CLINICAL AND MOLECULAR FEATURES OF HIGH-GRADE OSTEOSARCOMA

Jakob K. Anninga

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Zijn is de ziel, is luisteren, is wijken,
is kind worden en naar de sterren kijken,
en daarheen langzaam worden opgelicht.

Uit: "Hebben en Zijn", Ed. Hoornik

voor mijn geliefde Saskia
en mijn lieve kinderen, Nina en Lennard

CONTENTS

ABBREVIATIONS AND TERMINOLOGY

A = adriamycin, doxorubicin
M = methotrexate
Ifo = ifosfamide
P = cisplatin
E = etoposide
MAP = methotrexate plus adriamycin plus cisplatin
BCD = bleomycin, cyclofosfamide and actinomycin-D
MTP = liposomal muramyl tripeptide fosfatidylethanolamine or mifamurtide
OSS = high-grade osteosarcoma
CR = complete remission
PR = partial remission
RR = response rate (CR + PR)
COSS = Cooperative Osteosarcoma Studygroup
IOR = Istituto Ortopedico Rizzoli
IOR/OS = Istituto Ortopedico Rizzoli Osteosarcoma Study
SSG = Scandinavian Sarcoma Group
EOI = European Osteosarcoma Intergroup
FU = follow-up
OAS = overall survival
EFS = event free survival
pGR = pathologic good response
pPR = pathologic poor response

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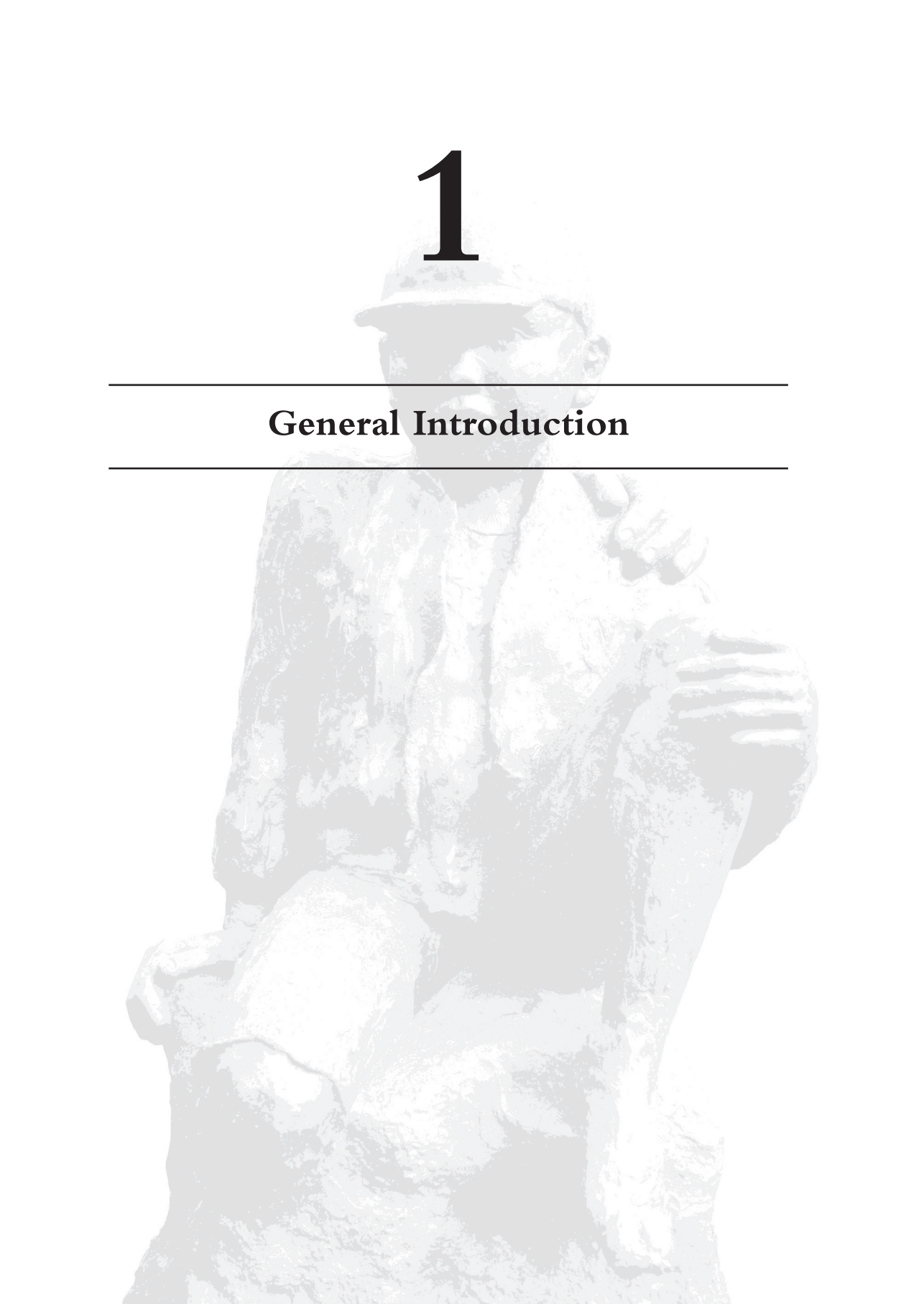
General Introduction



INTRODUCTION

Osteosarcoma is a primary, high-grade malignant spindle cell tumour, in which neoplastic osteoid or bone is produced by the proliferating malignant cells (1, 2). In this introduction epidemiology, genetics and pathology of osteosarcoma will be discussed against the background of this thesis, which focusses on clinical and pathologic aspects.

Epidemiology of Osteosarcoma.

Incidence and gender distribution of Osteosarcoma.

Cancer in general is a major health problem in the world, it is the 2nd leading cause of death for all ages (3). Not only primary cancer, but also the increased incidence of secondary malignancies (4-7) or side effects after treatment contribute to the health problem (8). Osteosarcoma is a rare type of tumour. The proportion of osteosarcoma among all cancers varies with age. In children up to 15 years, osteosarcoma comprises 2.3%(1.6%-2.6%) of all tumours (9-13), in adolescents 15-25 years, 2.6% (11, 14), but in patients older than 25 years, osteosarcoma represents less than 1% of all malignancies (3, 12, 13, 15-21). This variation in occurrence of osteosarcoma is reflected in table 1 and figure 1. Table 1 shows the incidence of osteosarcoma in patients younger than 25 years of age (14, 15, 18, 22-24). The highest incidence is found in children 10-19 year where osteosarcoma accounts for 8.6 new cases per 10^6 population per year (10, 14, 16). Conversely, osteosarcoma in children less than 5 years old is extremely rare. Among 6023 osteosarcoma patients, 105 (1.7%) children were less than 5 years old (25-28). In these young patients, osteosarcoma presents more often in the humerus (up to 32% of the cases), with the telangiectatic subtype more frequently diagnosed than in older patients, suggesting a possible difference in biology compared to osteosarcoma in the later age groups.

TABLE 1.

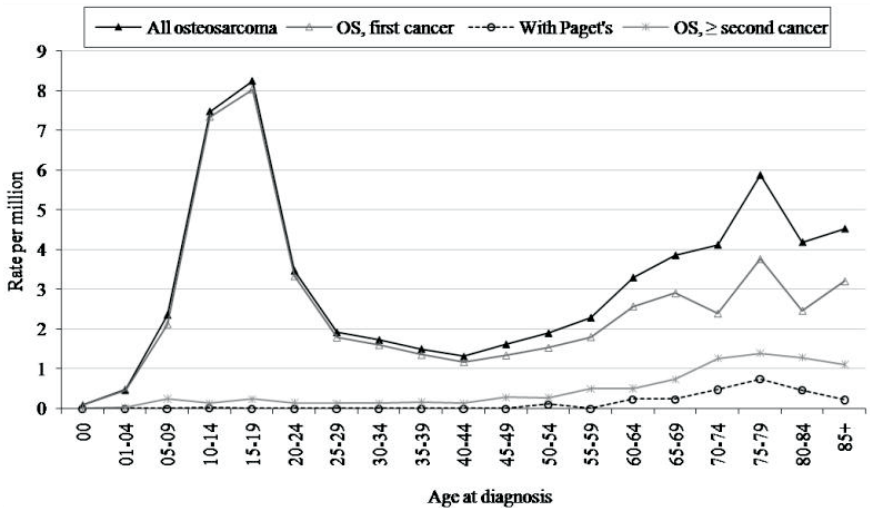
Age adjusted incidence rates of osteosarcoma, given as n/10^6/year^{-1}, as given by different authors in non-SEER series for patients to 25 years of age.*Patients 12-14 yr.

Author (ref)	0-4 y	5-9 y	10-14 y	15-19 y	20-24 y
Eyre (24)	0.4	2.6	5.7	-	-
van den Berg (23)	0.8	3.6	10.9	13.6	-
Stiller (22)	0.2	2.4	6.8	8.4	-
Birch (14, 18)	-	-	7.5*	7.7	3.3
McWhirter (15)	0.0	2.0	5.0	-	-

Figure 1 covers the distribution of osteosarcoma throughout all ages and is based on data from the National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) program (29). The incidence as number of patients per 10^6 population per year shows a triphasic pattern. After a steep raise in the age group of 5-14 years old the first peak of 8.4-8.6 cases per 10^6 persons per year is present in the age group of 15-20 years. This peak is followed by a plateau with a low incidence rate of on average $1.7/10^6$ per year in the age group 25 to 59 years. After 60 years the incidence gradually increases to a second peak, with an annual incidence of $4.9/10^6$ per year in patients of 77-79 years of age. In this age group secondary osteosarcoma and osteosarcoma in the context of Paget's disease contribute for 24% and 9% respectively and differ in localisation (see paragraph 1.2) (29).

FIGURE 1.

Incidence of osteosarcoma, showing a triphasic pattern with a peak during adolescence, a plateau during adulthood and a second peak in older patients (29).



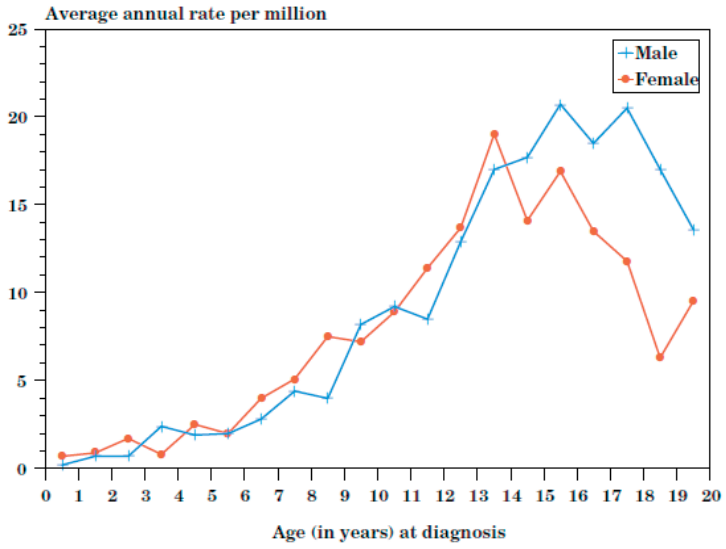
Consistent with other reports, osteosarcoma in older patients differs from that in the younger patients in localization (more non-extremity site), size (larger) and more metastatic disease at diagnosis or secondary osteosarcomas (30-35).

Gender and Osteosarcoma. The SEER data showed clear differences in male-female ratios in different age groups (17, 29). In patients younger than 15 years, the incidence of osteosarcoma in females is higher than in male (figure 2), but after 15 years, this ratio reverses to male

predominance (ratio male:female = 1.34:1). In patients 25-59 years still more males are affected by osteosarcoma (ratio male:female = 1.2:1), but after 60 years of age, osteosarcoma is less common in males (ratio male:female = 0.9:1), except in osteosarcoma in Paget's disease, that affects more males (ratio male:female = 1.58:1).

FIGURE 2.

Incidence of osteosarcoma in males and females, showing that in younger patients the incidence in females in higher than in males, which is reversed in the age group above the 15 years (17).

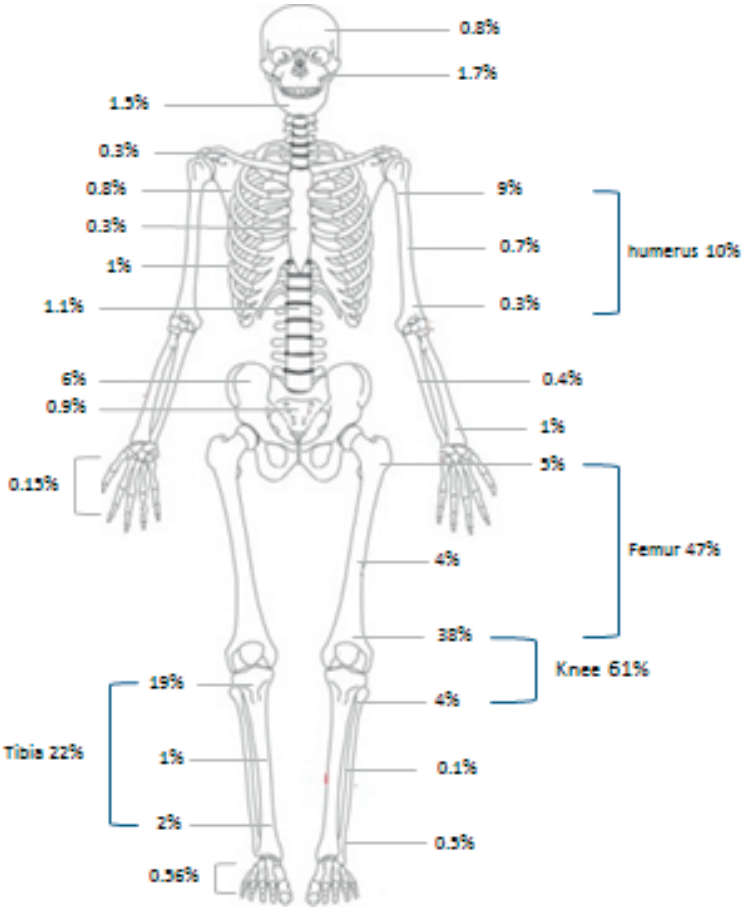


Localization of osteosarcoma

Osteosarcoma in patients is mainly localized in the long tubular bones, as is demonstrated in figure 3. The data of this figure are retrieved from more than 6.000 cases reported in 4 large studies (36-39) and in 2 atlases of bone tumours (40, 41). As can be seen, nearly 75% of the osteosarcomas are located in the long bones of the lower extremity, more than 60% in the metaphyseal region around the knee, and 10% in the long bones of the upper extremity. In the axial skeleton (vertebral column, sacrum, scapula and clavicle) 3% of all osteosarcomas are located, the chest accounts for 1.3%, the pelvis for 6% and the facial bones and skull for 4%. The location, found in the epidemiologic study of Mirabello shows different sites in the age groups, older than 25 years, whereas in the younger age groups the sites are similar to these in the large studies of figure 3.

FIGURE 3.

Distribution of osteosarcoma in the skeleton. Data from 6.454 cases of osteosarcoma (see text for references).



The variable distribution of osteosarcoma in the skeleton in the different ages (table 2) and the diverse histology of the different subtypes (29) indicates that osteosarcoma is not an uniform disease, and behaves different in younger people than in older patients.

