ES are defined by the presence of EWS-ETS and, as a substitute marker, by high $CD99^{mic2}$ expression levels on the genetic level (6).

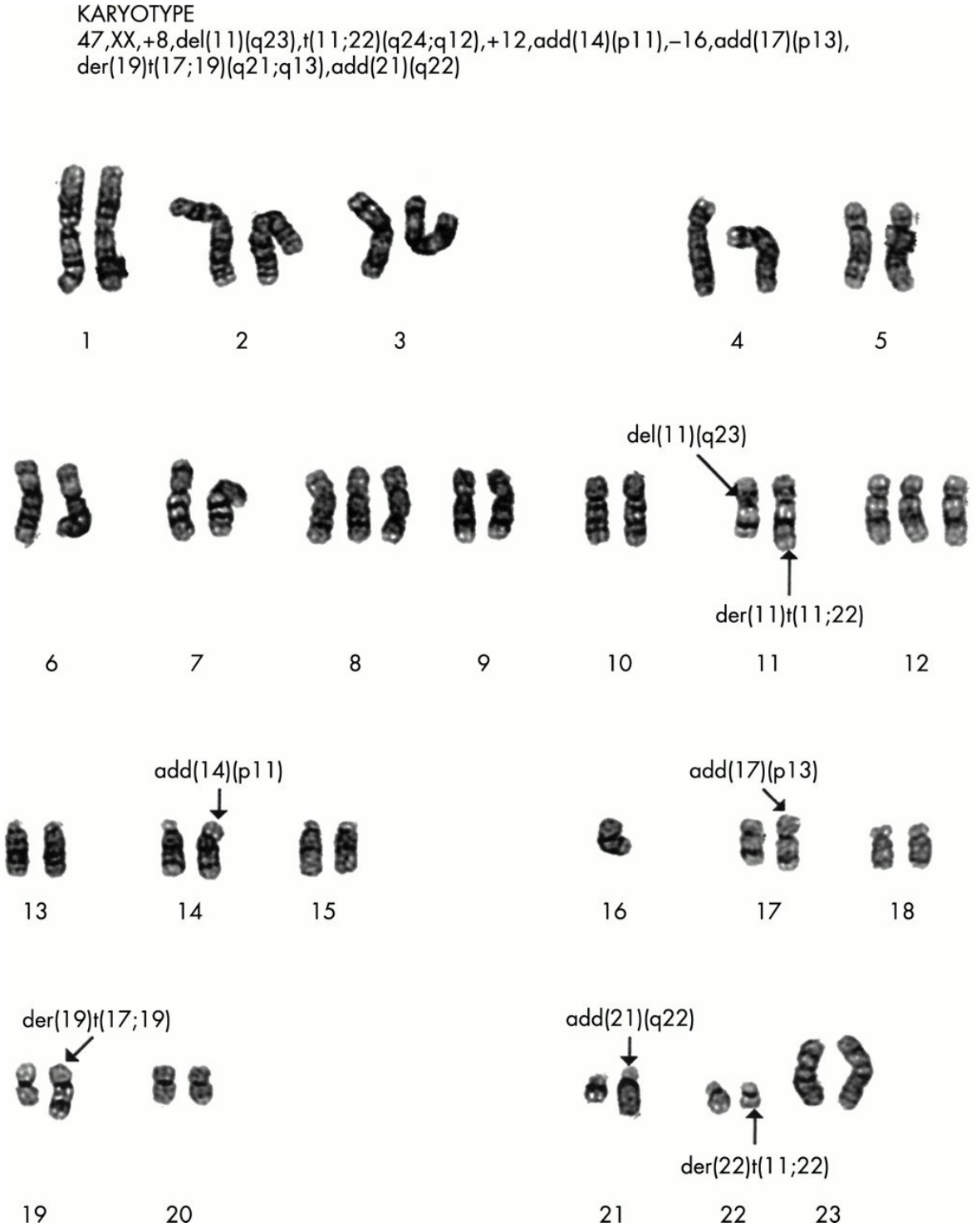

Figure 8 G banding of chromosomes from a ES showing the t(11.22)(q12.24) translocation (Burchill et al. 2003).

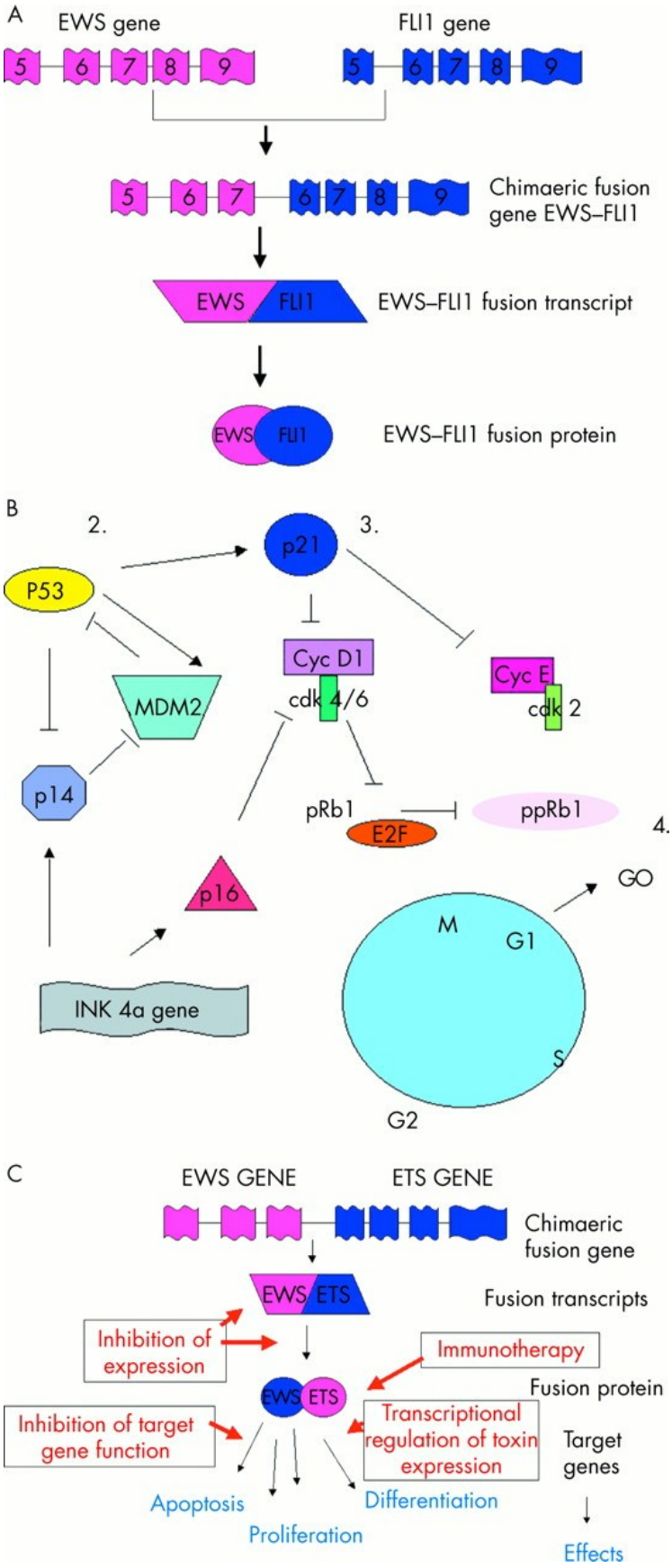

Figure 9 Diagrammatical representation of the t(11;22) (q24;q12) translocation resulting in the generation of the EWS-FL11 type 1 fusion transcript (Burchill et al. 2003).

Translocation	Gene fusion	Tumour type (% of tumours with this EWS gene rearrangement)
The presence of EWS–ETS gene rearrangements is increasingly used to define ESFT. The involvement of the EWS gene on chromosome 22q is consistent; this can partner with several different ETS gene family members located on various chromosomes, but most frequently with FLI1 on chromosome 11 in ESFT.		
t(11;22)(q24;q12)	EWS–FLI1	ESFT (85%)
t(21;22)(q22;q12)	EWS–ERG	ESFT (10%)
t(7;22)(p22;q12)	EWS–ETV1	ESFT (rare)
t(17;22)(q12;q12)	EWS–E1AF	ESFT (rare)
t(2;22)(q33;q12)	EWS–FEV	ESFT (rare)
t(12;22)(q13;q12)	*EWS–AFT1*	*Clear cell sarcoma*
t(11;22)(q13;q12)	*EWS–WT1*	*Desmoplastic small round cell tumour*
t(9;22)(q22;q12)	*EWS–CHN*	*Myxoid chondrosarcoma*
t(12;22)(q13;q12)	*EWS–CHOP*	*Myxoid liposarcoma*

Table 1 EWS fusion types described in ES sarcoma family of tumours (ESFT) and other sarcomas (Burchill et al. 2003).

1.6.5 Imaging

Imaging methods should be the first step in the assessment phase of a suspected tumour, consisting of plain radiography in two planes, computed tomography (CT) and/or magnetic resonance imaging (MRI). Patients with suspicious findings should be sent to a reference centre with experience in the disease before the biopsy is done (3).

Conventional radiographs of the primary tumour site are required as a first step and they are useful to determine the aggressiveness of the bone lesion (14).

Ewing sarcomas tend to be large, poorly marginated tumours, with over 80% demonstrating extension into adjacent soft tissues. It should be noted that PNET often extend into bone, making the distinction difficult (14).

The appearance of these tumours is very variable, with no specific radiographic features, but usually they have clearly aggressive appearance (9).

Common findings include indefinite osteolytic lesions, mainly involving the diaphysis of a long tubular bone with or without extension into adjacent soft tissue. The appearance can fluctuate from osteolytic to sclerotic (40%), but lytic bone lesions with moth-eaten permeative features are predominate in about 76%. In about 57% laminated or multi-layered periosteal reaction (onion skin-like) and, less frequent, perpendicular (sunburst type) reactive bone is found. Soft tissue calcification is uncommon, seen in less than 10% of cases (9).

Advanced imaging consists of MRI and/or CT imaging modalities to more precisely define the extent of the primary tumour and to assess its location relative to the adjacent bones, muscles, joints, blood vessels and nerves (14).

When performing a MRI or CT scan, the tumour volume should be estimated too (15).

A recent multi-institutional study found MRI and CT to be equally accurate for local staging of malignant bone and soft tissue tumours. The MRI is required due to its sensitivity to soft tissue contrast and multi-planar capabilities and it avoids the radiation that is used preforming a CT (14).

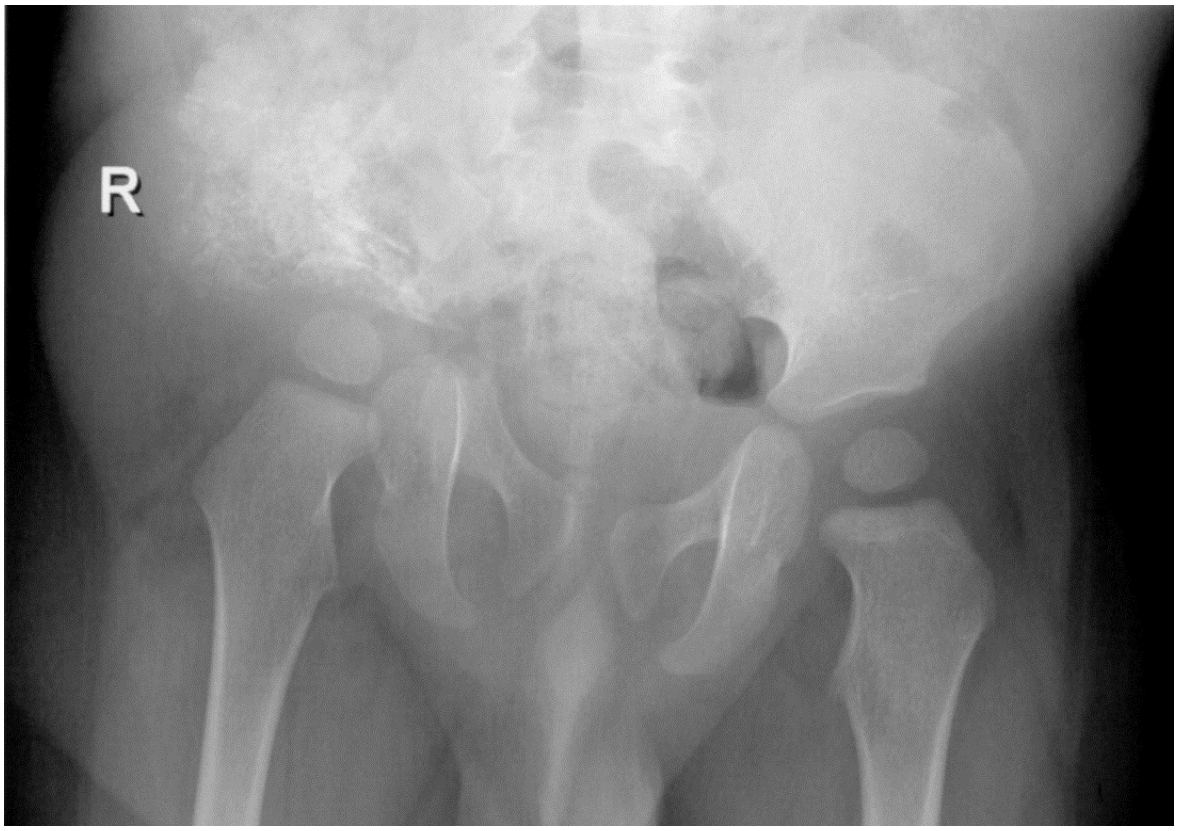

Figure 10 X-ray of an ES in the right ilium of a 2-year old boy.

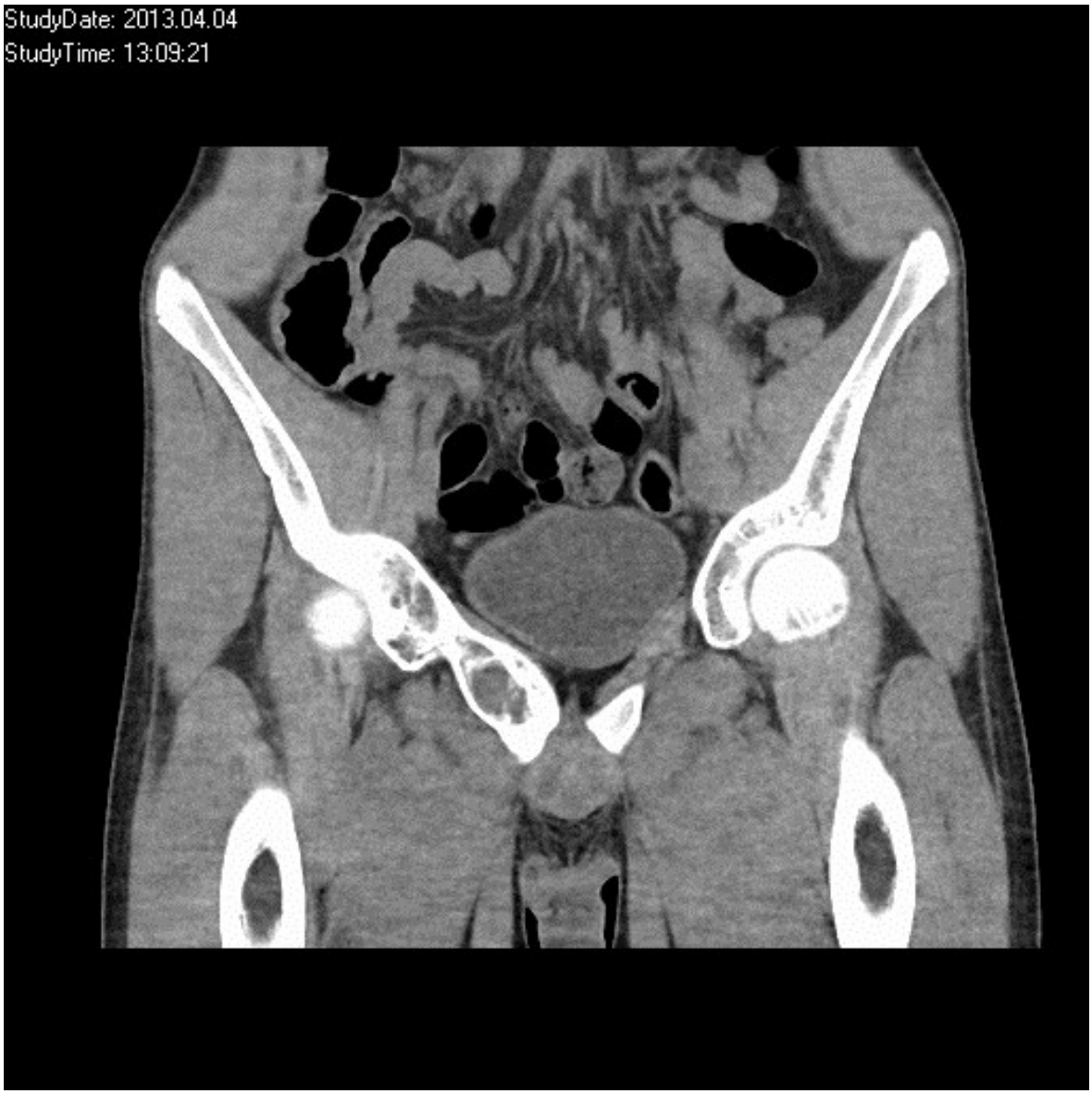

Figure 11 CT of an ES in the right os pubis of a 16-year old boy.

1.6.6 Biopsy

The definitive diagnostic test is, as for other malignant diseases, biopsy (3).
MRI should be done prior to biopsy to help determining the optimal site and to prevent falsification of the imaging findings by potential post-biopsy changes (14).
If possible, the surgeon who will do the local control surgery should do or participate the biopsy (5). Biopsy can be done by fine needle aspiration or by core needle biopsy technique, but the most adequate sampling is still guaranteed by open biopsy. Usually, the initial biopsy is incisional rather than excisional. The biopsy incision is longitudinal, so as to not violate

tissue flap planes and neurovascular structures. Sufficient material should be rapidly sent in a fresh state to a pathology department for further examination including conventional histology, immunohistochemistry and molecular biology (6).

1.7 Grading and Staging

1.7.1 Metastatic disease

ES has a strong potential to metastasize and the prognosis for patients suffering from metastatic disease still remains far poorer than in localized disease. At initial diagnosis between 20 and 25% of patients show metastatic disease (4). In 10% metastases occur in the lung or pleura (4). Pulmonary/pleural metastatic disease is evident if there is found one pulmonary/pleural nodule of >1cm, or more than one nodule of >0.5cm without any clear medical explanation of those lesions (15).
According to the ESMO Clinical Practice Guidelines, in another 10%, bone/bone marrow metastases are found (4). Bone metastases are evident if two or more sites are positive for uptakes in the bone scans. If so, further imaging by CT, MRI or PET of those sites is recommended. Bone marrow metastases are defined by light microscopic bone marrow (BM) involvement in any aspirate or biopsy sample (15).
Metastases to the liver, brain and to lymph nodes are rare (1). Suspected lymph node metastases should be confirmed by biopsy (15).

1.7.2 Staging investigations

According to international guidelines, following the diagnosis of a Ewing sarcoma, staging must be done (15,16). The stage is one of the most important factors in prognosis and in choosing treatment and is determined by the results of the following tests (15,16) :

a) Imaging of the primary tumour
- Plain radiograph
- MRI or CT scan

b) Skeletal system

- 99m Tc whole body scan
- PET scan
- FDG-PET scan
- MRI of sites suspicious in bone scan

c) Bone marrow

- Aspirates from _> 2 sites, biopsy from _> site:

Conventional cytology/ histology and RT-PCR for tumour specific chromosome 22 rearrangement.

d) Lung and Pleura

- Plain chest radiograph
- Chest CT scan

1.7.3 The Enneking System

In 1980 the surgical staging system of musculoskeletal neoplasms was initially suggested by Enneking et al. and got to be one of the most commonly used staging system for bone cancer. The Enneking system is based on the histological grade, the intraosseous or extraosseous extent of the tumour and the presence or absence of metastases (17).