TABLE 2.

Difference in osteosarcoma localisation in 3 age groups. This table clearly demonstrates more axial and extra-osseous location in older patients, compared to the younger patients (29).

site	0-24 yr	25-59 yr	≥ 60 yr
Lower Long bones	75%	43%	27%
Upper long bones	11%	10%	8%
Pelvis	4%	11%	19%
Facial bones/skull	3%	10%	5%
Chest	2%	4%	4%
Vertebral column	1%	4%	5%
Extra-osseal	< 1%	7%	19%

Survival in Osteosarcoma

The survival rates shown in figure 4 represent a general trend over the past 4 decades in children and adolescents. These American data do not differ from European studies, as is demonstrated in table 3.

FIGURE 4.

Five-year survival rates for children and adolescents with osteosarcoma, diagnosed during the period 1973-2002, and with follow-up until 2006. Data from the SEER-9 registries and Centers for Disease Control and Prevention (42).

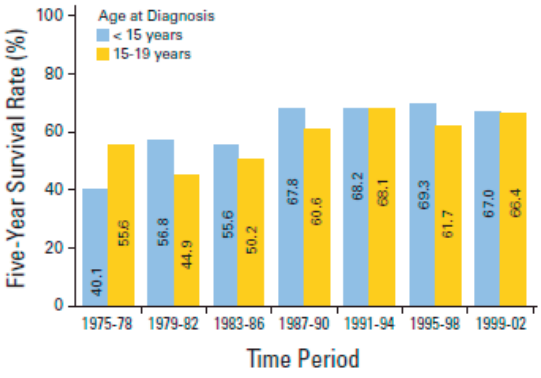


Figure 4 shows an substantial increase in 5-year overall survival from the mid 1970-ties onwards to the mid 1980-ties, due to the introduction of chemotherapy. Since the mid-eighties no further improvement in survival was achieved (9, 22, 29, 42-47). The effect of the development of chemotherapy on survival is discussed in **chapter 2** of this thesis. The mean 5-year overall survival compiled from these data bases is 62% (53%-77%). Overall survival after 5 year follow-up does not decrease much, because less than 5% of all osteosarcoma patients have a late relapse (48-50).

TABLE 3.
Population based studies reporting (5-year or more) overall survival (OAS) in children, adolescents or patients less than 40 years of age with osteosarcoma. These studies were selected on the basis of large international data bases, like SEER, EUROCARE and ACCIS.

Author (ref)	≥ 5 yr OS
Smith 2010 (42)	68%
Mirabello 2009 (29)	60%
Gatta 2009 (11)	77%
Arndt 2007 (47)	58%
Magnani 2006 (46)	61%
Stiller 2006 (22)	55%
Stiller 2006 (51)	53%
Gatta 2005 (9)	59%
Gatta 2003 (44)	66%
Stiller 2001 (43)	60%

Risk factors in Osteosarcoma from population based data.
Risk factors for osteosarcoma will be discussed as reported in population based data bases and in clinical treatment trials. Because this introduction is mainly focussed on the epidemiology of osteosarcoma, the patient- and tumour related factors will be discussed in more detail and for completeness, the treatment related factors will shortly be listed.

Patient related factors

Age

The age-dependent incidence pattern has been discussed earlier in the paragraph about epidemiology of osteosarcoma (paragraph 1.1). With respect to survival, children, younger than 5 years old have a survival of 52%-60% (25-27), but patients older than 60 years of age have reduced survival (22%-58%), due to secondary or Pagetoid osteosarcomas (30-35). It

seems that the presentation of osteosarcoma in the very young and elderly patients is different from the age group between puberty and 40 years of age on the bases of underlying biologic differences. This observation suggests a different biologic behaviour of osteosarcoma in the several age groups.

Length

The pattern of incidence of osteosarcoma (figure 1) suggests a relationship with pubertal growth and development of osteosarcoma. Since Fraumeni reported a relationship between large stature and osteosarcoma more than 40 years ago (52), other authors published a similar relationship between height and osteosarcoma in young patients (53-56). In a pooled analysis taller than average (51^{th}-89^{th} percentile) and very tall (≥ 90^{th} percentile) patients, mainly younger than 25 years, had an increased risk on osteosarcoma (57). The risks, expressed as odds ratios, were 1.35[(1.18-1.54) 95% CI] and 2.60[(2.19-3.07) 95% CI] respectively. A meta-analysis found that patients with osteosarcoma were 0.26 SD[(0.088-0.432) 95% CI] taller than the reference population and that 62% [(57%-67%) 95% CI] of the patients had a height above the median for the reference group (58). These data may suggest that particularly pubertal growth plays a role in the genesis of osteosarcoma.

Pre-malignant conditions as risk factors: Paget; fibrous dysplasia, chronic osteomyelitis and others

Osteosarcomas arising in Paget's disease and in fibrous dysplasia are more frequently occurring, but have to be distinguished from secondary osteosarcoma after radiotherapy or as second neoplasm after chemotherapy, because of a different causal relationship. Osteosarcoma arising in benign precursors, like chronic osteomyelitis, bone infarction and giant cell tumours of bone have rarely been reported (59, 60), and therefore will briefly be listed.

Osteosarcoma in Paget's disease of bone

Paget's disease of is a skeletal disorder, characterized by focal increased bone turnover, occurring in 1%-3.6% of the Caucasian population above the 55 years of age (61-64). The basic defect in Paget's diseases is an increased osteoclastic bone reabsorbtion, with reactive, disorganised bone formation. The cause is unknown, but based on familial history of Paget's disease, which occurs in 5%-40% (65-68), an autosomal dominant inheritance is assumed (63, 69-71). Other Paget's disease related syndromes have been recognized (70, 72), connected to each other by a activating mutation of the RANK-NF-κB pathway, which is the molecular basis of this bone disease (63, 70, 72, 73).

Osteosarcoma as complication of sporadic cases occurs in 0.4%-5.5% of the cases (56, 74-80). Most (80%) of these cases develop in the poly-ostotic form of Paget's disease (75, 76). Sparse case-reports of familial Paget's disease or related disorders documented the development of osteosarcomas (81-84), suggesting a shared susceptibility region on chromosome 18q21-22 between osteosarcoma and Paget's disease (83, 85-87).

Osteosarcoma complicating Paget's disease is localized in the deformed bones. Mainly the large limb bones (femur, humerus, tibia) or the flat bones (pelvis, skull, scapula) are affected in osteosarcoma, secondary to Paget's disease (59, 76, 78, 80). Survival in Paget related osteosarcoma is very low, with a median survival 8-11 months, and a 5-year overall survival of around 10% (78, 80).

Osteosarcoma in Fibrous Dysplasia

Fibrous dysplasia of bone is a focal bone disease where abnormal differentiation of osteoblasts, due to a mutation in the α-subunit of the G-protein on chromosome 20q13 (GNAS), causing a fibrous displacement of bone tissue (61, 88-90). Sixty percent presents as a mono-ostotic form, and 40% as a poly-ostotic disease. In less than 5%, the poly-ostotic form is associated with precocious puberty, other endocrine dysfunctions (hyperthyroidism, hypercortisolism, hyperprolactinaemia and renal phosphate wasting) and café-au-lait skin pigmentation, an entity which is called the McCune Allbright syndrome (89, 91). Symptoms of this bone disease are bone pain, deformations of the bones and in some cases pathological fractures, and occur in 80% before 15 years of age. Osteosarcoma occurs rarely, in 0.4%-1.6% of the cases of fibrous dysplasia in larger series (92, 93), either in the mono-ostotic or poly-ostotic form (56, 94-98). However, this proportion might be overestimated because almost the half of the patients were irradiated for fibrous dysplasia (93). Several case reports of osteosarcoma in the McCune-Allbright (99-102) or in fibrous dysplasia in association with muscular myxoma's, the Mazabraud syndrome have been published (103-106).

Osteosarcoma in other benign conditions

Osteosarcomas have sporadically been reported to occur in chronic osteomyelitis (107, 108), bone infarcts (109-111), Giant cell tumours (112-115), solitary (116-118) or multiple osteochondroma (119, 120), osteogenesis imperfecta (121, 122), aneurysmal bone cysts (108, 123, 124) or solitary bone cysts (108, 125). There is no explanation for a relationship between these benign conditions and the development of osteosarcoma (108).

Prognostic factors in Osteosarcoma

Prognostic factors in osteosarcoma are related to the *tumour features* (volume, location, histological subtype, pathological fracture and the presence of metastases at diagnosis) or *related to its treatment* (chemotherapy regimen, type and timing surgery, completeness of surgery of the primary tumour and, chemotherapy induced histologic response of the tumour). In this introduction large studies have been chosen to avoid missing significant prognostic factors (126)(table 4). In this table the results of the most powerful prognostic factors for overall survival are shown, as result from multivariate analysis in these studies (38, 127-132). An extensive list of molecular markers is not included in this table, because the emphasis of this introduction is clinico-pathological markers.

TABLE 4.
Clinico-pathologic factors related to overall survival in osteosarcoma found to be relevant after multivariate analysis in 7 large studies.

Author (ref)	No. patients	Prognostic factor	HR	95% CI	p-value	comment
Whelan (132)	1067	early time line surgery	1.80	1.17-2.76	0.007	stage IIb
		female gender	0.79	0.64-0.99	0.036	
		distal site tumour	0.66	0.51-0.87	0.003	
		good histol resp	0.48	0.38-0.61	<0.001	
McTiernan (131)	533	Nausea/vomiting gr 1-2	0.37	0.16-0.85	0.020	stage IIb
		good histol response	0.48	0.38-0.61	<0.001	
		Thrombocytopenia gr 1-2	0.49	0.27-0.87	0.016	
		0ral mucositis gr 3-4	0.51	0.29-0.91	0.023	
		distal site in bone	0.66	0.51-0.87	0.003	
		female gender	0.79	0.64-0.99	0.036	
Pakos (130)	1135	metastatic disease	6.59	4.77-9.09	< 0.001	all stages
		poor histol resp	1.67	1.29-2.16	< 0.001	patients > 1990
		surgery: amputation	1.56	1.20-2.03	0.001	
		site bone: tibia	0.66	0.51-0.88	0.004	CT (≥2 drugs)
Bacci (128)	789	protocol CT IOR 1/2/3	2.3/1.5/1.6	1.0-3.4	0.008	stage IIb
		AF elevated	2.1	1.6-2.7	<0.0001	stage IIb
		poor histol resp	2.0	1.6-2.6	< 0.0001	
		tumour volume ≥ 200 ml	1.4	1.1-1.8	0.01	
		surg margin inadequate	1.3	1.0-1.7	0.044	
		age ≤ 14 yr	1.3	1.0-1.7	0.044	
Petrilli (129)	225	poor histol resp	3.15	1.61-6.17	0.001	all stages
		metastatic disease	3.02	1.72-5.29	< 0.001	
		size T > 12 cm	1.93	1.20-3.12	0.007	
Smeland (127)	113	gender male	3.7	1.59-8.66	0.002	stage IIb
		volume T > 190 ml	2.4	1.18-5.05	0.017	
		mean MTX_{t24} > 4500 μM	0.4	0.21-0.88	0.017	
Bielack (38)	1702	residual T > surgery	4.01	2.66-6.04	< 0.0001	for all sites
		poor histol resp	2.44	1.98-3.01	< 0.0001	
		metastatic disease	1.88	1.33-2.65	0.0003	
		axial site	1.87	1.25-1.80	0.002	
		tumour size > 1/3	1.30	1.08-1.56	0.005	only sign for EFS

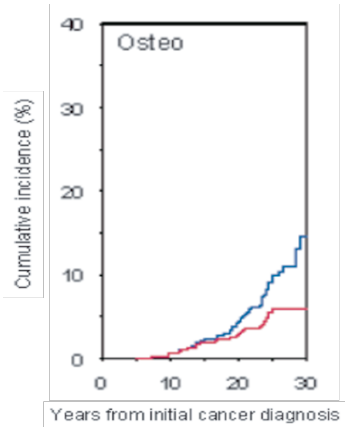
The most important *patient related* prognostic factors for poor overall survival include metastatic disease at diagnosis, large tumour volume and proximal or axial tumour site, whereas chemotherapy induced toxicity was prognostic favourable .
Treatment related prognostic factors are incomplete surgery resulting in residual disease, effectiveness of the chemotherapy regimen and a poor histologic response to pre-operative chemotherapy. Axial site is related to the difficulty in getting a complete resection (38), and early time-line surgery is often necessary in case of early progressive disease (132), which biases the outcome of the disease obviously. Young age as a relevant poor prognostic factor was relevant in the study of Bacci et al (128), but not in other studies (38, 132). Males had an unfavourable outcome relative to females in 2 studies (127, 132), but not in the 4 others (38, 128-130). Although histologic response to preoperative chemotherapy is one of the most important prognostic factors for survival of osteosarcoma, a recent trial from the European Osteosarcoma Intergroup (EOI) did not find a better event-free or overall survival in patients who were treated with a more dose-intensive chemotherapy arm despite the fact that this group showed a significant higher proportion of histologic good responders (133). These results called the use of histological response as measure for outcome into question. This issue will be extensively discussed in **chapter 2**.

Secondary malignancies, second malignancies following Osteosarcoma, and Osteosarcoma as 2nd malignant disease

Contemporary average survival in cancer in general is about 60% (3). In children the overall survival is higher, around 70%-80% (42). Due to the high survival, a growing population of survivors is at risk for the development of second malignancies. The cumulative risk for a second malignant disease is between 3.1% after 25 years to 7.9% after 30 years (6, 134-136). After treatment for cancer in childhood, a lifelong risk on a second malignancy was between 3.3 to 9.2-fold higher than in the general population (5-7, 134, 135). The cumulative incidence of subsequent malignant neoplasms following osteosarcoma is on average 1.3% after 10 years, 5.2% after 20 years and 6% after 30 years (figure 5) (6, 137-144), representing a 3.5-fold higher risk of a malignancy than the general population. Radiotherapy, female gender, genetic factors, such as the Li-Fraumeni syndrome or Rothmund-Thomson syndrome, young age at primary diagnosis and possibly chemotherapy, particularly alkylating agents are risk factors for the development of subsequent neoplasms.

FIGURE 5.

Cumulative incidence of 2nd primary malignancies after osteosarcoma. Blue line is all subsequent neoplasms, red line is subsequent malignant neoplasms (SMN). Estimated Cumulative incidence for a SMN is after 30 years is 6%(3.9%-8.1%) 95% CI. From ref (6).



Bone tumours in general account for 6.5% (3.3%-9.9%) of all second malignancies (5-7, 134, 136), representing a 20-30 fold excess risk compared to the normal population (6, 7, 134). Although the exact incidence of osteosarcoma as secondary malignancy is difficult to establish, because of the different results in the several data bases, the relative risk on a 2nd osteosarcoma after treatment for a previous malignancy is calculated to be 22-133 fold (6, 134, 139, 142, 143, 145-149).

Irradiation as risk factor for osteosarcoma has been recognized since 1929 in radium dial painters osteogenic sarcoma was observed as consequence of their work (150). Osteosarcoma after radiotherapy represents about 3.2% (1.0%-5.5%) of all osteosarcomas (40, 41, 147, 148, 151-161). Radiotherapy-induced osteosarcomas differ from conventional osteosarcomas in that the male-female ratio is equal (male:female = 0.98:1), and present more often in axial sites or skull, and less in extremity sites. In general, the same rate of metastases as in conventional osteosarcoma, around 13% have been reported in 5 studies (147, 154, 155, 157, 161). Two other studies documented an higher proportion of metastases rate at diagnosis, more than 20% (148, 160).

The average latency time between radiotherapy treatment and the occurrence of the subsequent osteosarcoma was 11.5 years but shorter latency times, for example 3-5 years, do not rule out radiotherapy induced osteosarcomas (151, 154-157). In most of these cases genetic causes, such as hereditary Retinoblastoma or the Li-Fraumeni syndrome contribute to the occurrence of these osteosarcomas (153). It also has been suggested that the latency was

shorter after concomitant use of chemotherapy than when radiotherapy was used as single treatment in some studies (145, 152, 156, 159, 160) or in younger patients (151).
Despite previous contradicting reports (148, 151, 162), aggressive treatment with neo-adjuvant chemotherapy and surgery leads to an overall survival of radiotherapy induced osteosarcoma between 40%-50% (147, 154, 157, 163), which is close to the outcome in primary conventional osteosarcoma. Adequate surgical margins are highly important for curative treatment in this subtype of osteosarcomas (147, 154, 160). Due to the localization of secondary osteosarcomas in the axial sites, adequate margins often are difficult to be achieved (147). Multi-agent chemotherapy has successfully been used in patients with secondary/ radiotherapy induced osteosarcoma despite the modifications that need to be given because of prior treatment with cytotoxic agents (147, 154, 160, 161).

Germline mutations and Osteosarcoma

It has been estimated that 1%-10% of all childhood cancers arise in the context of cancer predisposition syndromes (164, 165). Reports of familial clustering of osteosarcoma have been restricted to sparse case-reports and small series (table 5) (166-177).
Osteosarcoma is one of the cancers that has been associated with syndromes like the Li-Fraumeni syndrome, (hereditary) Retinoblastoma, and with RECQL-helicase mutation syndromes like the Rothmund-Thomson-, RAPADILINO-, Baller-Gerold-syndrome and others, as listed in table 6.

TABLE 5.
Case-reports of familial occurrence of osteosarcoma in the literature. Hillmann reviewed 41 other cases in the literature that were not listed in this table (166).

		Type	
Relation	**Age (year)**	**Osteosarcoma**	**Author (ref)**
sister-brother	11 and 12	(telangiectatic) OS	Ottaviani (167)
2 brothers	18 and 21	osteo-/chondroblastic	Chin (168)
son-father	13 and 44	HG conventional/osteoblastic	Longhi (169)
2 brothers	15 and 21	HG conventional/chondroblastic	
brother-sister	14 and 11	osteoblastic (both)	Hillmann (166)
brother-sister	11 and 14	osteoblastic/sclerosing	Danckwerth (170)
2 cousins	11 and 8	Telangiectatic	Nishida (171)
2 sisters	17 and 15	not specified	Miller (172)
sister-brother	11 and 9	not specified	Schimke (173)
daugther-father;	6 and 25	not specified	Swaney (174)
2 brothers	10 and 4	not specified	
daugther-father	13 and 40 (2x)	not specified	Epstein (175)
2 cousins	22 and 18	not specified	Robbins (176)
2 sisters, 2 brothers	11, 15, 20 and 22	HG conventional (3x), fibroblastic	Harmon (177)

Li-Fraumeni syndrome

The Li-Fraumeni syndrome (LFS) is a clinical and genetic heterogeneous cancer predisposition syndrome with multiple early onset sarcomas and other tumours within an individual (proband) and in first and/or second degree relatives in the same lineage (178, 179, 182); OMIM, MIM ID #151623. The most frequent tumours in LFS are osteosarcomas and soft tissue sarcomas, premenopausal breast cancer, brain tumours, adrenocortical carcinoma and leukemias (178, 182, 183).

TABLE 6.
Hereditable syndromes that have been related to the occurrence of osteosarcoma.

Disease/syndrome	Gene	Chromosome	Gene Function	Inheritance	Spectum of Cancers	References
Li-Fraumeni syndrome	p53	17p13.1	TSG	AD	(osteo-)sarcoma, breast cancer, adrenocortical carcinoma	(178-185)
Retinoblastoma	Rb	13q14.1-q14.2	TSG	AD	Retinoblastoma, osteosarcoma, soft tissue tumours	(186-192)
Rothmund-Thomson	RECQL-4	8q24.3	DNA-helicase	AR	osteosarcomas (32%), squamous cell carcinoma, basal cell carcinoma, myelodysplasia	(193-197)
RAPADILINO	RECQL-4	8q24.3	DNA-helicase	AR	mainly osteosarcomas	(198-200)
Baller-Gerold	RECQL-4	8q24.3	DNA-helicase	AR	one osteosarcoma	(201, 202)
Bloom	RECQL-3	15q26.1	DNA-helicase	AR	leukemia/lymphoma, carcinoma's (skin, breast, colon), osteosarcoma, Wilms' tumour	(203, 204)
Werner	RECQL-2	8p12-p11.2	DNA-helicase	AR	thyroid carcinoma, skin-, melanoma, soft tissue sarcoma; osteosarcoma (7%); meningeoma; hematologic	(204-207)
unknown	RECQL-1	12p12.1	DNA-helicase	?	unkown	(193)
Paget Sporadic	SQSTM1	5q35-qter	ubiquitin binding protein	AD	Osteosarcoma, undifferentiated sarcoma, fibrosarcoma	(78, 80, 208)
Paget Familial	TNFRSF11A	18q21-22	TNF-receptor super fam-11	AD	osteosarcoma, Kaposi sarcoma, chondrosarcoma	OMIM 601530, (82, 83, 85, 209)
Familial Expansile Osteolysis	TNFRSF11A	18q21-22	Rank signalling	AD	osteosarcoma	OMIM 174810,(84)
McCune-Albright (MAS)/ Mazabraud syndrome	GNAS	20q13.32	G-protein α- subunit	somatic mutation	osteo-and chondrosarcoma, breast- and thyroid cancer	(99-102, 104-106, 210)

TABLE 7.
Criteria for classical Li-Fraumeni syndrome (LFS), Li-Fraumeni-like syndrome (LFS-L), Chompret and revised Chompret criteria (185).

Classical LFS	Proband with sarcoma at age < 45 yr **AND**
	A first degree relative with any cancer at age <45 yr **AND**
	Another 1st or 2nd degree relative with either cancer at age < 45y **OR**
	a sarcoma at any age
Li-Fraumeni like	Proband with any childhood cancer or sarcoma, brain- or or adrenal cortical tumour at < 45 yr **AND**
syndrome (Birch)	First or 2nd degree relative with a spectrum tumour* at any age **AND**
	First or 2nd degree relative in the same lineage with any cancer < 60 yr
LFS-Chompret	Proband affected by spectrum tumour < 36 yr, **AND**
Criteria	≥ 1 first or 2nd degree relative with a spectrum tumour** < 46 yr or multiple primary tumours **OR**
	Proband with multiple primaries, 2 of which are spectrum tumours and the first at < 36 yr **OR**
	Proband with adreno-cortical tumour at any age
LFS-Chompret	Proband with spectrum (incl lung broncho-alveolar) tumour < 46 yr and ≥ 1 first or 2nd degree relative
criteria revised	with an spectrum tumour** < 56 yr or multiple primary tumours **OR**
	Proband with multiple primary tumours, 2 of which are spectrum tumours and the first at < 46 yr **OR**
	Proband with adreno-cortical tumour or choroid plexus tumour, irrespective family history
	* spectrum tumours are: bone or soft tissue sarcoma, pre-menopausal breast cancer, brain tumour, adrenal cortical carcinoma (leukemia/lymphoma); narrow spectrum cancer with hematologic cancers
	** other than breast cancer if the proband is affected by breast cancer

The classic criteria of for the LFS (178), the Li-Fraumeni-Like syndrome (LFL) (211), the Chompret criteria (212) and revised Chompret criteria (213) are listed in table 7, based on Ruijs (185). Birch reduced the age for families that were suspected for LFS (182). Chompret studied the incidence of unaffected mutation carriers or patients with multiple primary

cancers (212) and Tinat extended the Chompret criteria with respect to age at onset of the LFS-spectrum tumours in order to cover families with identified TP53 mutations (213). Malkin et al. demonstrated that germline mutations of the p53 gene were responsible for the excess of cancers in these families (179). Subsequent studies showed that 50%-85% of the families fulfilling the classical criteria for LFS harbour a germ-line TP53 mutation (180, 184). TP53 negative cases in classical LFS families are explained by de novo mutations, which occur in 7%-20% of the cases (214), posttranslational p53 alterations, abnormalities in regulation or modifier genes, or other genes that are of influence on the phenotype (for review see Malkin 2011(215)). The prevalence of osteosarcoma in families that met the classical LFS-criteria varied between 6%-16% (table 8) (178, 179, 183, 216, 217), not different from when other criteria are use, like the LFL-syndrome (182, 184, 218) or Chompret-criteria were used (181, 185).

TABLE 8.
Incidence of osteosarcoma (OS) in Classical Li-Fraumeni syndrome, Li-Fraumeni like syndrome (LFS/LFL; Birch criteria) and in the Li-Fraumeni syndrome according to the Chompret criteria (LFS-Chompret).

Author (ref)	Li-Fraumeni criteria	prevelance OS in LFS syndrome
Li (178)	Classical LFS	12.0%
Li (216)	Classical LFS	15.7%
Malkin (179)	Classical LFS	6%
Hisada (217)	Classical LFS	12.5%
Nichols (183)	classical LFS	12.1%
Varley (180)	LFS/LFL (Birch)	12.3%
Birch (182)	LFS/LFL	6.8%
Olivier (184)	LFS/LFL	14.9%
Chompret (181)	Chrompet	17.6%
Ruijs (185)	LFS/LFL/Chrompet	8.5%
	LFL/TP53 mut pos	0%
	LFL/TP53 mut neg	3.6%

However, Ruijs found the highest incidence of bone tumours in families with the typical Li-Fraumeni syndrome criteria (8,5%), which was higher than using the LFL-criteria of Birch (0-3.6%) (185). Mutant TP-53 was found in 26.3% of the sporadic osteosarcoma cases (table

9) (219-231), whereas only 4.9% of the investigated patients had a germ-line mutation (232-234). These germline mutations were often reported in patients without typical LFS history. This suggests that although TP53 alterations contribute significantly to the sarcomagenesis of osteosarcoma, familial cases are present in less than 5% of the cases.

TABLE 9.

Proportion of osteosarcoma patients with TP53 abnormalities, detected by southern blot (SB), (PCR)single strand conformation polymorphism (SSCP), immunehistochemistry (IHC), Microsatellite analysis (MSA) or DNA sequencing (DNA-seq). In 2 reports, no correlation with clinical features were reported (NR).

Author (ref)	Number OS	TP53 alteration (%)	Technique	Clinical correlation
Wunder (231)	196	19.4%	SB, SSCP	no
Entz-Werle (230)	54	53%	MSA, PCR	no
Kawaguchi (229)	23	21.7%	SSCP, DNA-seq	no; older age
Gokgoz (228)	272	22.1%	SSCP	no
Tsuchiya (227)	30	50%	SB, SSCP	EFS
Goto (226)	32	40.6%/28.1%	MSA, IHC	PR
Yokoyama (225)	17	23.5%	SSCP	no
Lonardo (224)	83	26.5%	IHC	no
Pellin (223)	19	21.1%	IHC and SSCP	no; older age
Miller (222)	42	30.1%	SB, SSCP	NR
Ueda (221)	18	27.7-61.1%	IHC	no
Toguchida (232)	76	23.7%	SB, SSCP	no
Miller (219)	60	18.3%	SB	NR

From table 9 can also be concluded that no relationship could be established between abnormalities of the TP53 and clinical features, e.g. progression of disease, survival or response on chemotherapy of osteosarcoma. Some authors found that TP53 rearrangements were more frequently encountered in older patients. This suggests that the TP53 is an early event in tumorigenesis, for example inducing chromosomal instability in osteosarcoma (235), rather than an indicator for tumour progression.

The lack of concordance between TP53 mutation and outcome might be related to germ-line variations in TP53 (215, 236) or polymorphisms in modifier genes, such as the MDM2 SNP309 variation (237) although most of the reports in table 9 ruled out normal variants.

Retinoblastoma and Osteosarcoma

Retinoblastoma (Rb) is a tumour of the retina and occurs in 3% of the childhood cancers (238). It occurs in 60% of the cases as a non-heritable and unilateral form, in 10%-15% as an unilateral but heritable disease and in 25%-30% as an heritable, bilateral disease (192, 239).

TABLE 10.

Number of patients with a secondary malignancy (SMN) and osteosarcoma (OS) as proportion of the 2nd maligancy in heritable Retinoblastoma (her Rb) or in sporadic Retinoblastoma (non-her Rb).

				SMN (number)		OS as SMN	
Author (ref)	**No Rb-pat**	**Hereditary RB**	**No SMN's**	**hereditary Rb**	**non-her Rb**	**hereditary Rb**	**non-her Rb**
MacCarthy (192)	1927	809	121	108	13	32	2
Marees (191)	668	298	74	62	12	15	0
Acquaviva (190)	1111	408	38	31	7	10	0
Mohney (189)	180	82	20	17	3	4	0
Wong (240)	1604	961	199	190	9	70	0
Fontanesi (186)	172	65	6	6	0	4	0
	5662	2623 (46.3%)	458 (8.1%)	414 (15.8%)	44 (1.4%)	135 (32.6%)	2 (4.5%)

Based on the fact that the Rb-gene is a tumour suppressor gene, mutations in this region implicate a higher incidence of cancer. Table 10 shows the 10-fold higher incidence of second malignancies in hereditary retinoblastoma than in sporadic retinoblastoma, which is explained by the genetic susceptibility of these patients for subsequent cancers. Osteosarcomas comprise nearly 1/3 of all second malignancies in long term survivors of retinoblastoma, and all but two patients with osteosarcoma fall into the group of hereditary Retinoblastoma. This suggests a relation between the genetic defect in hereditary retinoblastoma and osteosarcoma.
The risk of getting an osteosarcoma after hereditary retinoblastoma is around 500-fold (191, 241). The high risk on secondary osteosarcoma among survivors of retinoblastoma is for a part explained by the use of radiotherapy, although patients who were not treated with radiotherapy also have an high risk on getting an osteosarcoma during their life (242, 243). The different latency periods of osteosarcomas that develop inside and outside the radiation field suggest that multiple genes are involved in the radiation induced tumours in these patients, but one of these gene is the mutated Rb-gene (242). Retinoblastoma has been a model for carcinogenesis since the 2-hit hypothesis has been published by Knudson (244). The penetrance of this autosomal dominant disorder is complete in the bilateral disease, but not in the unilateral disease (245). Non-sense or frameshift mutations and splice mutations account for the most of the gene abnormalities in Retinoblastomas (246-248). Somatic

abnormalities in the Rb-gene were found in 14%-72% patients with osteosarcoma, using either LOH-analysis (234, 249-253), Southern or Northern Blotting (222, 249, 250, 254-256), Immunostaining or gel electrophoresis (table 11) (223, 250, 257). Some investigators found a correlation between Rb-LOH and clinical parameters as survival (234, 252) or metastases formation (257), whereas others could not confirm that (253).