Stage	Grade	Local Extent	Metastases
I-A	Low	Intracompartmental	None
I-B	Low	Extracompartmental	None
II-A	High	Intracompartmental	None
II-B	High	Extracompartmental	None
III	Any	Any	Present

Table 2 Musculoskeletal Tumour Society Staging System Enneking.

1.7.4 The AJCC System

The Enneking Staging System was evaluated and endorsed by the American Joint Committee on Cancer (AJCC) (17). The AJCC System describes all bone cancers, including skeletal Ewing sarcomas. Extraosseous ES are staged liked soft tissue sarcomas (16).
The AJCC staging system for bone cancers is based on 4 key pieces of information:

- **T** describes the size of the primary **tumour** and whether it appears in different areas of the bone.
- **N** describes the extent of spread to nearby (regional) lymph **nodes.**
- **M** indicates whether the cancer has **metastasized** to other organs of the body.
- **G** stands for the **grade** of the tumour, which describes how the cells from biopsy samples look.

T categories of bone cancer

T0: There is no evidence of a primary tumour.

T1: The tumour is 8 cm across or less.

T2: The tumour is larger than 8 cm across.

T3: The tumour is in more than one site in the same bone.

N categories of bone cancer

N0: There is no spread to regional lymph nodes.

N1: The cancer has spread to nearby lymph nodes.

M categories of bone cancer

M0: There is no spread to distant organs.

M1a: The cancer has spread only to the lungs.

M1b: The cancer has spread to other distant sites in the body.

Grades of bone cancer

GX: Grade can’t be assessed

G1-G2: Low grade

G3-G4: High grade

Stage grouping:

Stage IA*

T1, N0, M0, G1 to G2 (or GX): The tumour is 8 cm across or less (T1) and is low grade (or the grade can't be assessed). The cancer has not spread to nearby lymph nodes (N0) or to distant parts of the body (M0).

Stage IB*

T2 or T3, N0, M0, G1 to G2 (or GX): The tumour is either larger than 8 cm across (T2) or it is in more than one place in the same bone (T3). It is low grade (or the grade can't be assessed). The cancer has not spread to nearby lymph nodes (N0) or to distant parts of the body (M0).

Stage IIA

T1, N0, M0, G3 to G4: The tumour is 8 cm across or less (T1) and is high grade (G3 or G4). The cancer has not spread to nearby lymph nodes (N0) or to distant parts of the body (M0).

Stage IIB

T2, N0, M0, G3 to G4: The tumour is larger than 8 cm across (T2) and is high grade (G3 or G4). The cancer has not spread to nearby lymph nodes (N0) or to distant parts of the body (M0).

Stage III

T3, N0, M0, G3 to G4: The tumour is in more than one place in the same bone (T3). It is high grade (G3 or G4). The cancer has not spread to nearby lymph nodes (N0) or to distant parts of the body (M0).

Stage IVA

Any T, N0, M1a, any G: The tumour has spread only to the lungs (M1a). It has not spread to the lymph nodes or to other distant sites. (It can be any size or grade.)

Stage IVB (if either of these applies)
Any T, N1, any M, any G: The tumour has spread to lymph nodes (N1). It can be any size or grade, and may or may not have spread to other distant sites.
Any T, any N, M1b, any G: The tumour has spread to distant sites other than the lungs M1b). It can be any size or grade.

*All ES are classified as G4 (high grade), so they are never stage I bone cancers. Almost all ES fall into stages IIB or III (8,16).

1.8 Treatment

The treatment of ES is multimodal, including systemic chemotherapy combined with surgery and/or radiotherapy and depends on the localization and size of the tumour. Primary metastatic patients are treated by using the same approaches as used in patients with localized disease. Extra skeletal ES follow the same treatment principles like skeletal ES (4).

1.8.1 Chemotherapy

Chemotherapy in ES started in the 1960s using single agent therapy, followed by single-arm multi-agent chemotherapy and has been continuously modulated to the current state of the art, using a multimodality treatment concept (8). ES are sensitive to chemotherapy. Drugs, proven to be effective in ES are doxorubicin (DXR), cyclophosphamide (CPA), vincristine (VCR), actinomycin-D (ACT), ifosfamide (IFM), and etoposide (VP16) (8).
Following diagnosis, all ES patients receive chemotherapy, consisting of 3 to 6 courses of VIDE (vincristine, ifosfamide, doxorubicin and etoposide) chemotherapy as induction treatment. Following local therapy, another 6 to 10 cycles of chemotherapy are usually applied at 2-3 weeks intervals. Due to this, chemotherapy treatment duration is 10-12 months (4). In several studies high-dose chemotherapy with hematopoietic stem cell rescue (HDT) was applied to so called high-risk patients (patients with metastases or recurrence), but there has not yet been a controlled randomized clinical study that could prove an advantage using this therapy (8).

1.8.2 Surgery and Radiotherapy

Local therapy modalities including surgical resection of the tumour and/or irradiation remain controversial and must be decided individually (8). Though ES are radiation-sensitive, the number of patients treated with radiation alone has, due to the advances in orthopaedic surgery and the awareness of probable late effects of radiation, steadily declined over the past 3 decades (5). If pre-operative imaging suggests that the tumour resection is feasible, complete surgical resection without irradiation is the treatment of choice in primary ES. If radiotherapy is given alone there is a higher risk of local recurrence (4). If it is likely that surgery will not be possible with adequate resection margins, pre- and post- operative radiotherapy should be added (8). In large, unresectable primary tumours, like they are often seen in pelvic sites, radiotherapy alone might be applied. Studies showed, that patients who underwent intralesional resection followed by radiotherapy had no advantages in the local control rate compared to patients treated with radiation alone (4).

1.8.3 Targeted Therapy

New therapy strategies based on ES biology are tried to be developed, since conventional chemotherapy is ineffective in a quarter of patients with localized disease, and in three-quarters of patients with metastases. Promising targets are represented by studying the EWS-ETS fusion protein, IGF-1R and others (5).

1.9 Prognosis

All ES are high malignant tumours, showing a 5-year survival rate of less than 10% if treated with surgery or radiotherapy alone (3). The prognosis of ES have improved over the past decades, applying multimodality treatment including chemotherapy, achieving survival rates of about 60-70% in localize, but still only about 20-40% in metastatic disease (3). 5-year survival rates of <20% in patients with multiple bone metastases predict a poorer outcome than patients harbouring lung/pleura metastases which show 5-year survival rates of 20-40% (3). Tumour size or volume, serum lactate dehydrogenase (LDH) levels, axial localization or older age (>15 years) have been identified as other prognostic factors in several studies.

In 2000, Cotteril et al. published a study, including 975 ES patients, treated from 1977 to 1993, evaluating the prognostic factors and the improvements in relapse-free survival (RFS) (18). The results identified metastases at diagnosis as key adverse prognostic factor with a 5-year RFS of 22% in metastatic and 55% in non-metastatic patients (p>0.0001). In patients with no metastases at diagnosis primary site (axial vs. others) and age (>15 vs _>15) had significant influence on RFS (18). Once patients are relapsed, the 5-year overall survival rate in ES is poor and a late onset (<2 years) and a strictly localized relapse are prognostically favourable factors in relapsed patients (19). In 2010, Oberlin et al. analysed the event-free survival (EFS) and overall survival (OS) in 281 patients with primary disseminated multifocal ES. They showed an increased risk at diagnosis for patients older than 14 years, a primary tumour volume more than 200ml, more than one bone metastatic site, bone marrow metastases and additional lung metastases. This study suggests to invent a score that is based on these factors to may facilitate risk-adapted treatment (20).

However, the most significant prognostic factor is the presence or absence of metastases at diagnosis. It still remains unclear why patients with isolated lung metastases fare better than patients with bone or bone marrow metastases (7). A further prognostic factor is poor histological response to preoperative chemotherapy (3).

The relapse rate in ES is still high, with 30%-40% and the prognosis in these patients is, with less than 20% long time survival rates after recurrence, poor (7). The type of gene fusion could not have been declared to offer any prognostic value (9). The only reliable prognostic factor in relapse seems to be the time to relapse, with a better outcome for patients relapsing later than 2 years from initial diagnosis (3).

Specific Part

2 Bone marrow aspiration and biopsy (BMAB)

2.1 Background

Currently, one of the most useful adverse prognostic indicator in ES is the presence of metastatic disease at diagnosis. In the staging process of ES, the imaging investigations to detect metastases require radiographic and CT/MRT, chest scan and 99mTc bone scintigraphy. Each of these staging investigations provides complementary prognostic information, however the optimal combination is not clear (21). Besides these methods, the assessment for bone marrow (BM) metastases is light microscopically examination of bone marrow aspirates and biopsies (BMAB) (3).

According to the 2012 ESMO clinical practice guidelines, BMAB taken at sites distant from the primary tumour or known metastatic lesions are mandatory, although the added prognostic value of molecular positivity over light microscopic evaluation has not yet been proven (4).

Corresponding to the EURO Ewing 99 study protocol from 2006, BMAB examination is also required in the staging process of ES. Furthermore, they define BM metastases by light microscopic evidence of bone marrow involvement in any aspirate or trephine biopsy sample. Molecular evidence (i.e. by RT-PCR analysis) alone is, by definition of this protocol, not considered adequate for diagnosis of metastatic bone marrow disease (15).

The prognostic value of BM metastases is discussed controversially in different studies, as further prescribed in the literature search. Therefore the primary objective of this study was to retrospectively review BM samples from patients with ES.

2.2 Implementations

Bone marrow assessment includes bone marrow aspiration and biopsy (BMAB). BMAB is a frequently used medical procedure for diagnosis and staging of malignancies and other

diseases and is also used for the follow-up evaluation of patients receiving BM transplantation. BM trephine biopsy is still part in the initial staging of Ewing sarcomas and, if the results are positive, BM biopsy is also required (22).