TABLE 11.

Frequency of abnormalities in the Retinoblastoma (Rb) gene in patients with osteosarcoma. See table 9 for abbreviations. LOH is loss of heterozygosity. MI is microsatellite instability.

Author (ref)	patients	technique	mutation rate	comment
Heinsohn (253)	41	LOH	39%	LOH not progn sign
Patiño-Carcia (234)	76	LOH	37%	LOH Rb associated with reduced (E)FS
Benassi (257)	39	IHC; EF	53%	pRb- associated with metastases ($p<0.05$)
Pellin (223)	19	IHC; EF	26%	no progn correlation with clinical parameters
Belchis (251)	18	LOH	72%	LOH or MI in 14/18; MI in 8/18 (44%); LOH in 72%
Feugeas (252)	34	LOH	71%	EFS $Rb^{-/-}$ 43%, EFS $Rb^{+/+}$ 100% ($p=0.008$) and $Rb^{-/+}$.
Miller (222)	37	SB	19%	60% had alterations in Rb and/or p53
Wadayama (250)	63	LOH	63%	LOH not necessarily correlated with
		SB	29%	inactivation Rb gene at protein level
		IHC	54%	
Scholz (256)	14	SB	14%	1 DNA abn samples also had no prot exp
		NB	4/8 -, 2/8 -/+, 2/8?	5 prot def samples had no Rb abn
Araki (254)	23	SB; NB	35%	1 $SB^{-/+}$ had also $NB^{-/+}$; 4 cases $NB^{-/-}$ were $SB^{+/+}$
Wunder (255)	12	SB; NB	50%	All $Rb^{-/-}$ or $Rb^{-/+}$ abnormal Rb-RNA expression
Toguchida (249)	30	LOH	64%	40% of the OS patients has Rb abn.
		SB	43%	LOH and SB do not correlate

RECQL-Helicases mutation syndromes

RECQL-helicases are a highly conserved family of genes and proteins that have an important role in adapting to cellular stress, and thereby maintaining genomic stability, preventing epigenetic drift and early senescence (204, 258-260). Examples of abnormal repair in humans

are present in rare genetic disorders of the RECQL genes as listed in table 6. Of the 5 known RECQL-genes in humans, 3 are known with a recessive inherited gene mutations, leading to the Werner syndrome (RECQL-2 mutations) (261), Bloom syndrome (RECQL-3 mutations) (203) and the RECQL-4 mutation spectrum syndromes: Rothmund-Thomson (RTS), RAPADILINO and Baller-Gerold syndrome (reviewed in Lindor 2000 (193)). Mutations in the RECQL-1 and RECQL-5 genes are not known to cause syndromes or diseases, particular no cancer. Particularly in the RTS, the incidence of osteosarcoma is extremely high (194-197). Osteosarcoma in patients with RTS behaves almost similar as in non-syndrome patients. Age at presentation was lower in some series (195, 262, 263), but location, response to pre-operative chemotherapy and outcome was like sporadic osteosarcoma. However the proportion metachronous or secondary tumours in RTS patients was 17% compared to the 2.6%-5.4% in sporadic osteosarcomas, reflecting the genetic basis in syndromatic patients (263).
Not all germline RECQL-mutations have the similar genetic consequences for the development of osteosarcoma. In the Werner syndrome (RECQL-2 mutation), the incidence of osteosarcoma is 7.6% of 157 cancers (205), the peak age was 35-55 years and the osteosarcomas were located in unusual sites in the skeleton, for example the patella, the radius or the foot (205-207, 264). The incidence of osteosarcoma in the Bloom syndrome (RECQL-3 mutation) was low, not more than 2%, but still higher than in the general population (203). Overall, these syndromes with germline mutations in RECQL helicases 2,-3 and -4 predispose to an increased risk of osteosarcoma (265).

Paget's disease, Familial Osteosarcoma and the McCune-Allbright/Mazabraud syndrome
For osteosarcoma in Paget's disease, see paragraph 1.4.2., for familial osteosarcoma see the introduction of this paragraph. Osteosarcoma in McCune-Allbright/Mazabraud syndrome, see paragraph 1.4.2.2.

Pathology
The term osteosarcoma historically developed from osteogenic sarcoma (266) which encompassed all tumours derived from bone. From the period after 1946 osteosarcoma is defined as a primary, intramedullary high-grade bone tumour producing malignant osteoid (267, 268).
Since the first 2 editions of the WHO classification used a similar framework, based on histologic criteria (270) progress in biological and genetic understanding of these malignancies was made. In 2002 the third revision of the classification of bone and soft tissue tumours was published, which integrated morphological data with tumour specific cytogenetic and molecular data (269). Table 12 shows the different subtypes of osteosarcoma, based on the WHO-classification 2002. The unusual histological forms of high-grade conventional osteosarcoma will be discussed here in more detail, because in **chapter 6** of this thesis some of these rare subtypes are more present among the patients.

TABLE 12.
Subtypes of osteosarcoma according to the site in the bone (269)

SITE IN BONE	GRADE of MALIGNANCY	TYPE	SUBTYPE
Intramedullar	*High*	Conventional OS	Osteoblastic
			Chondroblastic
			Fibroblastic
		Unconventional OS	Osteoblastic-sclerosing
			Osteoblastoma resembling
			Chondromyxoid fibroma-like
			Chondroblastoma-like
			Clear-cell
			Malignant fibrous histiocytoma-like
			Giant cell rich
			Epitheloid
		Teleangiectatic OS	
		Small Cell OS	
		Secondary Osteosarcoma	M.Paget
			Post-Irradiation
			In various bone diseases
	Low	Low Grade Central OS	
Surface	*High*	High-Grade Surface OS	
	Intermediate	Periosteal (Juxta Cortical Chondroblastic OS)	
	Low	Parosteal (Juxta-cortical OS)	
Intra-cortical	*High*		
Extra-Skeletal	*High*		

Conventional High-Grade Osteosarcoma

The proportion of high-grade conventional osteosarcoma is between 70%-90% of all osteosarcomas in larger studies (2, 269, 271, 272). Histologically, the malignant cells of an osteosarcoma consist of anaplastic, pleomorphic spindle cells, although other forms can be present like epitheloid, plasmacytoid, ovoid, round or fusiform cells or the tumour may contain multinucleated giant cells. The malignant osteoid, formed by the pleomorphic tumour cells, is highly variable in thickness and ranges from tiny amounts to a frank ossifying tumour, as is visible on a plain radiograph. Besides osteoid, high-grade conventional osteosarcoma can also produce cartilage and/or fibrous tissue. Depending on the amount of matrix, conventional osteosarcoma is divided into osteoblastic (50%), chondroblastic (25%) and fibroblastic (25%) (see also table 12). This subdivision has no prognostic value, because the outcome in these three subgroups did not differ, despite a significant better response rate among the fibroblastic group (271).

Unconventional types of high-grade conventional Osteosarcoma

Sclerosing subtype Osteosarcoma

The sclerosing subtype of osteosarcoma has been classified by most authors under multifocal osteosarcoma (273-281). This multifocal variant was diagnosed in young patients, age 5-16 years, and is characterized by multiple foci of high-grade osteosarcoma, sclerotic on the radiographs and all localized in the metaphyseal part of the long tubular bones. The clinical behaviour of this variant is very aggressive, most patients died within 1 year after diagnosis with widespread disease. However, not all sclerosing variants are clinically highly malignant (282-289). These variants of sclerosing osteosarcoma are of low to intermediate grade, occur generally in older patients, are located predominantly in multiple sites of the axial skeleton and skull, either with or without involvement of long bones or occur as recurrent disease. This subtype is mentioned here only for completeness to define this subtype of osteosarcoma appropriately.

Osteoblastoma-like subtype of Osteosarcoma

The osteoblastic resembling subtype of osteosarcoma has been estimated to occur in less than 1.5% of all osteosarcomas (290). The localization in 33 cases differs from HG conventional osteosarcoma. Thirty nine percent of the cases are found in the axial skeleton and skull, and 61% in the appendicular skeleton (290-296). The most common involved bone was the tibia, and this subtype presents often in unusual sites like the foot (290, 291, 296) or rare locations in the bone, like the condyles of the femur (294, 295). As other high-grade osteosarcoma, osteoblastoma-like osteosarcoma has a similar tendency to metastasize as the conventional subtype of osteosarcoma (290).

Chondromyxoid fibroma like Osteosarcoma
This type has been described in 2 case reports (297, 298) and by Mirra (299). Although this subtype has been described as a low grade osteosarcoma (297, 299), local and systemic recurrences were described in both patients, with an unusual metastases in the left atrium (297).

Chondroblastoma-like Osteosarcoma
Schajowicz published one case of chondroblastoma-like osteosarcoma in the tibia of a 12-year old boy with a tumour located in the diaphysis of the femur (300). Clinically, the tumour was highly malignant as was demonstrated by a fast local growing and recurrence tendency and the development of pulmonary metastases, one months after resection.

Clear Cell type Osteosarcoma
Four cases of the clear cell type osteosarcoma have been described, in 3 children and one adult patient (301, 302). All lesions were located around the knee, in the meta-epiphyseal part of the distal femur in 3 cases or proximal tibia in one case. Two of the 4 patients died from metastases, and the follow up of the other 2 was less than 1 year.

Malignant Fibrous Histiocytoma type of Osteosarcoma
This subtype of osteosarcoma needs to be distinguished from Malignant Fibrous Histiocytoma of bone (MFH) (303-306). Nine patients were reported, aged 8-75 years, with lytic lesions in the meta-epiphyseal part of the distal femur (n=6) or the proximal tibia (n=2) (304, 305). MFH-like osteosarcoma has no p53 overexpression and a low Ki-67 labelling index compared with conventional osteosarcomas or MFH of bone (306). The reports are conflicting with respect to clinical aggressiveness. Whereas Ballance reports the development of pulmonary metastases in 4 of 6 patients within one year (304), Naka finds an 5-year overall survival of 67% in 7 cases (306).

Giant cell rich Osteosarcoma
Giant cell rich osteosarcoma has been reported to occur in 0.6%-3% of all primary osteosarcoma and is defined as an undifferentiated sarcoma with scanty tumour osteoid and an abundance of osteoclast-like giant cells (41, 307, 308). Fifteen cases were described in detail (307-311). All but one showed ill-defined lytic lesions, with a wide zone of transition, located in the meta-diaphyseal part of the femur(n=10) or tibia (n=4) and one was located in the navicular bone of the foot (311). The mean age was 21(6-41) years, older than in conventional high-grade osteosarcoma, but younger than in giant cell tumours of the bone. The differential diagnosis is high-grade conventional osteosarcoma with giant cells (more abundant osteoid), telangiectatic osteosarcoma (septae with sarcomatous cells), giant cell tumours of bone (epiphyseal location in the bone) and aneurysmal bone cyst (no malignant cells) (307, 308, 311). Prognosis is difficult to give, because the few well documented cases, but seems to be similar as in conventional high-grade osteosarcoma (307, 312)

Epitheloid Osteosarcoma
In the epithelial subtype of osteosarcoma, epitheloid-differentiated osteoblasts are arranged in nesting or gland-like structures, admixed with osteoid producing malignant spindle cells, forming a biphenotypic tumour (313, 314). The histological picture resembles (metastatic) carcinoma (315, 316). Variable immunohistochemical expression of cytokeratins, vimentin or epithelial membrane antigen have been reported in these cases (313, 316-320). Overall a male predominance is observed, an average age of 29 years, ranging from 4.5-66 years, and most often, the osteosarcomas are located in the femur (313, 315-323). Apart from a poor outcome in the Rosette-formed epithelial subtype, prognosis is similar as in high-grade conventional osteosarcoma (324).

Teleangiectatic Osteosarcoma
This variant of osteosarcoma is defined as a one forming tumour, characterized by large spaces, filled with blood with or without septa (325). This type has been reported to occur in 5% (0.9%-11%) of all cases of osteosarcoma (2, 40, 325-330). Radiologically, it is an aggressive, purely lytic lesion, with destruction of the cortex, periosteal reaction, soft tissue invasion and a relatively high proportion of pathological fractures (40, 325, 328, 330, 331). Mineralization, typical for osteosarcoma, is scant on plain films but can best be shown by CT. MRI is effective in distinguishing a telangiectatic osteosarcoma from other types or benign blood filled lesions by marrow replacement on T1-, and high signal on T2-weighted images (40, 332). With contemporary neoadjuvant chemotherapy, outcome in patients with this variant are similar (328, 330, 333) or even better (329) than conventional osteosarcoma.

Small cell Osteosarcoma
The small cell variant of osteosarcoma is composed of small cells with a variable degree of osteoid production (334). The mean incidence rate from 4 different studies is 2.2% (1.1%-4%) (335-337). Males and female were equally affected in 147 cases, dissimilar like conventional osteosarcoma (40, 335-337). Age distribution shows the highest incidence in the adolescent and young adult group, and the localization in the skeleton were similar like in conventional osteosarcoma, with a relatively a high proportion (18%) located in the humerus. This variant of osteosarcoma has to be distinguished from Ewing sarcoma, another small cell tumour of bone, which is sensitive for radiotherapy, in contrast to the small cell osteosarcoma. This can be done using the characteristic translocation t(11;22) in Ewing sarcoma, which is not present in small cell osteosarcoma. Although small cell osteosarcoma seems to be sensitive for platinum analogs (335), survival is worse than in HG conventional osteosarcoma, although this is based on older reports, with less effective medical treatment (335, 337, 338).

Low-grade central Osteosarcoma
A low grade central osteosarcoma is a well differentiated subtype, arising from the medullary cavity of the long tubular bones (339). This subtype has a better prognosis than its high-grade counterpart but also has another location than other non-high-grade subtypes, parosteal and

peri-osteal osteosarcoma (339-341) and accounts for approximately 1-2% of all osteosarcomas (40, 339-341). The age at presentation is generally around the 3rd decade, and patients have a prolonged history of symptoms of on average 1 year of nonspecific pain with or without swelling in the diaphyseal site of the femur or tibia. Occasionally, a low grade central osteosarcoma is diagnosed in the small bones of the hand or foot (340-342) or in the flat bones of the ribs (340, 342, 343) or skull (340, 344). Histological, the low grade osteosarcomas are hypo-to moderate cellular spindle cell tumours with slight atypia and occasional mitotic figures, irregular bone formation in a parosteal, desmoid or fibrous dysplasia like pattern (340). If this tumour is inadequately excised, progression into higher grade of malignancy occurs in 15% of the patients with recurrence (340, 341, 345, 346) with the potential for developing distant metastases and leading to death. Dedifferentiation not only occurs at recurrence, but has been reported at diagnosis in rare instances (347-349).

Surface Osteosarcomas

Surface osteosarcomas arise by definition from the surface from the bone, and can be of high-grade (high-grade surface osteosarcoma, also known as juxtacortical osteosarcoma), intermediate grade (periosteal osteosarcoma; juxtacortical chondroblastic osteosarcoma) or of low grade (parosteal osteosarcoma or juxtacortical osteosarcoma) (350-352).

TABLE 13.
Characteristics of 3 types of surface osteosarcomas. FU follow-up; LR local relapse; SR systemic relapse; OAS overall survival; DOD death of disease; OS osteosarcoma.

	Parosteal	Periosteal	High-Grade Surface
incidence	4%	1.5%	< 1%
male:female	1:1.4	1.4:1	4:1
peak incidence (decade)	3rd	2nd	2nd-3rd
symptoms	swelling ≥ pain	swelling ± pain	pain + swelling
duration of symptoms	1-5 yr	0.1-2 yr	< 1 yr
involved bones	femur, tibia	tibia, femur, ilium	femur, tibia, radius
involved site bone	distal meta-diaphysis	shaft, prox/dist	shaft/dia-metaphyseal
specific X-feature	broad based sclerotic, lobulated mass	small, cortical, lytic, extending soft tissue	broad based T, cortex destruction, periost ++, medullary involvement
metastases at Diagnosis	no	no	no
metastases during FU	30% LR, 6% SR	LR in 22%; 17% SR	12-26% LR, 5y-OAS 46-82%
preferred treatment	wide excision	wide resection	as HG conventional OS
prognosis	91% 5y OAS	17% DOD; 10y OAS 84%	as HG conventional OS
typical histology	hypocellular stroma with absent to moderate atypia (≤ 20%) between well formed bony trabeculae	appearance of moderately differentiated chondroblastic OS	high-grade anaplastic tumor cells, as in HG conventional OS

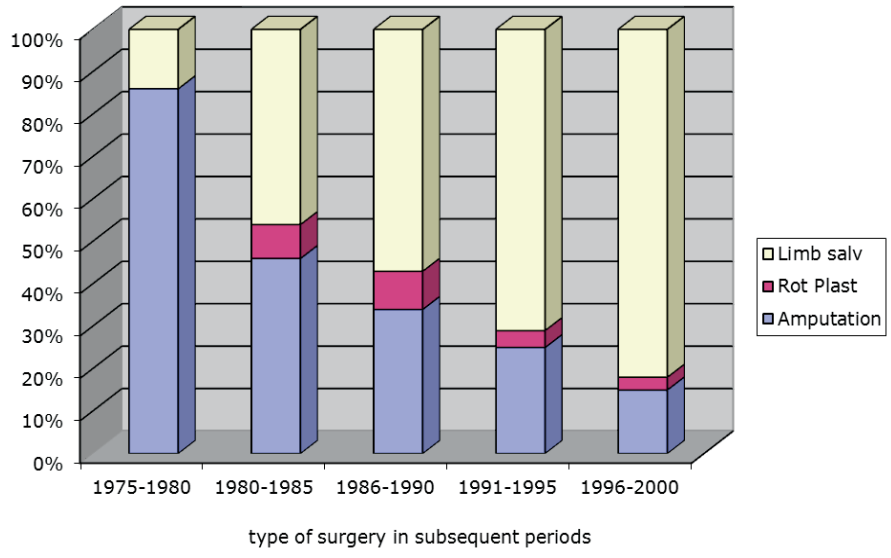
Table 13 shows the clinic-pathological differences between the subtypes of surface osteosarcoma. As is shown in this table parosteal osteosarcoma is the most frequent type (353-356), whereas the periosteal osteosarcoma and high-grade surface osteosarcoma accounts for 1.5% (357, 358) and less than 1% respectively (359, 360).
Despite the lower grade of parosteal and periosteal osteosarcoma, focal dedifferentiated areas into higher grade have been described(353, 355, 361-363), which occur more often in recurrences in these tumours(355, 356, 364, 365).

Treatment of Osteosarcoma

Modern treatment of osteosarcoma consists of pre-operative and postoperative (neoadjuvant) chemotherapy providing systemic tumour control in conjunction with adequate resection of the tumour. Because an extensive meta-analysis of chemotherapy is presented in **chapter 2**, the surgical treatment will only be discussed shortly here.
For local tumor control, limb salvage has replaced ablative surgery as surgical option in the majority of patients (figure 6), it became clear that chemotherapy contributes to local treatment as well (163, 366-370).

FIGURE 6.

Different types of surgery in subsequent 5-year periods.



Limb salvage is a challenge due to the diversity of sites in which the tumours arise, the extension of the tumour to adjacent soft tissue, the proximity of neurovascular structures and the age (356, 371-373). This raises the question about safety in terms of local recurrence and survival after such a procedure.
Table 14 shows an average rate of local recurrence of 6% among more than three thousand three hundred patients, nearly 2x higher after limb salvage (7.8%) than after ablative surgery (4.0%) or rotation plasty (3.8%).

TABLE 14.
Local recurrence rate (LR) among osteosarcoma patients who underwent an amputation, limb salvage surgery or and rotation plasty.

	No.	LR rate (%) after			
Author (ref)	**patients**	**amputation**	**limb salvage**	**rotation plasty**	**total**
Bacci (163)	1126	2.8	6.3	5.6	5.3
Rodriguez-Galindo 2004	397	6.7	6.0	-	6.5
Weeden (374)	559	2.4	10.2	0	7.5
Brosjo (375)	223	3.1	10.5	-	6.3
Bielack (368)	440	2.9	8.8	incl Amp	5.2
Rougraff (376)	227	5.5	10.9	-	7.4
Glasser (366)	279	1.9	9.2	-	6.5
Eckardt (377)	116	7.8	3.8	-	5.2
total	3367	4.0	7.8	3.4	6.1

Survival after local recurrence is poor, on average 21% (5-41%), especially in the presence of concurrent systemic metastases (163, 370, 378-380). However, table 15 shows that the outcome after limb salvage does not fare worse compared with amputation or rotation plasty, indicating limb salvage is a safe procedure.

TABLE 15.
Five year survival in 5 studies of patients who underwent an amputation, a limb salvage procedure or an rotation plasty.

		5-year overall survival			
Author (ref)	**No pats**	**amputation**	**limb salvage**	**rotation plasty**	**p-value**
Bacci (381)	1.148	53%	61%	58%	< 0.001
Bielack (38)	1.702	66%	70%	-	0.089
Weeden (374)	559	42%	61%	59%	< 0.01
Rougraff (376)	227	51%	48%	-	0.84
Glasser (366)	279	73%	80%	-	-
total /mean	3.915	62%	64%	59%	

Factors that significantly relate to the risk of local recurrence were the application of chemotherapy (367, 368, 380), histologic response on pre-operative chemotherapy (163, 367, 368, 379), tumour volume (379) and surgical margin (163, 367, 368, 379, 382). An adequate (radical or wide) surgical margin has the lowest risk for local recurrence, whereas inadequate (marginal or intralesional) margins have high local recurrence rates in most studies, up to 24%(163, 383). Poor histological response has a high additional risk for local recurrence. Particularly when also inadequate margins are present, local recurrence rates can raise to 16%-31% (163, 368, 383). In these studies nearly all patients had chemotherapy, which was essential in limb salvage.
It can be concluded that limb salvage surgery is feasible in contemporary osteosarcoma treatment, but only after pre-operative chemotherapy has been given, and adequate surgical margins can be achieved.

AIM OF THIS THESIS

Despite the enormous number of papers about osteosarcoma that has appeared last decades, there are still many unanswered questions about this bone tumour. One question is whether or not osteosarcoma is one disease or has to be considered as a complex of different disease entities. If osteosarcoma consists of different disease entities, the consequence of that conclusion would be not only a different treatment approach, but it raises then also the question how these different forms are related to each other. For example, osteosarcoma is considered as a high-grade malignant disease, and modern treatment protocols are based on surgical excision of the tumour in combination with neo-adjuvant chemotherapy. However,

16% of the patients will survive, despite the fact that they were treated with local treatment only (384, 385). Around 20% of the patients with recurrent disease can be cured with surgery only (386), while relapsed disease is considered as one of the most disastrous presentations of osteosarcoma. These clinical experiences may suggest that there exist some subgroups of osteosarcoma, that have a less malignant behavior than others. If indeed a less malignant subgroup could be defined, the next question should be whether or not chemotherapy could be reduced or even avoided in this group, in order to prevent the serious side effects of chemotherapy, stressing the importance of this question.
So far, we are not able to distinguish osteosarcomas with unfavorable or favorable outcome by clinical parameters. It might be asked if that would be possible, based on different molecular signatures. Questions about the molecular behaviour of osteosarcoma are not only important from scientific point of view, but may reveal insight in the development of new treatment options. Studies about the pathophysiology of osteosarcoma are hindered by complex genetic changes in this tumour (387-393). Although concerns were raised about the use of *in vitro* models in osteosarcoma research (394, 395), recently it was demonstrated that research on osteosarcoma cell lines is representative for clinical osteosarcoma (396). However, it remains important to understand the complete picture of osteosarcoma that clinical data, filtered by statistical systems need to be transferred into the laboratory and the other way around.
The present study was undertaken to meet some of these questions in order to understand the evaluation of treatment for high-grade conventional osteosarcoma at usual and unusual sites in an attempt to get evidence from clinical and pathological point of view if therapy can become more tailored. Furthermore, we wanted to study a possible relationship between benign osseous lesions and high-grade malignant osteosarcoma using a high throughput method.
To meet these questions, in **chapter 2** the background of chemotherapeutic treatment was investigated with emphasis on chemotherapy. Evidence was found that the drugs that are used in modern treatment protocols indeed are valuable, but are limited to four effective drugs. In this study trials in stage IIb osteosarcoma were investigated, in order to get as much homogeneity as possible. Treatment in metastatic or relapsed osteosarcoma is poorly defined, experience based but no randomized trials were found in these subgroup of patients. In this meta-analysis a new statistical tool, a multivariate random effects analysis with survival data at several time points (Fiocco et al) was applied, heading the heterogeneity of the used studies for this paper. The value of salvage of patients with a poor response the pre-operative chemotherapy is also critically reviewed in this chapter.
Chapter 3 goes deeper to the genetic basis of osteosarcoma. In this study, we tried to find a RNA-expression profile which distinguishes histologic response on pre-operative chemotherapy and/or outcome of patients. A small size of patient samples was available for this study due to the limited amount of tumour tissue at diagnosis, necrosis of tumour in patients with good histological response and uniformity of treatment of patients. In this study RNA expression of tumour tissue was compared with the expression profile of benign osteoblastomas, mesenchymal stem cells that were altered into osteosarcomas and mesenchymal stem cells that were altered into osteosarcomas. Being at the start of the this high

output techniques, we were faced with problems of the analysis and interpretation of these approach. However, some important issues were concluded from this pilot and the technique is nowadays more routine.

Chapter 4 deals with the background of the statistical analysis is given in a paper of Goeman et al, who is working on the department of biostatistics in the Leiden University.

Chapter 5 describes the transition from clinical to laboratory investigations. In this chapter, the expression of the *HER*-2 oncogene in osteosarcoma is critically reviewed. The aim of this study was to investigate whether or not the *HER*-2 oncogene can be demonstrated by several techniques, as Real-time PCR (RT-PCR), Immunohistochemistry (ICH) and Fluorescent in situ Hybridisation (FISH). The importance of this oncogene lies in the fact that treatment whit the monoclonal antibody *Herceptin*, which is regularly used in Breast carcinoma, is supported when the results of such an investigation confirm the presence of this receptor on the membrane surface of the osteosarcoma cells.

In **Chapter 6** a study is presented which comprises osteosarcoma in rare localizations: the hand and the feet. It is not well-known if osteosarcomas in these unusual sites need a similar treatment approach than osteosarcoma elsewhere in the body. Is surgery in addition to chemotherapy acquired for these sites? The assumption may be that local excision only will be sufficient to treat osteosarcomas in the hand and foot, because it can be recognized quite early. However, the outcome and factors that influence the outcome of osteosarcomas in these locations are not exactly known, and no definite conclusions about appropriate treatment can be given before such is better described.

Chapter 7 is concerned with factors that determine the outcome of patients with pulmonary metastasized osteosarcoma. Our questions in this single institute experience were mainly if repeated surgery is valuable in these patients and if factors could be defined for resectability of the tumours in these patients. Unfortunately the series was too small to make a definite conclusion about the role of repeated chemotherapy, but a suggestion was done about the value, based on the relationship between vitality of the tumours and the outcome.

In **Chapter 8** a summary of the chapters is given and some concluding remarks about future research are made, which is in Dutch in **chapter 9**.

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2

Chemotherapeutic adjuvant treatment for osteosarcoma: where do we stand?

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ABSTRACT

Aim

Since the introduction of chemotherapy, survival in localised high-grade osteosarcoma has improved considerably. However there is still no worldwide consensus on a standard chemotherapy approach. In this systematic review evidence for effectiveness of each single drug and the role of response guided salvage treatment of adjuvant chemotherapy are addressed, whereas in a meta-analysis the number of drugs in current protocols is considered

Methods

A systematic literature search for clinical studies in localised high-grade osteosarcoma was undertaken, including both randomized and non-randomized trials. Historical clinical studies from the pre-chemotherapy era were included for comparison purposes.

Results

Nine historical studies showed a long-term survival of 16% after only local treatment. Fifty single agent phase II studies showed high response rates for adriamycin (A, 43%), ifosfamide (Ifo, 33%), methotrexate (M, 32%), cisplatin (P, 26%) but only 4% for etposide (E). In 19 neo-adjuvant studies the mean 5-year event free survival (EFS) was 48% for 2-drug regimens and 58% for ≥ 3 drug regimens, with an 5-year overall survival (OAS) of 62% and 70%, respectively. Meta-analysis showed that ≥ 3 drug regimens including MAP(Ifo) had significant better outcome (EFS: HR=0.701 (95% CI: 0.615 – 0.799); OAS: HR=0.792 (95% CI: 0.677 – 0.926) than 2-drug regimens, but there was no significant difference between MAP and MAPIfo (or plus etoposide). Salvage of poor responders by changing drugs, or intensifying treatment postoperatively has not proven to be useful in this analysis.

Conclusion

Meta-analysis in patients with localised high-grade osteosarcoma shows that 3-drug regimens, for example MAP are the most efficacious drug regimens.