BM aspirate material allows cytochemical stains, cytogenetic and molecular analysis, flow cytometric evaluation and other specialized investigations. They are useful for differential cell counts and the evaluation of individual cell morphology. Material from BM biopsy can be stained by the immunoperoxidase or other techniques and is valuable for the evaluation of marrow cellularity, determination of the number of megakaryocytes and the detection of focal lesions. Because BM aspirates and biopsies provide supplementary information, both are usually carried out as part of the same procedure (23). Before the BM collection is performed, examination of a blood film, assessment of results of a full blood count in the patient history must be regarded. It is also important to know, if the patient has recently been taking any medication that may have an influence in the blood count or BM cytology (24). Before the BMAB is done, a local anaesthetic, mainly using lidocaine, is injected into the subcutaneous tissue to numb the area and reduce pain. Anxious patients may get diazepam and lorazepam and in children often a general anaesthesia is needed (25).

The typical site for obtaining BMAB is the posterior superior iliac crest. In thin persons, the anterior iliac crest is also satisfactory. Although the iliac crest is safer, in some patients this is not possible. The sternum can be used for BAMB in adult patients with pelvic tumours, previous pelvic irradiation, very obese or immobile patients (24).

If the BMAB is done on the posterior iliac crest, the patient is placed into a right or left lateral decubitus position. The bone marrow biopsy is usually, but not always, performed first, because procurement of the aspirate specimen prior to the trephine core can cause hypocellularity and contamination of the core with sinusoidal blood. Although it is not needed, many physicians perform a small skin incision to ease the insertion of the needle. A needle is then introduced through the skin into the bone, and a sharp pain may be felt as the needle is propelled into the bone cavity. The stylet is then unlocked and slowly removed and a 10 ml syringe is attached. It is pulled back to aspirate about 0.3 ml of marrow, which causes a possible unpleasant sensation for the patient. If so, slowing the rate of aspiration is indicated. Bone marrow is collected by applying manual pressure to propel the needle through the bony cortex. This is performed by using a larger trephine biopsy needle to collect a cylindrical sample of solid bone marrow. Ideally the length of the biopsy specimen from an adult should measure at least 20 mm in length. Normally, the biopsy core is dark red with

fine white trabecular network. Finally, the needle is removed, and pressure is applied to the biopsy site to stop possible bleeding (22-24).

BM aspirate specimens should be smeared quickly and because aspirates may clot anticoagulants should be used. To assess BM films first they should be examined under low power (x10 objective) to evaluate the number of fragments, the cellularity, and the number of megakaryocytes. It is also possible to detect carcinoma cells. The films should then be examined in detail using a ×40 or ×50 objective. A systematic assessment of the cellularity and contents of fragments, megakaryocyte number, and morphology and cytological features of other lineages should be done. Fine cytological details should be assessed using an oil immersion ×100 objective (24). BM biopsy specimens should be stained with haematoxylin and eosin and for reticulin and examined systematically with low, medium and high power objectives. If needed, other specialized cytochemical or immunohistochemical stains may be done also (22).

As BMAB collection is an invasive procedure, although rare, complications may occur (26). The major adverse event is haemorrhage and is more likely in patients with myeloproliferative disorder, aspirin treatment, platelet dysfunction and thrombocytopenia. Further, there are reports of incidents of needle break offs and in some of these cases surgical removal was required (27). Infections may also occur, as well as allergic reactions to given medications, such as lidocaine or diazepam and, not to forget, the costs and the time needed to proceed BMAB. To prevent patients from errors and complications, BMAB should be done in a referring centre and carried out by trained individuals (26). Although BMAB generally is a safe procedure it is not to forget that they are not completely free of risk and that can have considerable impact on individual patients (25).

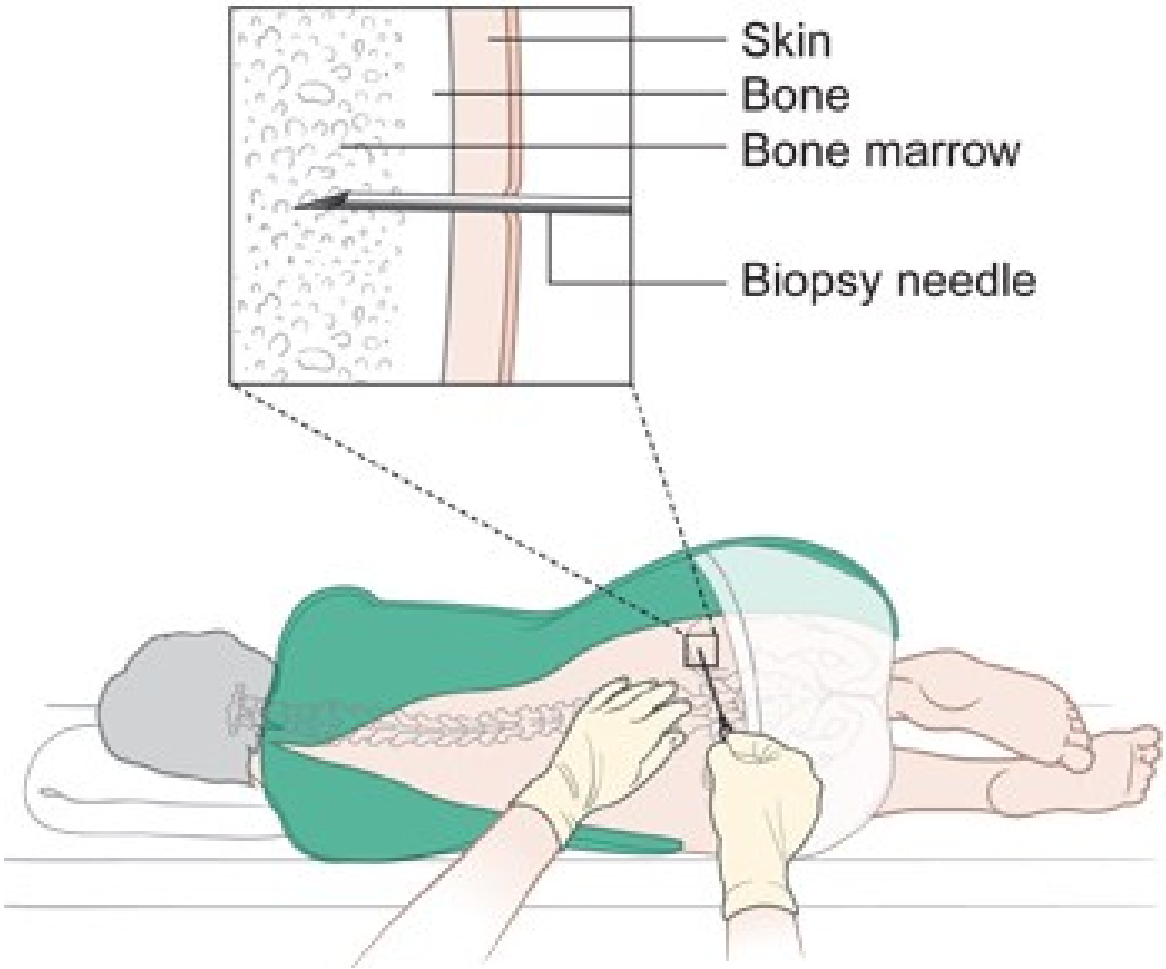

Figure 12 Site for BMAB (Cancer Research UK).

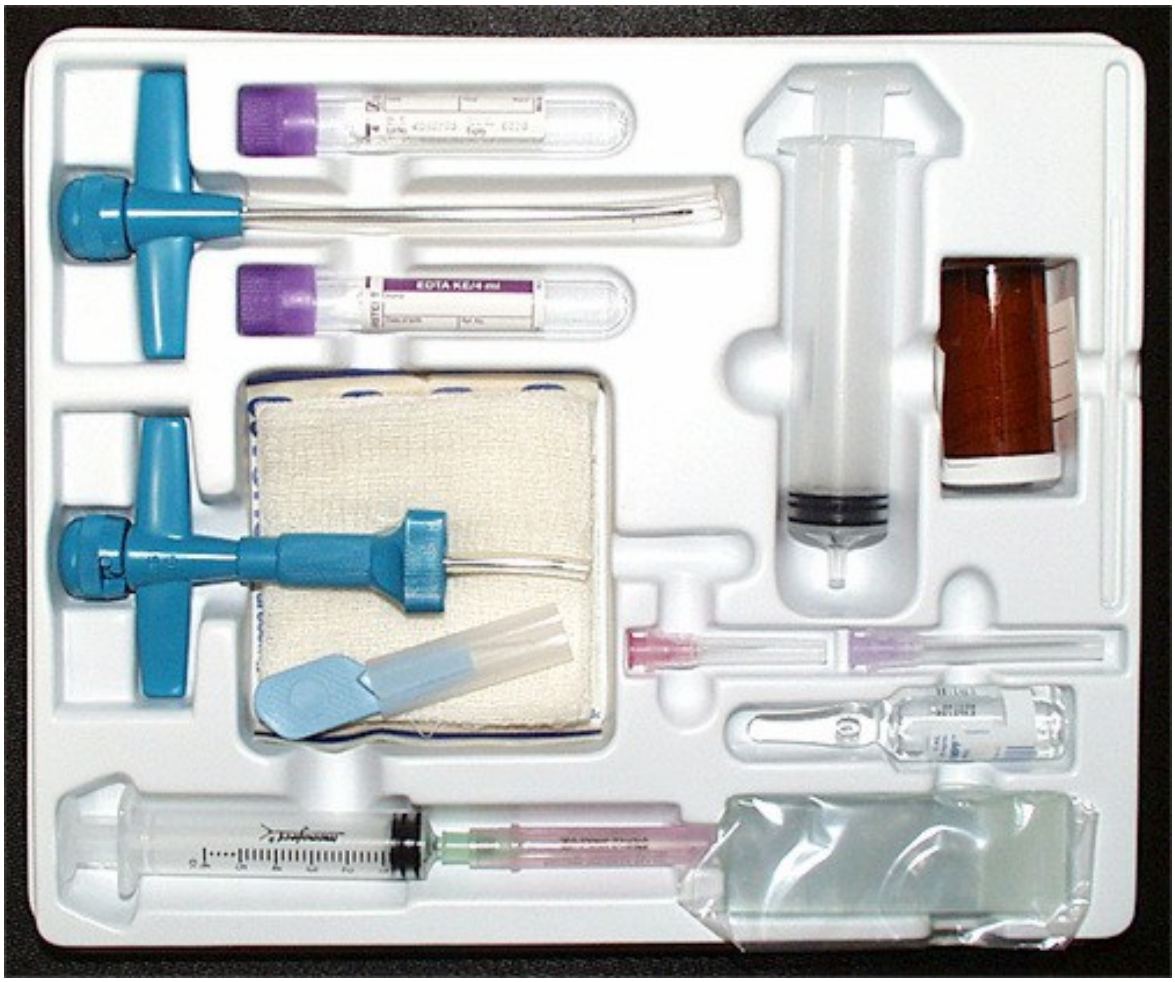

Figure 13 BMAB kit (Cancer Research UK).