INTRODUCTION

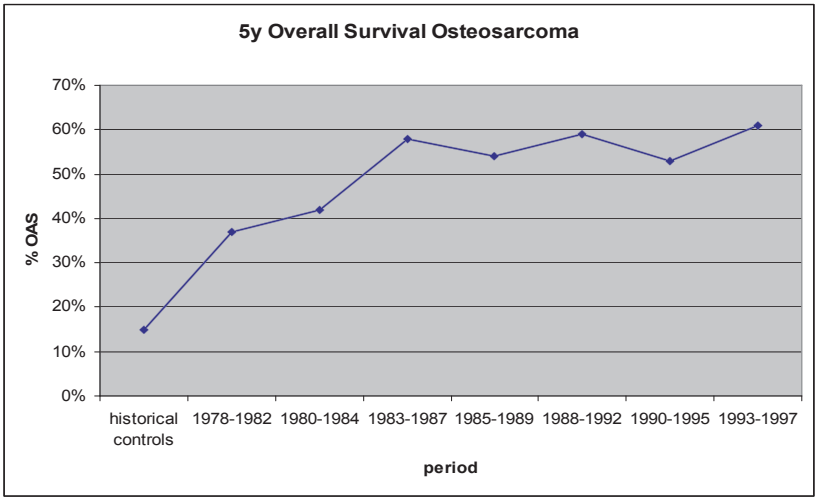
High-grade osteosarcoma is the most frequent primary malignant bone tumour (1) and occurs predominantly during puberty with a second peak in the elderly (2-4). The annual incidence rate is on average 4.4 per 10^6 people aged 0-24 years, 1.7 per 10^6 people aged 25-59 years and 4.2 per 10^6 in people ≥ 60 years. Osteosarcoma typically is a tumour of the extremities: 78% is localized in the lower extremity, with 64% around the knee and 10% localized in the humerus (5-10). Long term survival in localised osteosarcoma has increased substantially from 10-20% when surgery as single treatment was given before the 1980's up to 50%-60% from 1985 onwards. However, since then no substantial further improvement of survival is observed (4, 11-16) (Fig 1). Children have a 5%-10% better survival rate than patients up to 50 years, while patients older than 60 years have a survival rate of only 24% (4, 15, 16). The improvement in survival has been attributed to the use of intensive multi-agent chemotherapy given in combination with advanced surgery. In modern treatment schedules, usually a combination of doxorubicine (adriamycin (A)) and cisplatin (P), with or without high-dose methotrexate (M) and/or ifosfamide (Ifo) and/or etoposide (E) are being used.

Our aim is to address several questions. What is the evidence for the effectiveness of each of these drugs as single agent? How many drugs should at least be given to accomplish the most effective treatment regimen? What is the value of increased dose intensity or salvage treatment after a poor pathological response on preoperative chemotherapy?

Due to the presence of heterogeneous studies including the design, regimen, follow-up or definitions of histological response, a random effects meta-analysis was employed on a number of selected studies (17). The ultimate goal of the analysis was to define the most efficacious treatment in localised osteosarcoma.

FIGURE 1.

Reported 5y-overall survival (% OAS) during subsequent periods. Data from Stiller (n=1324) (15) and Magnani (n=3502) (14). Overall survival since 1970, when chemotherapy was introduced in addition to surgery (historical controls). This curve demonstrates clearly that OAS reaches a plateau phase from 1985 onwards.



MATERIALS AND METHODS

Literature search strategy

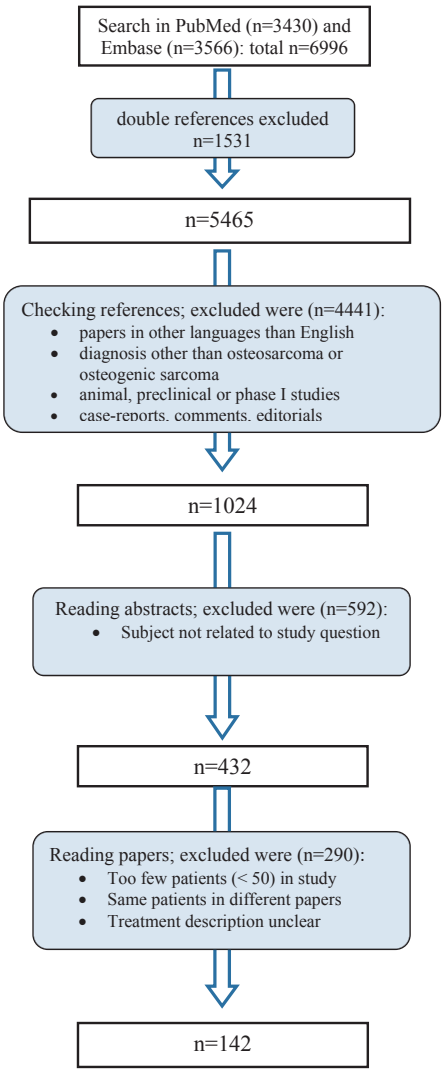
To assess the efficacy of the different chemotherapy regimens a Pubmed and EMBASE search was performed in January 2010, with osteosarcoma, osteogenic sarcoma, bone sarcoma and the drug names methotrexate, doxorubicin, adriamycin, cisplatin, ifosfamide and etoposide as search terms. Only papers in the English language were accepted for this review. Letters, abstracts or review papers were not included for reason of incomplete data of the studies or follow-up or duplication (fig 2). If reports were published more than once on the same patient population, the most mature data were used.

Phase II studies on the aforementioned 5 drugs were included. For the historical pre-chemotherapy era studies additional studies were retrieved from the references. Only studies with an appropriate definition of osteosarcoma and non-metastatic stage were used. Phase III studies of patients with localized disease only, were selected to have included at least 50 patients and with at least 5 years of follow-up. For the included studies, the following data were collected: study period, patient number and characteristics, chemotherapeutic regimens

(drugs, dose, frequency) as well as type of surgery, histological response, duration of follow-up (FU), event free (EFS) and overall survival (OAS).

FIGURE 2.

Search strategy for papers in this review.



Definition of results and outcome

Histological response was defined according to the proportion of viable tumour cells after induction chemotherapy: good pathological response (pGR) was defined if <10% are viable and poor pathological response (pPR) if ≥ 10% of the tumour cells are viable. Response rate, event free survival (EFS) and overall survival (OAS) were taken from the original publications. In phase II studies, a drug was considered effective when the response rate was ≥ 20%.

Statistical analysis: meta-analysis

The meta-analysis performed here is based on a new methodology for pairs of survival curves under heterogeneity and cannot be casted in the classical meta-analysis where the well-known forest plot is used to illustrate the results of the meta-analysis. A multivariate random-effects model for a joint analysis of survival proportions reported at different times in the different studies has been used in this manuscript in order to be able to use all information available in each paper included in the meta-analysis. For each study included in the meta-analysis where the same two treatments are compared, published EFS and OAS at a predetermined set of time points during follow-up and accrual information are known. Data in each study consist of disease free survival and overall survival probabilities every 6 months for the first 5 years after treatment. Two separate meta-analysis are performed. First the survival rates of patients who received a 2-drug regimen are compared with those who received a 3-drug regimen. Then the survival rates of patients, treated with 3-drug versus 4-drugs were compared. The techniques described by Parmar (18) and Fiocco (19) were used to reconstruct the number of patients at risk, the number of deaths and the number of censored patients during the time intervals in each arm and each trial. Using these aggregate data, the treatment effect and the overall survival curves for the two arms were estimated by applying a Poisson correlated gamma frailty model as described in Fiocco (17). Using this model, we were able to incorporate also studies with only one arm, while the traditional approach can be applied only when information concerning both treatment arms are given. This adds more efficiency to the results based on the statistical model.

RESULTS

Pre-chemotherapy era studies

Nine historical studies were retrieved from 43 papers on treatment of localised osteosarcoma before the chemotherapy era (table 1).

Long term survival of the combined 1555 patients after local tumour control without chemotherapy was 16% (9-23%). The typical course of the disease in these patients is reflected by the pattern of metastases, with 85% of patients developing pulmonary metastases, half of these within 6-8 months after local treatment (Fig 3). With (neo)-adjuvant chemotherapy, survival was higher, time to metastases was on average 1.5-2x longer, less pulmonary metastases but more extra-pulmonary metastases were observed compared with the historical group (14, 20-26).

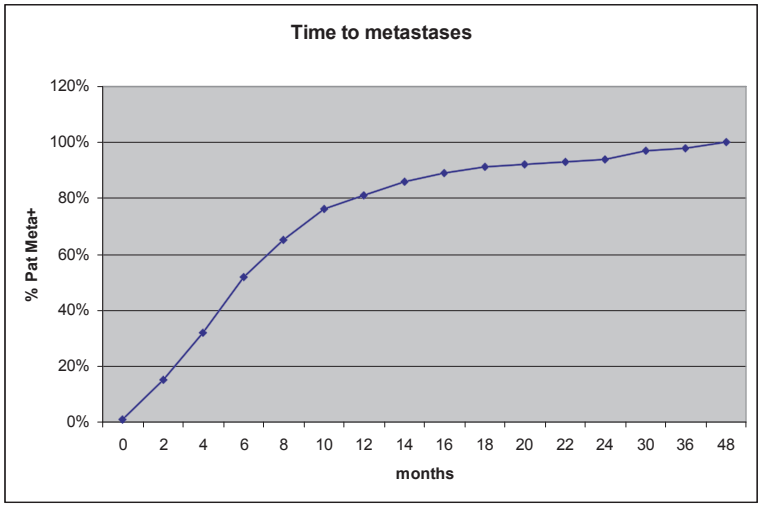
TABLE 1.

Selected studies from treatment of localised osteosarcoma patients before the chemotherapy era. Nine papers with the total of 1555 patients with surgery or/and radiotherapy and follow-up of at least 5 year or more were selected out of more than 40 papers.

Institute	study-period	number patients	Overall survival ≥ 5 year (%)	reference
Karolinska Hospital Sweden	< 1956	86	17	(27)
Westminster Hospital London	1951 - 1962	92	22	(28)
MSKCC New York	1949 - 1966	145	17	(29)
Mayo Clinics Rochester	1900 - 1966	465	18	(30)
Radium Hospital Oslo	1938-1964	102	18	(31)
Bristol Bone Tumor Register	1946 - 1972	149	17	(32)
Rizzoli Bologna	1959 - 1979	127	10	(21)
MD Anderson Cancer Center	1950 - 1974	213	9	(6)
EORTC	1962 - 1969	176	23	(7)

FIGURE 3.

Pattern of clinical detectable metastases in patients with local treatment only (historical data). In 80-90% of all patients with osteosarcoma metastases develop in the lungs, other bones and rarely in lymph nodes and other organs. Half of the metastases develop between 6-8 months after local therapy, 75-90% occur within 1 year, and after 2-2.5 year the curve of development of metastases flattens.



Single drug phase II studies

In order to get evidence for responsiveness of drugs, which are commonly used in osteosarcoma, phase II studies of M, A, P, Ifo and E as single drugs in pre-treated, relapsed or refractory patients were retrieved from literature. Among 140 papers, 50 were selected for this review (Table 2). Patients, entered in these studies, had relapsed or refractory disease. The data from studies showed high response rates of 43% for A, 33% for Ifo, 32% for M and 26% for P, all well above the predefined 20% threshold. E was included because some modern trials included this drug. However, E had a response rate of only 4%.

TABLE 2:

Drugs with a response rate (CR plus PR) of ≥20%. Etoposide is included to demonstrate the response rate in a small number of studies.

	dose range	N	N responding patients		response	References
Drug	mg/m²/course	patients	CR	PR	rate (%)	
Adriamycin	35-90	108	14	32	43	(33-44)
Ifosfamide	5.000-15.000	246	30	50	33	(45-59)
Methotrexate	80-15.000	164	26	26	32	(60-70)
Cisplatin	60-150	174	18	28	26	(69, 71-78)
Etoposide	120-625	27	0	1	4	(79-82)

DESCRIPTION OF NEO-ADJUVANT CHEMOTHERAPY STUDIES

1. American OSS studies (Table 3)

a. Memorial Sloan Kettering Cancer Center (MSKKC).

The first neoadjuvant (Rosen's) T-5 protocol enabled limb salvage after shrinkage of the tumour by pre-operative MA (83). The M-dose was escalated when no clinical or biochemical response were present (84) and based on the excellent results after 2 years, chemotherapy was further intensified in T-7 (84, 85) and T-10 (86) by giving M at weekly intervals and replacement of cyclophosphamide by BCD. To salvage pPR in the T-10 protocol, drugs post-operatively differed from those used preoperatively (86, 87). The response rate in T-10 was lower than in the previous trial, due to early planning of surgery, but the EFS was similar as in T-7 (88). In the last randomized study (T-12), a higher response rate in the more intensified arm resulted in a similar EFS (78%) to the control arm (73%) and to the previous (T-10) trial (89). Again, despite an increased response rate, no improvement in EFS was achieved.

b. MD Anderson Cancer Center

In three subsequent studies (TIOS I-III), the response of preoperative chemotherapy was used to design postoperative treatment in 98 patients (90). Sixty seven patients were treated with M,A and P containing regimens, depending on the response on preoperative chemotherapy, but 31 patients refused surgery and were treated with chemotherapy only. These patients had a significant lower 5y-EFS (23%) compared to those who were treated with surgery and chemotherapy (5y-EFS 62%) (90, 91), confirming that patients with localized osteosarcoma cannot be treated with chemotherapy alone (91) Intra-arterial P was more effective than M in a subgroup of these patients (90, 92).

c. Children's Cancer Group CCG782.

The objectives of CCG-782 were to improve EFS compared to the adjuvant protocol CCG 741and to evaluate the value of a grading system for histological response, using a T-10 based regimen (93, 94). Although the outcome was significantly better than in CCG 741, the response rate and survival were lower than in Rosen's T-10 study (86). However, because CCG-741 was less intensive, the conclusion that the neo-adjuvant approach was better than adjuvant chemotherapy could not be generalized. pPR was a significant higher risk for an adverse event than pGR (relative risk 0.23, $p<0.0001$).

d. Pediatric Oncology Group POG 8561.

This randomized study compared immediate and delayed surgery after an induction of 2 cycles MAP (95). Outcome was not significantly different between both arms. Patients, who had < 10% viable tumour after induction, had a significant better EFS (73%) than patients with pPR. It was concluded that timing of surgery did not influence outcome and that a better response was not translated into a survival benefit.

e. South West Oncology Group SWOG 9139.

In order to assess the efficacy of additional Ifo, 63 patients were treated with a regimen consisting of A and P, alternated with Ifo (96). With a response rate of nearly 50% and 5y-OAS of 58%, the authors concluded that this 3-drug regimen did not improve outcome compared with prior regimens of A and P alone and that the value of increasing dose intensity by adding drugs in osteosarcoma is limited.

f. Children's Oncology Group Intergroup study INT0133.

In a randomized 2x2 factorial study INT0133 the value of Ifo as a 4th drug compared with MAP and the addition of the immune modulating agent liposomal muramyl-tripeptide (MTP) to chemotherapy were investigated (97, 98). Analysis after 4 year follow-up suggested an interaction between Ifo and MTP but re-analysis after 6 years FU showed no evidence of interaction (98). A significant ($p=0.03$) improvement of OAS when MTP was added to chemotherapy (6y-OAS 78% vs 70% in chemotherapy alone) was observed while outcome of MAPIfo vs MAP were similar. Due to the complex design and interaction concerns of this study, the relevance of these conclusions have been challenged (99).

TABLE 3.
American OSS groups. In this table are included only studies with more than 50 patients (for drug names see list of abbreviations). BOTG: Brazilian Osteosarcoma Treatment Group. In addition: Ctx: Cyclophosphamide, Vc: Vincristin, Epi: Epirubicin, Ca: Carboplatin. LR and HR represent low risk (patients without HR criteria) and high risk (patients requiring an amputation or tumors > 12 cm) respectively.