3 Retrospective Study

3.1 Objective

According to clinical guidelines, BMAB examination is still mandatory in the staging process of Ewing sarcomas, but the utility of these investigations and the prognostic value of positive tested BM is discussed controversially.

Therefore, the primary objective of this study was to retrospectively review BM samples from ES patients that have been treated at the Department for Orthopaedics and Orthopaedic Surgery, at the University Hospital of Graz.

3.2 Materials and Methods

3.2.1 Dataset

This retrospective study was approved by the Ethics Committee of the Medical University of Graz (EK-Nr.: 25-525 ex 12/13) and is based on analysis of a dataset including Ewing sarcoma patients that were diagnosed between 2000 and 2014 and treated at the above named institution. This dataset provided patient information enclosing age, gender and data about diagnosis and treatment of the tumour. The main attention was paid to the data concerning BMAB.

3.2.2 Patients

This retrospective study included ES patients with molecular secured ES and patients who underwent BMAB. The only excluding criteria was the lack of secured ES and BMAB evaluation. Based on this criteria, study population consisted of 31 newly diagnosed ES patients. They were between 1 and 25 years of age (median age, 13.83 years). 21 were male and 10 were female. 27 patients had skeletal ES and in 4 patients the tumour was localized in the soft tissue. Metastases at diagnosis were present in 5 patients. In 3 of these 5 patients the lungs were the only site of metastases. In 1 of 5 patients the metastases occurred in the bone and 1 of 5 patients had a combination of lung and bone metastases.

Patient Nr.	Sex	Age at diagnosis	Localization Primary tumour	Metastases at Diagnosis
1	M	14	Pubis	-
2	F	3	Metacarpal bone	-
3	M	8	Ilium	-
4	M	23	Soft Tissue (Thigh)	-
5	F	13	Tibia	-
6	M	18	Femur	-
7	M	14	Ulna	-

8	M	19	Rib	Bone
9	F	20	Rib	-
10	M	8	Calcaneus	-
11	F	12	Tibia	-
12	M	1	Ilium	Lung
13	F	25	Femur	-
14	M	11	Humerus	-
15	F	17	Fibula	-
16	M	15	Humerus	-
17	M	14	Humerus	-
18	M	14	Clavicula	-
19	F	22	Tibia	-
20	M	13	Femur	-
21	M	10	Tibia	-
22	M	15	Soft Tissue (Lower Leg)	-
23	M	15	Sacrum	Lung
24	M	15	Scapula	-
25	F	10	Tibia	-
26	M	18	Ilium	Lung + Bone
27	F	18	Soft Tissue (Iliopsosas)	-
28	M	10	Rip	-
29	M	10	Tibia	-
30	M	16	Sacrum	Lung
31	F	8	Soft tissue (Upper leg)	-

Table 3 Patient characteristics.

3.3 BM assessment

All BM samples were obtained at diagnosis, before treatment. All samples were collected from the iliac crest and were morphologically and immunohistochemically examined by the pathologists. These findings were searched and screened for the presence or absence of BM metastases.

3.4 Molecular analysis

Furthermore, in 15 of the 31 patients BM samples were still available and were reanalysed at the Department of Pathology at the University Hospital of Graz, using nested PCR. 5 of the 15 retested BM samples were available from the left and the right side.

3.4.1 Nested PCR

(1) Total RNA was extracted from formalin-fixed paraffin embedded (FFPE) tissues, using the Maxwell® Instrument LEV RNA FFPE Kit.

(2) Then the RNA was rewritten into cDNA by means Superscript® III and was processed according to the c-DNA synthesis Superscript III protocol.

(3) Both, the rewriting of the RNA in the cDNA, as well as the quality of the RNA was checked by the control PCR HPRT (hypoxanthine-guanine phosphoribosyl transferase = housekeeping gene). The detection of the translocation t(11;22)(q24;q12) FLI1/EWSR1 was carried out by amplification of the fusion products of the gene FLI1 (11q24) with the gene EWSR1 (22q12) applying nested PCR.

Reagents for the master mix were from the company Qiagen®.

(4) The following primers are used for nested PCR:

Ewing ERG	**5` nach 3`**
EWSR-US1	GGATCCTACAGCCAAGCTCCAAGTC

EWSR-US2	ACAGAGCAGCAGCTACGGGCA
ERG-LS1	GGAGTTGGAGCTGTCCGACAGG
ERG-LS2	CAGGAGCTCCAGGAGGAACTGC
ERG-LS1N	CACTGTGGAAGGAGATGGTTGAGC
ERG-LS2N	TGGTTGAGCAGCTTTCGACTGG

Ewing FLI	**5` nach 3`**
EWSR-US1	GGATCCTACAGCCAAGCTCCAAGTC
EWSR-US2	ACAGAGCAGCAGCTACGGGCA
EWSR-L-US1	GCTGGAGAGCGAGGTGGCTTC
EWSR-L-US2	CGAGGTGGCTTCAATAAGCCTGG
FLI1-LS1N	AGGATCTGATACGGATCTGGCTGG
FLI1-LS2N	GCTGGGGCCGTTGCTCTG

Table 4 PCR-Primers.

3.4.2 Agarose gel electrophoresis

The PCR product was identified by gel electrophoresis on the basis of its size, being allowed to run with a specific control, a negative control and a standard with known DNA fragment lengths. The following results for ES sarcomas are possible with this method:

In the examination of paraffin material from isolated RNA by RT-PCR, using primers specific for the FLI1 and EWSR1 gene, the gel electrophoresis may whether detect or not detect a fusion product. Sometimes, due to inadequate RNA quality, the result cannot be evaluated.

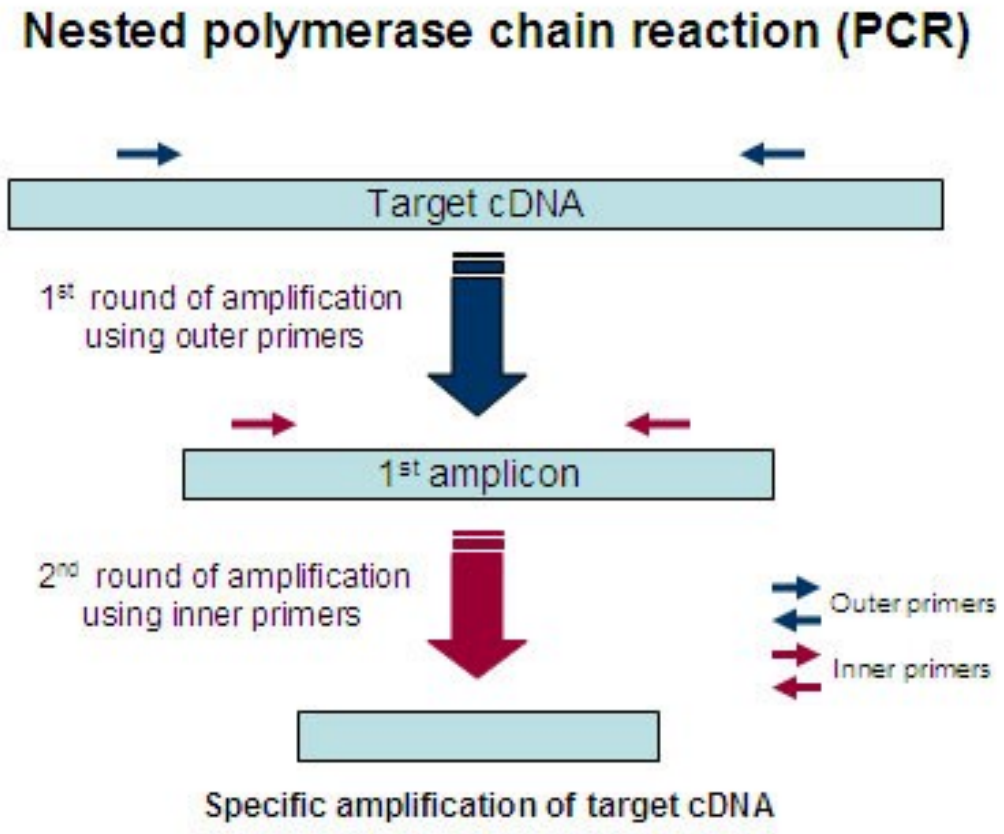

Figure 14 Nested PCR.

3.5 Results

Morphologically there was no evidence of bone marrow infiltration by the known Ewing sarcoma in any of the histopathological findings of our 31 patients. Furthermore the samples underwent immunohistochemical studies, performed at the unfixed material. In none of our 31 patients immunohistochemistry any evidence of bone marrow infiltration by the known Ewing sarcoma was found. All 31 patients, also the 5 with metastases at diagnosis were BM negative using morphological and immunohistochemical methods.

PCR results were negative in all of our 15 retested patients. These 15 patients included two patients with metastatic disease (1 with bone and 1 with lung metastases) and even among them, PCR was negative.

Patient Nr.	**Metastases at diagnosis**	**Morphological and Immunohistochemical results**	**PCR results**
1		neg	neg
2		neg	neg
3		neg	neg
4		neg	neg
5		neg	neg
6		neg	neg
7		neg	neg
8	Bone	neg	neg
9		neg	neg right neg left
10		neg	neg right neg left
11		neg	neg right neg left
12	Lung	neg	-
13		neg	neg
14		neg	-
15		neg	-
16		neg	-
17		neg	-
18		neg	-
19		neg	-
20		neg	-
21		neg	-
22		neg	-
23	Lung	neg	-
24		neg	-
25		neg	-
26	Lung and bone	neg	-

27		neg	neg right neg left
28		neg	neg
29		neg	-
30	Lung	neg	neg right neg left
31		neg	-

Table 5 Study results.

4 Literature Research

4.1 Objective

Regarding the prognostic value of BMAB results in ES, contradictory results were reported. Therefore, the secondary objective of this study was to review published literature concerning BM examination in ES.

4.2 Materials and Methods

4.2.1 Searching Parameters

PubMed database was searched iteratively to identify relevant literature, published from 1995 to July 2015, including the terms: “Ewing sarcoma”, “bone marrow”, “bone marrow metastases”, “bone marrow aspiration/biopsy”, “staging”, “predictive potential” and others. Papers providing quantitative results were included, whereas review articles were excluded. Papers classified as “relevant” were divided into groups, based on their findings concerning the value of BM positivity in ES.

4.3 Results

15 relevant studies were identified and summarized.
Following, an overview of these studies will be given:

In 7/15 studies no clear conclusion could have been drawn whether positive tested BM has any influence on prognosis in ES patients:

1.1. In 1995 Pfleiderer et al. (28) applied reverse transcriptase-polymerase chain reaction (RT-PCR) to monitor ES cells circulating in the peripheral blood or infiltrating bone marrow in order to identify patients with disseminated disease. Peripheral blood, BM samples and peripheral blood stem cell collections (PBSC) from 16 ES patients were analyzed. All of these samples were aspirated from tumor free sites of the pelvis or the sternum before the start of chemotherapy.
At diagnosis BM specimens from 6/16 patients with tumours located in the central axis contained tumour cells detected by RT-PCR. 5/6 of these patients presented with clinically evident metastases. In only 2 cases with disseminated ES no tumour cells were detected in the BM samples. Comparison of cytological and molecular data of the BM samples showed that tumour cells were detectable by light microscopy in at least one of the specimens derived from different aspiration sites in 4/6 RT-PCR positive cases. Only 1 peripheral blood specimen was tested positive for ES chimeric RNA. 4 patients were monitored during therapy and tumour cells were detected by RT-PCR in the BM samples from 2/4 patients, collected after 6 and 12 weeks of treatment, although previously aspirated samples were negative.
This study showed that ES cells infiltrating the BM or circulating in peripheral blood can be identified by RT-PCR. The sensitivity of the RT-PCR screening method for the presence of tumor cells was determined to be $1x10^{-6}$. The clinical implications of these findings has not been evaluated in this study.

1.2. In 1995, Oberlin et al. (29), performed a systemic BM investigation in newly diagnosed or relapsed ES patients to assess their BM status, saying that the detection of BM involvement in ES at diagnosis is a critical matter whenever high dose chemotherapy and autologous BM transplantation in poor prognosis patients is planned.

The aim of the study was to improve the detection rate BM involvement and to find the most accurate and rapid technique for routine investigations.

From January 1985 to May 1989, in 59 untreated patients and 5 patients at the time of relapse BM investigation was done. It consisted of 2 aspirations and two biopsies.

The aspirates were examined for cellular morphology by the classical technique of smearing and compared to the method of centrifugation of the pool of BM samples after density separation.

BM was involved in 13/59 untreated patients. Only 1/13 had BM involvement without any other metastatic spread, whereas 12/13 had known extra medullar metastases.

The incidence of BM involvement was high in patients with bone metastases detected by bone scan (9/13 = 69%). From 10 patients with lung metastases only 3 showed BM involvement and among the 3 patients with bone and lung metastases, two had BM involvement. 46/59 patients had a negative BM investigation and clinical characteristics were compared to those 13/59 with positive BM results. 40% of the patients with a pelvic primary had BM involvement, but only 11% of the patients without a pelvic primary had BM metastases. Furthermore, 42% patients with fever (>38 degrees C for at least 3 days) but only 13% of the patients without febrile condition at diagnosis had a positive BM study. These data was were available for 47/59 patients.

A stronger positive correlation existed between metastases in the BM and in other sites. The other factors had no independent predictive value of a BM involvement.

The study showed discrepancies between the results of BM aspirates or BM biopsies and also between the results of the sites explored according to the number of aspirates or biopsies, emphasizing the usual focal distribution of BM involvement.

In 39/59 patients BM screening was done by conventional smearing of each sample and smearing from the pool of samples. The comparison between these two cytological techniques was too small (7 patients) to draw definite conclusions.

Though the number of patients with BM involvement (13/58) was small, the study indicates BM involvement as a frequent event in metastatic ES patients (52%) and that it is often multifocal and therefore requires extensive BM investigations.

1.3. Also in 1995, Peter et al. (30) described the method, based on nested-PCR amplification of the EWS/FL-1 or ERG fusion transcripts, which enables the detection of small numbers of Ewing cells in biological samples.

This technique was applied to detect these fusion transcripts from mononuclear cells isolated from blood, BM and peripheral stem cell harvests. The study population consisted of 36 EWS patients treated from 1992-1993. 16/36 were studied at diagnosis, 18/36 after induction therapy and 2/36 patients were analyzed at the time of relapse. 3 could be studied at diagnosis and at the time of stem cell harvest. 28 primary sites and 51 peripheral samples from the 36 patients were investigated.

Tumour cells were found in 4/18 blood samples, 2/18 peripheral stem cell harvests and 4/15 BM aspirates. In all cases the fusion transcripts were identical to that detected in the primary site. 2/4 positive BM samples were detected in patients with localized disease at diagnosis, 1/4 after induction therapy and 1/4 at the time of relapse. None of these patients hat detectable metastases evaluated by chest CT scan and cytohistological examination of the BM.

This study concludes, that regional disease can be accompanied by circulating tumour cells or occult BM metastases and that a progressive increase of tumour cells might be indicative of a relapse.

1.4. Another study from 1995, performed by Zoubek et al. (31) used RT-PCR technique and immunofluorescence to detect minimal metastatic and minimal residual disease in patients with ES.

BM samples from 14 patients were taken before therapy and examined by RT-PCR and MIC2/CD99 analyzes. 6/14 showed RT-PCR BM positivity. Among the 6 patients who were positive, 4/6 had clinically evident metastases and 2/6 were non-metastatic.

3/6 also showed BM involvement verified by light microscopy. In contrary, 8/14 patients were BM positive by using MIC2/CD99 analyses, whereas only 4 of these 8 were also positive by RT-PCR.

This study showed the discrepancies between the results of those 2 detection methods and therefore no consequences in BM positivity in localized ES should be drawn.

1.5. In 1997 West et al. (32) determined the feasibility of detecting fusion transcripts by RT-PCR in ES or peripheral primitive neuroectodermal tumours (PNET) in blood and BM samples.

In this study patients were divided into two groups: newly diagnosed patients with non-metastatic disease (no evidence of distant tumor spread at diagnosis, assessed by bone scan, chest CT scan and histologic examination of a single BM aspirate and biopsy sample) and patients with metastatic or relapsed disease (distant tumor spread by at least one of the studies).
None of the patients had histological evidence of BM involvement.
Patient collective consisted from a total of 28 patients. 14/28 had both, BM and peripheral blood (PB) samples available, 8/28 had BM only and 6/28 had PB only. 16/22 BM aspirates and 10/20 PB samples derived from newly diagnosed, non-metastatic patients and 6/22 BM aspirates and 10/20 PB samples from metastatic or relapsed patients. Among non-metastatic patients, 3/16 were positive in BM and 3/10 were positive in PB. Patients in this group, who were positive in either PB or BM were 4/16 (25%). Among metastatic or relapsed patients, 2/6 were positive in BM and 5/10 were positive in PB. Patients in this group, who were positive in either PB or BM were 6/12 (50%).
This study marks that not all patients had tumour cells detected by RT-PCR in PB or BM, not even those with metastatic disease and that the true biological meaning or clinical relevance of detecting tumour cells in PB or BM of ES and PNET patients is unknown.

1.6. In 2001, Sumerauer et al. (33) investigated the incidence of BM infiltration in Ewing sarcoma family of tumours (ESFT).
BM aspirates were taken at the time of diagnosis from 22 patients (21 newly diagnosed and 1 recurrent disease). Besides RT-PCR detection, BM smears were also evaluated by light microscopy, but they were all negative, even in 2 patients with multiple bone metastases.
16/22 patients presented with localized disease. Using RT-PCR, 5/16 (31%, altogether 27 samples/7 positive) non-metastatic patients were positive for the marker mRNA in BM. Among the 6/22 patients that had distant metastases (3 in the lung only, 1 in the lymph nodes and 2 had lung and bone metastases) at the first presentation, 3/6 (50%, altogether 13 samples/6 positive) were tested positive. In both, patients with localized and metastatic disease, 8/22 (36%) showed BM infiltration, altogether there were 13/40 (33%) samples tested positive.
This study showed the possibility to detect transcripts by RT-PCR in BM of ES patients with high sensitivity.

In this study more than one quarter of the patients presenting with localized disease had minimal BM infiltration, but the presence of a low number of tumour cells in the BM in these patients, detected by RT-PCR, is not a reason to apply more intensive therapy.
It also says that the clinical significance of the minimal disease detected at molecular level remains unknown, but that RT-PCR evaluation may help to divide ES patients into risk groups in the future.

1.7. In a study from 2001, Athale et al. (34) compared RT-PCR findings with those of morphology-based methods in pediatric alveolar rhabdomyosarcoma (ARMS), desmoplastic small round cell tumours (DSRCT) and ESFT.
Patient collective consisted of 47 patients, 13/47 suffering from ARMS, 3/47 with DSRCT and 31/47 with ES. This study is further summarized only with regard to the BM analyses from ES patients. From a total of 31 ES patients 26 BM samples were available, 18/26 were taken at the time at diagnosis and 8/26 at the time of relapse.
In ES patients RT-PCR analyses were positive for metastatic disease in 7/26 (26.9%) BM samples. 5 of these 7 were diagnosed by both methods (morphologic methods and RT-PCR), 1/7 was positive by RT-PCR alone and 1/7 was positive by morphology alone. From the total of 26 BM samples 19 were tested negative by both methods.
For ES the sensitivity of RT-PCR was 83.3% in BM. BM disease was detected by RT-PCR in 37.5% of ES patients with metastatic disease, but, importantly, none of the samples from patients with localized disease had BM micrometastases detected by RT-PCR.
This study concludes that the clinical significance of molecularly detectable disease remains unknown and that further studies are needed.