Study	Patient	Drug Regimen				
period	number	pre-operative	post-operative	GR (%)	EFS (%)	OAS (%)
MSKCC T10	153	M_{x4}	*pGR:*$[M_{x4}+A+BCD]_{x4}$;	34	77^{3y}	82^{3y}
1978-1981			*pPR:* $M_{x4}+A+BCD+[AP_{x2}-BCD]x3$			
MSKCC T12	Pilot (n=51)	$M_{x6}+BCD_{x2}$	*GR:*M-BCD; PR:AP_{x6}	41	75^{2y}	76^{2y}
1986-1993	MBCD (n=26)	$M_{x4}+BCD$	*pGR:*$[M_{x2}+A+BCD]_{x4}$; *pPR:*$[M_{x2}+AP+BCD]_{x2}+[AP_{x2}+BCD]_{x2}$	39	73^{5y}	78^{5y}
	MAPBCD(n=26)	$M_{x2}+AP_{x2}+BCD$	*ALL:* $[M_{x2}+A+BCD]_{x3}+BCD$	44	67^{5y}	73^{5y}
MD-Anderson	65	All		43	62^{5y}	-
1979-1989	TIOS-1	M_{x12}vs P_{iartx7}	Resp: MAP_{x6}; non-Resp: MA or MAP			
	TIOS-3	P_{iartx7}	A_{450} or A_{450}-Cycl or A_{450}-VAC			
CCG 782	231	$M_{x4}+BCD$	*pGR:*$[M_{x4}+A+BCD]_{x4}$;	28	53^{8y}	60^{8y}
1983-1986			*pPR:* $M_{x4}+ABCD+[AP_{x2}-BCD]x3$			
POG 8651	100	ALL			65^{5y}	78^{5y}
1986-1993	*neoadjuvant (n=45)*	$M_{x4}+AP_{x2}$	$M_{x8}+A+AP_{x2}+BCD_{x5}$	62	61	76
	adjuvant (n=55)	-	$M_{x12}+A+AP_{x4}+A+BCD_{x5}$		69	79

SWOG 9139	63	$AP_{x2}+AI_{x2}$	$AP_{x2}+AI_{x2}$	48	41^{5y}	58^{5y}
1992-1996						
INT 0133	677	ALL		45	64^{6y}	74^{6y}
1993-1997	MAP (n=340)	$M_{x4}AP_{x2}$	$M_{x8}+AP_{x2}+A_{x2}$ -/+ L-MTP	43	63	73
	MAPIfo (n=337)	$M_{x4}AI_{x2}$	$M_{x8}+AP_{x2}+AIfo_{x2}+P_{x2}+Ifo_{x2}$ -/+ L-MTP	48	64	75
			MAP(Ifo) - MTP		61	70
			MAP(Ifo) + MTP		67	78
BOTG	225			29	39^{10y}	47^{10y}
1991-1996 (study III)	96/105	IfoEpiCax2	LR: IfoEpix3+IfoCa+EpiCa; HR: LR+Mx6	47	40	50
1996-1999 (study IV)	113/120	APCax3	IPx2+IAx2+ACa+ICa	18	38	44

f. Brazilian studies.

Both the EFS and OAS were lower in a regimen that did not contain M, but Ifo and Epirubicin plus Carboplatin (study III) (100), both considered less active drugs in osteosarcoma. In Study IV, A was added to the regimen of study III, without better results.

2. European OSS study groups.

a. Cooperative Osteosarcoma Study Group (COSS) studies (Table 4).

The first neoadjuvant study of the COSS (COSS-80) demonstrated a significant better survival compared with the COSS-77 adjuvant study (101, 102). Randomization in this study did not show any difference between P and BCD and Interferon-β was of no additional benefit (102). The following trial, COSS-82, investigated the reduction of intensity of pre-operative chemotherapy and salvage of poor responders. The overall results were worse than the previous trial and M-BCD not only showed a significant lower response rate compared with AP, but the pPR had also a significant worse survival (103). It was concluded from this randomized trial that salvage by changing drugs failed (104). Therefore, in COSS-86, chemotherapy was intensified by adding Ifo to an already aggressive regimen of MAP for high risk (definition risk groups: see Table 4) patients (105). Furthermore in a controlled way the question was addressed whether intra-arterial administration of P would yield a higher response rate, hence a better outcome. With a long term EFS of 66%, these results were the best published so far by COSS (104, 105). In both high and low risk patients, the response rate was nearly similar, and like the previous studies, pGR had a significant better survival than pPR. No benefit of the intra-arterial use of P on tumor reponse or survival was seen (105, 106).

TABLE 4.

COSS results. Overview COSS-studies from 1979 until 1988. The first 2 studies were randomized. The subscript figures in the rows with chemotherapy indicate the number of courses of the particular drug or drug combinations (drug names see list of abbreviations). pGR is pathologic good response, pPR is pathologic poor response. GR is proportion of good responders, in most cases ≥ 90% TCN (tumour cell necrosis). The superscript figures in the survival rows indicate follow-up period in years.

Study	patient	Drug Regimen				
period	numbers	pre-operative	post-operative	pGR (%)	EFS (%)	OAS (%)
COSS 80	116			53	58^{10y}	67^{10y}
1979 - 1982		M_{x4}+A+BCD	M_{x10}+A+BCD_{x3} -/+ Ifn		59	69
		M_{x4}+A+P	M_{x10}+A+P_{x3} -/+ Ifn		56	65
COSS 82	125			43	50^{10y}	64^{10y}
1982 - 1984		M_{x4}+BCDx2	*pGR:*M_{x4}+BCD_{x2}; *pPR:*AP_{x6}	26	46	59
		M_{x4}+APx2	*pGR:*M_{x4}+AP_{x2}; *pPR:*$IfoP_{x3}$+BCD_{x3}	60	55	68
COSS 86	171			69	66^{10y}	71^{10y}
1986 - 1988		LR: A+M_{x2}+P_{x2}	*pGR:*A_{x3}+M_{x10}+P_{x2}; *pPR:*A_{x4}+M_{x12}+$PIfo_{x3}$	68	66	75
		HR: AMx2PIfo x2	A_{x4}+M_{x12}+$PIfo_{x3}$	69	67	72

b. Istituto Ortopedico Rizzoli (IOR/OS) studies (Table 5).

In the first IOR/OS study it was shown that high-dose M regimens had a significantly better outcome than low-dose M and that salvage of pPR by changing drugs failed (107, 108). Subsequently, a greater response rate and better salvage therapy by more intensive pre-operative chemotherapy and the addition of Ifo and E for pPR respectively, resulted in a significant better EFS in the next trial, IOR/OS-2 (109, 110). The following trial demonstrated that the cumulative dose of A safely could be reduced to 390 mg/m2, and Ifo alone instead of Ifo plus E could be used to salvage for pPR (111). IOR/OS-4 succeeded in increasing the response rate to 77% by further intensifying pre-operative chemotherapy, which was not translated into a better outcome (112). Finally the effect of giving all 4 effective drugs at maximum dosages was feasible but did not yield a superior outcome compared with standard Ifo dose (113, 114). The value of the intra-arterial administration of P was investigated in the IOR-studies as well, but despite a higher response rate in the less intensive IOR-OS-3 study, no effect on the EFS or surgical procedure was present (115). In the more intensive IOR-OS-4 both administration routes were equally efficient.

TABLE 5.

Istituto Orthopedica Rizzoli (IOR) results. Successive chemotherapeutic protocols of IOR (drug names see list of abbreviations). The first study randomized between low dose M (0.75 g/m^2) and high dose M (7.5 g/m^2). M-doses are noted by superscript in pre-operative column, and are post-operatively the same. TN is total necrosis, No-TN is group without TN.

Study	patient	Drug Regimen				
period	numbers	pre-operative	post-operative	pGR (%)	EFS (%)	OAS (%)
IOR/OS 1	127			52	46^{12y}	53^{12y}
1983-1986	*MDMTXn=60)*	$M^{0.75}P_{x2}$	*pGR:* $A+MAP_{x3}$; *pPR:* $A\text{-}BCD_{x5}$	*42*	38^{12y}	45^{12y}
	HDMTX (n=67)	$M^{7.5}P_{x2}$		*62*	52^{12y}	61^{12y}
IOR/OS-2	164	$M^{8}AP_{x2}$	*pGR:* $A+MAP_{x3}$; *pPR* $A+MAPIfoE_{x3}$	71	63^{5y}	75^{5y}
1986-1989						
IOR/OS-3	95	$M^{10}AP_{x2}$	*pGR:* $A+MAP_{x3}$; *pPR:* $A+MAPIfo_{x3}$	56	54^{7y}	69^{7y}
1990-1991						
IOR/OS-4	162	$M^{12}APIfo_{x2}$	*No-TN:* $MAPIfo_{x3}+AM$; *TN:* $MAPIfo_{x2}+AM$	77	56^{7y}	71^{7y}
1993-1995						
ISG/SSG-pilot	68	$M^{12}APIfo_{x2}$	*pGR:* $MAPIfo_{x2}$; *pPR:*$MAPIfo_{x2}$+MIfoP	56	73^{4y}	87^{4y}
1995-1997						
ISG/SSG-1	182	$M^{12}APIfo_{x2}$	*pGR:* $MAPIfo_{x2}$; *pPR:* $MAPIfo_{x3}$	60	64^{5y}	77^{5y}
1997-2000						

c. Scandinavian Sarcoma Group (SSG) studies (Table 6).

In study SSG-II, the results of Rosen's T-10 protocol could not be confirmed (116, 117). The modest response rate (17%) and low outcome of pPR patients indicated an insufficient effect of single agent M as induction treatment and the salvage of pPR by changing drugs. The next study SSG-VIII was a MAP based induction, with change to IfoE to salvage pPR (117, 118). The response rate increased to 57%, but long term survival and EFS for pPR were not different compared to SSG-II, indicating that a better response rate was not translated into a survival advantage and salvage for pPR by changing drugs failed.

TABLE 6.
Scandinavian Sarcoma Group (SSG) results. Summary of the results of the SSG since 1982 (drug names see list of abbreviations).

Study period	patient number	Drug Regimen pre-operative	Drug Regimen post-operative	GR (%)	EFS (%)	OAS (%)
SSG-II 1982-1989	97	$M^{12/8}_{x4}$	*pGR:*M_{x16}+BCD_{x4}; *pPR:*M_{x4}+$AP_{x6}BCD_{x4}$	17	56^{5y}	66^{5y}
SSGVIII 1990-1997	113	$M^{12}_{x4}AP_{x2}$	*pGR:*$M_{x2}AP_{x3}$; *pPR:*$IfoE_{x5}$	58	61^{5y}	74^{5y}
ISG/SSG-1 1997-2000	182	$M^{12}_{x2}AP_{x2}Ifo_{x2}$	*pGR:*$MAPIfo]_{x2}$; *pPR:*$[MAPIfo]_{x2}$+[MIfoP]	60	64^{5y}	77^{5y}

d. European Osteosarcoma Intergroup (EOI) trials (Table 7).

The EOI compared, in 2 randomized trials, the role of AP based regimens with multidrug regimens (119, 120). EFS in the AP-arm of study 80831 was significantly (HR = 0.63; 95% CI(0.42-0.94)) better than in the MAP arm, but no difference in OAS was observed (HR = 0.69; 95% CI(0.43-1.09)) (119). In the next trial (80861) outcome was similar in the AP and multi-drug arm and the AP-regimen was preferred because of the better tolerability (120). However in the 80831 trial, the total dose intensity of AP in the MAP-arm was reduced to 2/3 of AP in the 2-drug arm (119). In 80861 the received dose intensity of P and A in the multidrug arm were 52% and 62% respectively, whereas in the 2-drug arm this was 78% for both drugs (120). In the 80931study it was possible to increase the dose intensity by shortening the interval between subsequent cycles of chemotherapy, using G-CSF, by 30% (121). This resulted in a significant (p=0.003) higher proportion of pGR. However, outcome was similar in both arms, suggesting that the increased histological response rate was reflecting the given pre-operative dose and not translated into better survival.

e. French OSS studies (Table 8).

The first single centre study aimed to reproduce the findings of Rosen's T-10 protocol and showed similar results (122). The next study was MAPIfo based, resulting in a better response rate, but no improved survival (123). The last trial SFOP-OS94, was a randomized comparison between MIfoE and MA (124) and showed a better response rate in the IfoE arm, but the outcome was not statistically different.

TABLE 7.

EOI results. Summary of results of the 3 randomized EOI trials since 1983 (drug names see list of abbreviations). The number of patients in each arm is given between brackets. In the column "Patient number" arms C and DI represent the conventional dose and the dose intensive regimen respectively. All M doses are 12 g/m², the number of courses are indicated by the subscript figures.

Study	Patient	Drug Regimen				
period	number	pre-opreative	post-operative	GR (%)	EFS (%)	OAS (%)
EORTC 80831	179					
1983-1986	AP (n=99)	AP_{x3}	AP_{x3}	41	57^{5y}	64^{5y}
	MAP (n=99)	MAP_{x2}	MAP_{x2}	22	41^{5y}	50^{5y}
EORTC 80861	391					
1986-1991	AP (n=199)	AP_{x3}	AP_{x3}	30	44^{5y}	55^{5y}
	multidrug (n=192)	$M_{x4}A$	$M_{x4}A+BCP_{x4}AP_{x6}$	29	44^{5y}	55^{5y}
EORTC 80931	504					
1993-2002	C (250)	AP_{x2}	AP_{x4}	36	39^{5y}	55^{5y}
	DI (254)	AP_{x3}	AP_{x3}	51	41^{5y}	58^{5y}

TABLE 8.

Other European study groups. Studies from France and (former Eastern) Germany (for drug names see list of abbreviations). IGR: Institute Gustave Roussy, SFOP: Société Française d'Oncologie Pédiatrique, HELP: Holoxan (Ifo), Eldesine (Vindesine, V), Cisplatin (P) with A.

Study group	patient	Drug regimen				
period	number	pre-operative	post-operative	% GR	EFS (%)	OS (%)
T-10 IGR-Paris	70	M_{x7}+BCD+A	*pGR:* $[M_{x4}A\text{-}BCD]_{x3}$; *pPR:* $[AP_{x2}\text{-}BCD]_{x3}$	56	68^{7y}	74^{7y}
1981-1986						
SFOP-HELP	62	$M_{x7}+Ifo_{x2}+V_{x2}+AP_{x2}$	$M_{x6}+Ifo_{x2}+V_{x2}+AP_{x2}$	64	59^{5y}	77^{5y}
1989-1993						
SFOP-OS94	234				62^{5y}	76^{5y}
1994-2001	MA (n=116)	$M_{x7}+A_{x2}$	*pGR:* $M_{x12}+A_{x3}$; *pPR:* $IfoE_{x5}$	43	58	75
	MIfoE (n=118)	$M_{x7}+IfoE_{x2}$	*pGR:* $M_{x12}+IfoE_{x3}$; *pPR:* AP_{x5}	64	66	76
Berlin	53	$[APCtxVc]_{x3}$	$[APCtxVc]_{x6}$	45	59^{10y}	67^{10y}
1986-1992						

f. Berlin study (Table 8).

Tunn et al. demonstrated in a small cohort of 53 patients that a multidrug regimen without M achieves similar survival rates to M-based schedules (125).