2. 5/15 studies correlated BM positivity with poorer outcome.
(2/5 in metastatic patients: 2.1., 2.2.)
(1/5 in non-metastatic patients: 2.3.)
(2/5 in both, metastatic and non-metastatic patients: 2.4., 2.5.)

2.1. In 1998, Fagnou et al. (35) studied patients with ES for the presence of tumour cells in PB and BM by RT-PCR, respectively and compared the results with clinical parameters and with the patients follow up.

This study is further summarized only with regard to the BM analyses from ES patients.
In 67 patients, RT-PCR was performed in 43 BM specimens and 14/43 (33%) had positive BM. 15/43 had metastases and 28/43 did not show metastases.
In the metastatic group 8/15 were RT-PCR positive and 6/28 in the non-metastatic group were RT-PCR positive. The presence of circulating tumour cells was not obviously associated with the size nor the localization of the primary tumour but it was associated with the presence of clinically detectable metastases.
In univariate analyses the estimated overall survival was lower in the group of patients with RT-PCR positive BM compared with the group with RT-PCR negative BM (p=0.002; mean follow up: 12 months). RT-PCR positive BM was associated with lower survival (p=0.01). Multivariate analyses would be necessary to show if this marker is independent from the existence of metastases. RT-PCR positive BM was frequently found in patients with metastatic disease and these study data suggest that the monitoring at diagnosis of BM by RT-PCR might be an important criterion for the staging in ES patients.
In the absence of metastases it could not be appreciated that a positive RT-PCR BM result is a prognostic factor.

2.2. In a study from 2006, Oberlin et al. (36) examined 97 patients with untreated metastatic bone ES/PNET.
Metastatic sites at diagnosis were:
44/97 in the lung only, 23/97 had BM involvement (3/23 had isolated BM metastases), 22/97 showed bone metastases without BM involvement (9/22 had isolated bone metastases) and 8/97 had other metastases.
BM was assessed by multiple aspirates and two biopsies.
Treatment consisted of seven cycles of induction chemotherapy, local therapy and high-dose chemotherapy (HDCT) with autologous stem cell transplantation (SCT).
Univariate analyses of potential prognostic factors for event free survival (EFS) and overall survival (OS) were performed.
Among the 97 patients, 75 received HDCT and the 5 year EFS rate after HDCT was 47%.
The EFS for the 44 patients with lung metastases only was 52% and 36% for the 22 patients with bone metastases without BM involvement. Among the 23 patients with BM metastases the 5 year EFS was only 4% and only 1/23 patients survived.
Further, multivariate analyses were performed and identified three independent prognostic factors for EFS, including BM metastases:

Age 15 years or older, fever at diagnosis and BM involvement at diagnosis.

2.3. In 2003, Avigad et al. (2) studied the prognostic potential of positive RT-PCR BM and PB results in non-metastatic ES patients during a long follow-up period (median, 61 months).
26 pediatric patients who were diagnosed with localized ES were analysed. This study is further summarized only with regard to the BM analyses from ES patients.
At diagnosis, 6/14 (43%) patients were RT-PCR BM positive, with no correlation to tumour progression (p=0.3). For statistical analyses, the last sample from each patient was examined. 8/14 (57%) BM samples showed positive RT-PCR results and were correlated with disease progression (p=0.02).
This study suggests that serial monitoring with RT-PCR is recommended for the prediction of disease recurrence.

2.4. In 2011, Ash et al. (37) used multiparameter flow cytometry (MPFC) to detect tumour cells in BM of ES patients at diagnosis by the combination of CD99+/CD90+/CD45- and to evaluate the prognostic significance of CD56 expression in BM samples.
All 46 BM samples (35/46 non metastatic, 11/46 metastatic) were evaluated positive for micrometastatic tumor cells assessed by CD99+/CD90+/CD45- expression, indicating EWS as a systemic disease.
According to the level of BM involvement no statistical differences could be shown in the OS and progression free survival (PFS), nor was any correlation with clinical parameters found. 27/45 (60%) BM samples were found to harbor high CD56 expression. High or low expression of CD56 was defined as higher as or lower than the cutoff of 22%, defined by ROC analyses. There was no correlation between CD56 expression and clinical parameters, such as: age, primary site and metastases at diagnosis. However, a significant correlation between disease progression and CD56 expression was found. Patients with low/negative CD56 expression had a significant better PFS.
This was the first study in ES patients reporting CD56 as an independent prognostic marker for relapse.

2.5. In 2003 Schleiermacher et al. (38) used RT-PCR to search for tumour cells in BM and PB in ES.
This study is further summarized only with regard to the BM analyses from ES patients.

In this retrospective study RT-PCR BM analyses from a total of 131 patients, 39/131 metastatic and 92/131 non-metastatic, were examined.
36/131 (27%) patients were scored positive for tumour cells.
RT-PCR positivity was found in 18/39 (46%) metastatic patients versus 18/92 (19%) in non-metastatic patients, indicating that BM involvement is strongly associated with the presence of clinical evident metastases (p=0.0018), but RT-PCR positivity was not associated with tumor volume nor with the histological response to primary chemotherapy.
Univariate analyses of the whole study population showed that RT-PCR positivity in BM predicted a poorer disease free survival (DFS) rate (p=0.0003). Among patients with localized disease, BM involvement also predicted a poorer DFS rate (p=0.043).
The study concludes that the search for occult tumour cells should be included in the staging of patients with ES.

3. 3/15 studies did not show a negative correlation over positive tested BM and disease progression and 2/3 (3.2., 3.3.) even suggest the elimination of BMAB in non-metastatic ES patients.

3.1. In 1998, Zoubek et al. (39) presented a study where BM samples from ES patients were analyzed by RT-PCR to evaluate the incidence and the prognostic impact of BM involvement.
35 newly diagnosed ES patients, treated between 1993 and 1996 were included.
23/35 patients had localized disease and 12/35 showed metastases, detected by routine clinical measurements (CT scans of the lungs, Tc99 bone scan, BM aspiration and trephine biopsy investigated by light microscopy). In the 12 patients with metastatic disease 6/12 showed lung metastases only, 2/12 had additional bone or BM involvement and 4/12 had lungs, bones and BM metastases. All samples were taken at diagnosis, before treatment.
Among the patients with localized disease, 7/23 (30%) were BM positive and after a median observation time of 30 months, 3/7 of these patients have relapsed. 2/16 PCR-negative patients in the non-metastatic group have relapsed. This result had no statistical significance (p=0.208). Among the patients with metastatic disease, 9/12 (75%) were BM positive. 3/6 (50%) of the patients with lung metastases only were RT-PCR positive, whereas 6/6 (100%) patients with bone metastases showed BM positivity. After a median observation time of 32 months, in the group of metastatic patients, 4/9 BM positive patients and 1/3 BM negative

patients have relapsed. This result was also of no statistical significance (p=0.943). Furthermore, the 16 patients who showed RT-PCR positivity in their BM at diagnosis had a control examination which was performed after application of 3-5 chemotherapy cycles.
All 16 initially BM positive patients were tested negative during treatment.
The results of this study excluded a correlation of RT-PCR positivity and early relapse in ES.

3.2. In 2013, Newman et al. (21) performed a retrospective study, comparing different staging modalities in the metastatic evaluation of ES.
Staging investigations included chest computerized tomography (CT), technetium bone scintigraphy (bone scan), F18-fluorodeoxy-D-glucose-positron-emission tomography (FDG-PET) scan and bone marrow aspiration and biopsy (BMAB), but the ideal combination of those methods is not clear.
This study compared the findings of FDG-PET and bone scans and additionally the results of imaging methods compared to bilateral and unilateral BMAB.
Only staging investigations done before treatment were included. To identify osseous metastases, the imaging methods, bone scan and FDG-PET were compared by using the discordant and concordant results. The concordance rate between the two imaging methods for detecting osseous metastases (examination-based concordance rate) and identifying regions of osseous metastases (region-based concordance rate) was calculated. 63 patients had both, bone scan and FDG-PET scans. The examination based concordance rate was 98%, with only one patient having a positive FDG-PET scan but a negative bone scan.
The region- based concordance for all patients using both modalities was 97% and 63% for metastatic cases. To assess BM involvement, unilateral and bilateral BM samples, all taken from the iliac crest were examined. Bilateral BM samples results from BMAB were divided into: positive for metastatic BM disease bilaterally, unilaterally, or negative.
Additionally, left to right samples were compared and each pair was classified as concordant or discordant. These results were used to calculate the right versus let concordance for BMA and BMB. Using the totals, the ipsilateral BMA/BMB concordance rate was calculated. 59 patients had a bilateral BMA and 62 patients had a bilateral BMB.
Metastatic disease was found in 3 patients sampled bilaterally, 1/3 BMA was positive bilaterally and 2/3 BMB were positive bilaterally. Bilateral BMA showed a 97% and BMB 98% concordance rate. From a total of 75 patients (58 bilateral, 17 unilateral) the matched ipsilateral concordance rate was 98%. 4 BMAs were positive and also had positive ipsilateral

BMB, whereas 3 positive BMBs had negative ipsilateral BMA, what indicates that BMB was more sensitive to detect metastases than BMA.
In all cases where BM metastases were detected by BMA or BMB osseous metastases were also found using bone scan and FDG-PET.
This study concludes, that FDG-PET is slightly superior to bone scan in detecting osseous metastases and that BMB is better than BMA in detecting BM metastases. Bilateral versus unilateral sampling is slightly better to detect metastatic BM disease.
BM sampling did not detect BM metastases unless osseous metastases were found by other imaging modalities and therefore this study suggest BM sampling only if osseous metastatic disease is detected by other staging modalities.

3.3. In 2014, Kopp et al. (40) retrospectively reviewed newly diagnosed ES patients with initial staging including imaging and BMAB.
Bilateral BMAB for initial staging in pediatric ES patients is currently standard.
Because the usefulness of BMAB as part of the initial staging is not well defined this study analysed the concordance of positive BMAB results with positive or negative metastatic status by imaging modalities.
It was hypothesized that patients deemed non-metastatic by imaging are unlikely to have BM involvement and therefore do not need BMAB.
BM was considered positive if ES cells were found in BMA or BMB either on H&E stain or by immunohistochemistry.
The patient collective consisted of 116 patients: 85/116 with non-metastatic disease and 31/116 with metastatic disease by imaging at diagnosis.
None of the 85 non-metastatic patients by imaging had a positive BMAB.
13/31 (42%) of metastatic patients by imaging had a positive BMAB.
Patients with bone metastases had a high correlation of a positive BMAB (p=0.002), whereas patients with lung metastases had a low correlation with a positive BMAB (p=0.017).
None of the patients with metastases had BM metastases as the only site of metastatic disease and a pelvic primary was not correlated with BM involvement.
This study suggests, that in pediatric ES patients with localized disease by imaging, BMAB may be eliminated in as part of the initial staging.

Nr.	Study	Testing method	Nr. of all patients	Nr. of BM + patients	Nr. of BM – patients	Nr. of BM + in metastatic patients	Nr. of BM + in non-metastatic patients
1	Pfeiderer et al. 1995	RT-PCR	16	6/16	10/16	5/6	1/6
2	Oberlin et al. 1995	Morphological	59	13/59	46/59	12/13	1/13
3	Peter et.al 1995	Nested-PCR	15	4/15	11/15	4/4	0/4
4	Zoubek et al. 1995	RT-PCR	14	6/14	8/14	4/6	2/6
5	West et al. 1997	RT-PCR	22	5/22	17/22	2/5	3/5
6	Sumerauer et al. 2001	RT- PCR	22	8/22	14/22	5/8	3/8
7	Athale et al. 2001	RT-PCR	26	7/26	19/26	7/7	0/7
8	Fagnou et al. 1998	RT-PCR	43	14/43	29/43	8/14	6/14
9	Oberlin et al. 2006	-	97	23/97	74/97	23/23	-
10	Avigad et al. 2003	RT-PCR	14	6/14	8/14	-	6/6
11	Ash et al. 2001	MPFC	46	46/46	0/46	11/46	35/46
12	Schleiermacher et al. 2003	RT-PCR	131	36/131	95/131	18/36	18/36
13	Zoubek et al. 1998	RT-PCR	35	16/35	19/35	9/16	7/16
14	Newman et al. 2013	-	75	6/75	69/75	6/6	0/6
15	Kopp et al. 2014	-	116	13/116	103/116	13/13	0/13
	Results	**9/15 RT-PCR 1/15 Nested-PCR 1/15 MPFC 1/15 Morphological 3/15 Unknown**	**731 100%**	**209 28.59%**	**522 71,40%**	**127 60.76%**	**82 39.23%**

Table 6 Results of 15 studies concerning BM examination in ES.

5 Discussion

Corresponding to clinical guidelines, BMAB examination is still mandatory in the staging process of Ewing sarcomas.

As the prognostic value of BMAB in ES patients is discussed controversially we tried to better specify the need of this investigation in the staging process.

Also, the ideal method to examine BM is not clear. Although, according to the 2012 ESMO clinical practice guidelines, BMAB taken at sites distant from the primary tumour or known metastatic lesions are mandatory, they say that the added prognostic value of molecular positivity over light microscopic evaluation has not yet been proven (4). Wagner et al. reviewed other methods to asses BM involvement in ES. Additional to morphological and immunohistochemical methods, BM evaluation by using PCR is a very common molecular method (41). 10 of 15 studies described in the literature research above also performed PCR, but, corresponding to the EURO Ewing 99 study protocol molecular evidence (i.e. by RT-PCR analysis) alone is not considered adequate for diagnosis of metastatic bone marrow disease (15). As described by DuBois et al. a new method to detect ES cells in BM is flow cytometry, where BM cells are stained for CD99 and CD45 in order to detect CD99+ CD45- (42). Ash et al. reported the use of multiparameter flow cytometry (MPFC) to detect ES cells in BM. This method identifies tumour cell expressing CD99 and CD90 and being negative for CD45 and other hematopoietic panels (37). This study, performed in 2001, is the only one where all BM samples (46/46) were tested positive for micro metastatic tumour cells. Furthermore, in this study, 27/45 (60%) BM samples were found to show high CD56 expression and a significant correlation between disease progression and CD56 expression was described (37). Another potential method to asses BM in ES patients may be the use of FISH, which identifies translocations involving the EWS gene, but, there are no current studies available in regard to this method (41). A non-invasive method to assess bone and/or BM involvement is FDG-PET as demonstrated in the study by Newman et al. where 0/57 non metastatic patients showed BM involvement by FDG-PET (21). In our study 0/31 ES patients (5/31 with metastases) showed BM involvement using morphological and immunohistochemical methods and also the nested-PCR results were negative in all of our 15 retested patients (2/15 with metastases) at the time of diagnosis. These results are different to the 10 /15 studies that also used PCR methods to examine BM, because in all of them positive BM samples were found (2,28,30-35,38,39). The cohorts of these 10 studies were,

like ours, also rather small, including 14 to 131 ES patients. Neither the size of our study, nor the used method seems to be the reason that no positive BM sample was found in our patients.

Altogether the 15 studies included 731 (100%) ES patients and BM involvement was found in 209/731 (28.59%) patients. 127/209 (60.76%) BM positive patients had known metastases, whereas BM positivity was seen in only 82/209 (39.23%) patients without distant metastasis. According to this results BM involvement seems to be more frequent in metastatic patients. Fagnou et al. also concluded that RT-PCR positivity is frequently found in metastatic ES patients and suggested that the monitoring of BM at diagnosis might be an important criterion for the staging (35). None of the tested patients without metastatic diseases showed BM positivity in 4/15 studies (21,30,34,40).

Currently, the ideal method for BM assessment in ES seems to be unknown.

As far as a negative effect of positive tested BM in ES is concerned, studies provide controversial results. Only 3/15 studies, identified by the literature search, found a negative effect of positive BM results (2,37,38). Avigad et al. showed, that positive RT-PCR BM results were correlated with disease progression and recommended the serial monitoring with RT-PCR for the prediction of disease recurrence (2). Schleiermacher et al. also recommended the search for occult tumour cells in the staging of ES patients (38).

In contrary to these recommendations, Zoubek et al. excluded a correlation of RT-PCR BM positivity and early relapse in ES patients (39). Moreover, in the study by Kopp et al., 0/85 patients without any imaging evidence of osseous metastases had BM involvement. These data indicate futility, not utility of BM metastases examination and the authors suggest that BMAB may not be further required in the initial staging process of ES patients considered non metastatic by imaging (40). Referring to this study, Peter M. Anderson published an article evaluating the findings of BM examination in ES. Anderson therefore recommends to first do modern imaging studies like chest CT and FDG-PET and if no metastases are found by these imaging modalities, BM analyses can be considered unnecessary. In those patients with metastatic disease the utility of BM analyses is uncertain (44). In addition, Valvi et al. came to the conclusion, although it is still recommended in ongoing clinical trials, to stop BMAB in non metastatic ES patients (45).

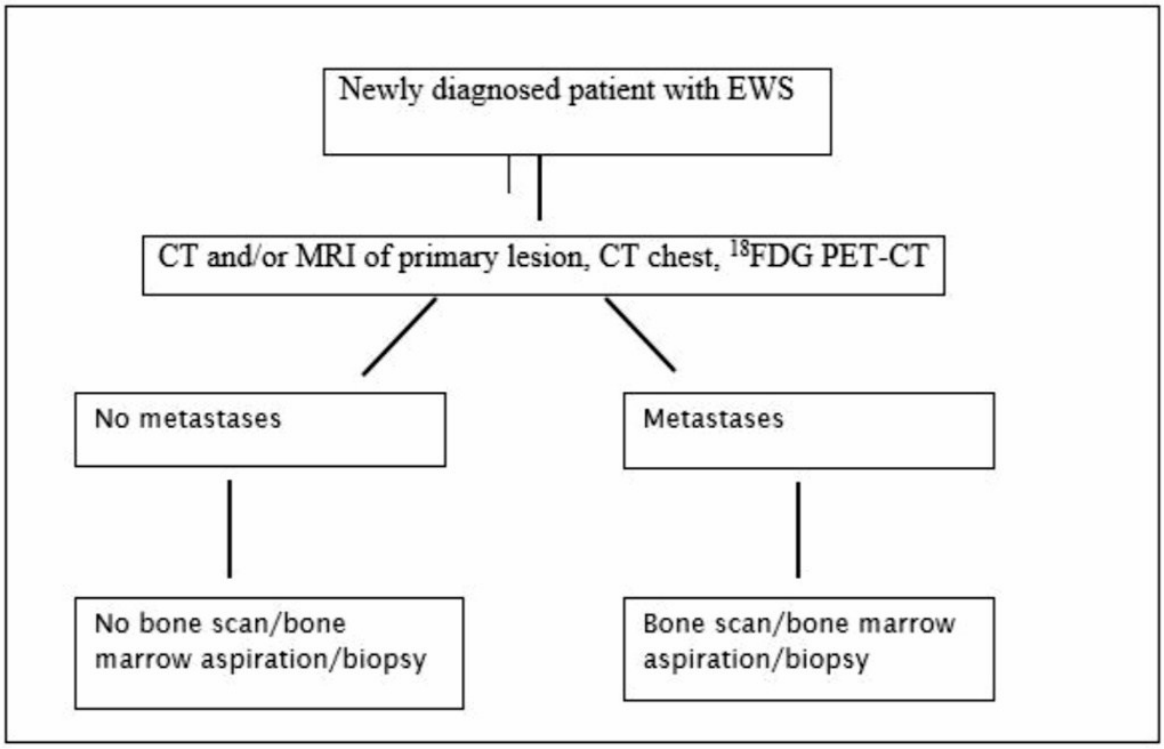

Figure 15 Staging in ES (Valvi et. al. 2015).

5.1 Conclusion

Our study did not show BM involvement at diagnosis in any of our 31 ES patients, neither using histologic, nor with molecular methods. Furthermore, even for the 5 patients with known metastases, all tests were negative. Ours, and other studies, concerning this topic, are limited by the small cohort and by the retrospective study design.

According to our results and due to the previous findings of Kopp et al. we would, like Anderson and Valvi et al., also suggest the elimination of BMAB in the initial staging process of newly diagnosed paediatric and young adult ES patients. Furthermore, the prognostic value of BMAB examination in metastatic patients is also discussed controversially and needs to be further researched.

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