Statistical results and meta-analysis

Two drug, 3-drug and 4-drug regimens as listed in table 9 were used for meta-analysis, according to Parmar (18) and Fiocco (17, 19). For each study-arm multiple EFS and OAS corresponding to a predetermined set of time points (0.5,1,1.5,2,2.5,3,3.5,4,4.5,5,10 years) were known. The meta-analysis on EFS shows an improvement in survival by employing a three instead of two-drug regimen, which is significant (HR: 0.701, 95% CI: 0.615 – 0.799; fig 4). The same was demonstrated for the OAS as is shown in figure 5 (HR: 0.792, 95% CI: 0.677 – 0.926). Treatment effect was not significant different between regimens with 3 drugs and 4 drugs with respect to either EFS (HR: 0.956; 95% CI: 0.779 – 1.177) or OAS (HR: 1.043; 95% CI: 0.851 – 1.280). Figure 6 and 7 illustrate the estimated means survival for EFS and OAS respectively.

TABLE 9.

Studies included in the meta-analysis to estimate survival (EFS and OS) at different time points. From these aggregate survival data, the difference between 2-drug and 3-drug regimens was estimated by employing a Poisson correlated frailty model (see text for details and reference). Two drug regimens used for analysis were AP from the EOI-80831, EOI-80861, both AP-arms from study EOI-80931 and the MA-arm from SFOP-OS94. Three drug regimens used in the analysis were the MAP regimens from the randomized EOI-80831, COSS-80, COSS-82, INT-0133 and SFOP-OS94 studies, as well as the non-randomized IOR/OS-2 and -3 and SSG-VIII studies. The four-drug regimens which were used in the meta-analysis were the multi-drug arm of EOI-80861, the high-risk patients of COSS-86, the IOR/OS-4, ISG-SSG studies, the 4-drug arms of the randomized INT-0133 study and the POG-8651 multidrug study.

2-drug regimens	3-drug regimens	4-drug regimens
EOI-80831	EOI-80831	EOI-80861
EOI-80861	COSS-80	COSS-86
EOI-80931	COSS-82	IOR/OS-4
SFOP-OS94	IOR/OS-2	ISG-SSG-I
	IOR/OS-3	INT 0133
	SSG-VIII	POG 8651
	INT 0133	
	SFOP-OS94	

FIGURE 4.

Estimated events free survival (EFS) based on meta-analysis of 5 two-drug regimens versus 8 three-drug regimens. Mean values of EFS are estimated along with their confidence intervals: HR = 0.701; 95% CI(0.615 – 0.799).

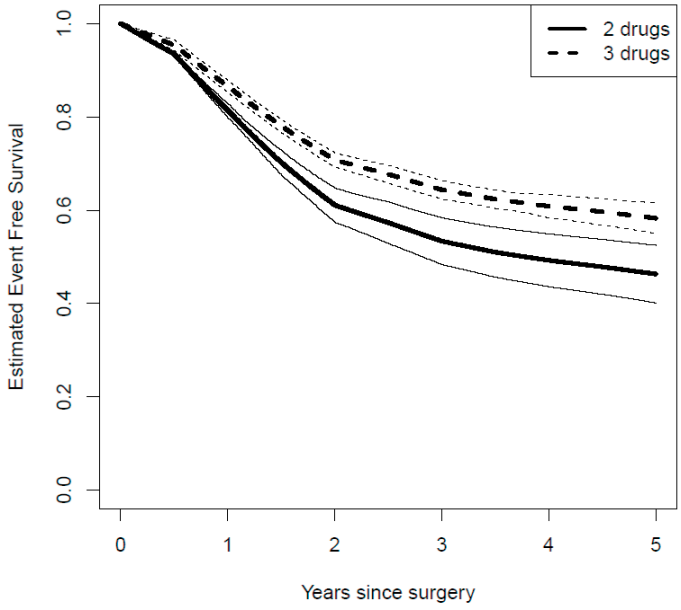


FIGURE 5.

Estimated overall survival (OAS) based on meta-analysis of 5 two-drug regimens versus vs 8 three-drug regimens. Mean value of OAS are estimated along with their confidence intervals: HR = 0.792; 95% CI(0.677 - 0.926).

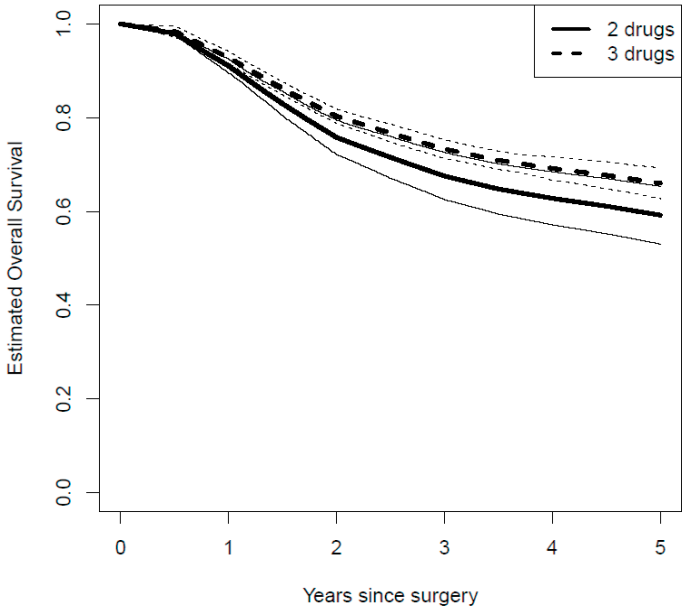


FIGURE 6.

Estimated EFS curve based on the meta-analysis of 8 three-drug regimens versus 7 four-drug regimens. As illustrated, the survival curves are completely overlapping. HR = 0.956; 95% CI(0.779 - 1.177).

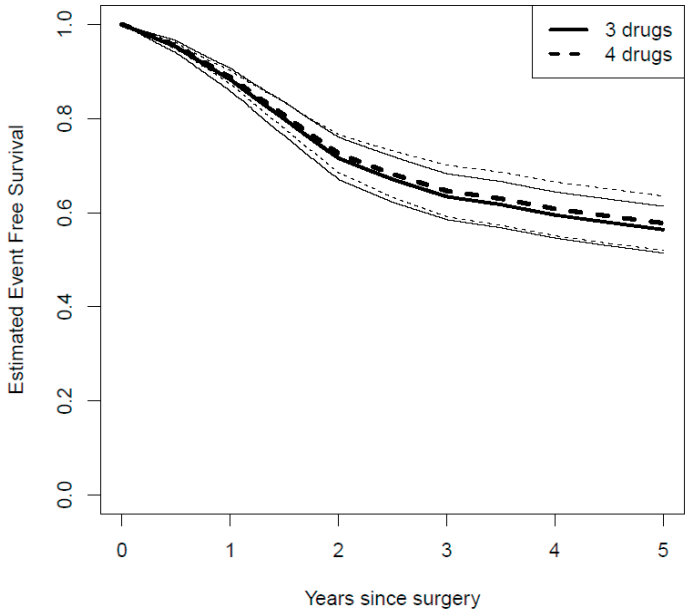
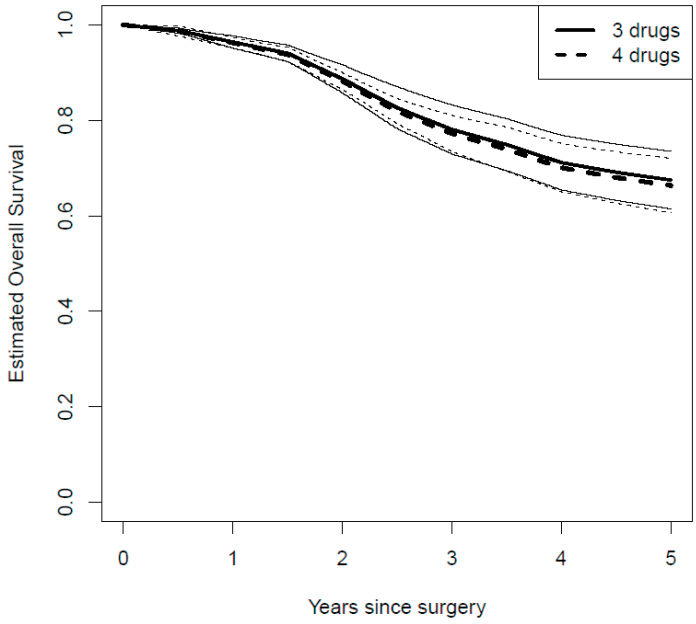


FIGURE 7.

Estimated OAS curve based on meta-analysis of eight 3-drug regimens versus seven 4-drug regimens. Similar as in fig 6,, the survival curves are overlapping, indicating no difference between both arms. HR = 1.043; 95% CI(0.851 - 1.280).



DISCUSSION

Data from single agent phase II studies in osteosarcoma patients for M, A, P and Ifo show response rates ≥ 20%, indicating the effectiveness of these drugs. Several investigators confirmed the importance of A in a sufficient dose, for example 390-450 mg/m^2, to be included in regimens for osteosarcoma (103, 104, 107, 111, 126-128). A number of studies addressed the question whether or not high-dose M is essential for adequate treatment of osteosarcoma (96, 100, 119, 120, 125). Survival outcomes of the SWOG, the Brazilian Osteosarcoma Study group and the EOI without M all are around 40% to 55% (96, 100, 119-121), lagging behind the results of the M containing regimens of the COSS, IOR/OS, SSG and INT0133. The conclusion of the EOI that AP was superior (119) or equal (120) to M-based regimes must be interpreted with caution because of the inequalities in total dose intensity (119, 120, 129).

To cope with heterogeneity between studies a Poisson correlated gamma frailty model has been used in this analysis. The results show a significant (p=0.03) different 5y-EFS in 2-drug regimens (46%) compared with 3-drug regimens (54%) (fig. 4). The five year-OAS of the 2-versus 3 drug regimens were 60% and 66%, respectively (p=0.04; fig. 5), justifying 3-drug regimens in current osteosarcoma protocols. Whether or not a fourth drug has to be added to MAP remains an unsolved question. The meta-analysis comparing 3-drug regimens (n=9) with 4-drug regimens (n=6) did not show a difference in EFS and OAS between the 2 arms (fig. 6 and fig. 7). This indicates that there is no benefit of a fourth drug in treatment regimens.

The question how to salvage patients who respond poorly on preoperative treatment cannot simply be answered. Using different drugs and/or intensification after surgery has not shown to been beneficial (88, 103, 104, 107, 117). Because in many studies histological response has been an highly important prognostic factor, intensifying pre-operative chemotherapy not only increases the response rate (104, 105, 107, 118), but also leads to better survival in most studies (105, 111, 130). Although getting a higher intratumoral drug concentration by intra-arterial infusions in possible, resulting in a high fraction of tumour cell necrosis (69, 78, 106, 115, 131-133), this route of administration does not result in a better survival than when given intravenously (78, 105, 106, 115, 134). Therefore, intensifying chemotherapy beyond a certain level does not improve outcome, neither for histologic poor responders and for salvage histologic poor responders (89, 95, 113, 114, 118, 121). Probably the results of the EURAMOS-1 study will give an answer whether or not patients with a pPR benefit from Ifo and E, added to MAP (www.euramos.org). As was suggested by Meyers in 1992 (88), intensive upfront treatment to increase the proportion of pGR has shown that the response rate improves, but this is not necessarily accompanied with better survival, which has been shown in other studies as well (89, 105, 112, 114, 118, 121, 123, 130, 135, 136). Limitations of treatment due to toxicity (114, 123) and lack of efficacy despite maximal dosages (105, 114, 121, 123, 137) prevent further improvement in outcome. Therefore, new approaches have to be investigated, such as immune modulating agents as MTP (97, 98, 138, 139) or interferon (140, 141) as well as molecular approaches (142). International large collaborative

randomised studies in the last decennia, did regrettably not result in further improved survival. Our opinion is that Bayesian designed rapid turnover trials with biological endpoints should be encouraged to explore the field of new ways of treatment of this resistant disease. It is emphasized here that this kind of studies only can be successful in international collaboration. In summary: early phase-II trials demonstrated that A, M, P and Ifo have a proven single agent efficacy against osteosarcoma. Meta-analysis showed an significant advantage of 3-drug over 2-drug regimens, but the use of a fourth drug is not better than 3 drugs. Whether or not dose intensification after a poor response to preoperative chemotherapy improves survival remains an open question.

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3

Profiling of high-grade central osteosarcoma and its putative progenitor cells identifies tumorigenic pathways

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ABSTRACT

Background

Osteosarcoma is the most prevalent primary malignant bone tumor of children and young adults, with poor survival in 40%. In order to identify signaling pathways involved in tumorigenesis we compared gene expression in osteosarcoma versus its presumed normal counterparts.

Methods

Genome wide expression profiles were generated from 25 high grade central osteosarcoma pre-chemotherapy biopsies, 5 osteoblastomas, 5 MSC populations and these same MSCs differentiated to osteoblasts. Genes that were differentially expressed between were analyzed in the context of the pathways in which they function using the GenMAPP program.

Results

MSCs, osteoblasts and osteosarcomas clustered separately and thousands of differentially expressed genes were identified. Most significantly altered pathways are involved with cell cycle regulation and DNA replication. Several upstream components of the Wnt signaling pathway are down regulated in osteosarcoma. Two genes involved in degradation of β-catenin protein, the key effectors of Wnt signaling, Axin and GSK3-β show decreased expression, suggesting that Wnt signaling is no longer under control of the regular signals. Comparing benign osteoblastomas with osteosarcomas identified cell cycle regulation as the most prominently changed pathway.

Conclusion

These results show that up-regulation of the cell cycle and down-regulation of Wnt signaling play an important role in osteosarcoma genesis. Gene expression differences between highly malignant osteosarcoma and benign osteoblastoma involve cell cycle regulation.

INTRODUCTION

Osteosarcoma is the most common primary bone malignancy, with a yearly incidence of approximately 6 per million children and 2 per million adults (1). The peak incidence occurs in late puberty, with 50% of the patients being between 10-20 years, and 60% younger than 25 years. Osteosarcoma in patients over 40 years of age is in a substantial number of cases generally considered secondary, such as after exposure to irradiation, or it arises in areas of pre-existing Paget's disease of bone (2). It might thus be considered as different disease than osteosarcoma in young patients.
Several histological subtypes are distinguished, of which conventional high-grade central or intramedullary osteosarcoma is the most common (75%) (3). The etiology of high-grade central osteosarcoma in young patients is elusive. No benign-, or malignant precursor lesions are known. These tumours recapitulate osteogenesis, compliant with their capacity to produce osteoid, alkaline phosphatase, osteocalcin, osterix and bone sialoprotein.
The outcome for patients with high-grade osteosarcoma has improved substantially since the introduction of multimodal chemotherapy, with present overall survival rates, ranging 65-75%. However, this improvement has reached a plateau despite several trials opting for intensifying dose or applying alternative chemotherapy schedules. Increasing the dose of chemotherapy prior to surgery only improved response rate, but not survival (4, 5). In order to treat patients that are refractory to chemotherapy or those that relapse alternative targets for therapy are required which can be identified through knowledge on molecular biological characteristics of the tumor.
Molecular studies on osteosarcoma are greatly hampered by the enormous genetic instability, that obscures the identification of genetic loci involved in osteosarcoma genesis (6), furthermore by the lack of benign precursors and no certainty on the normal counterpart or the progenitor cells. Osteoblastoma is a benign bone tumor occurring at the same site, but this tumor has never been reported to progress to osteosarcoma. A potential cell-of-origin of osteosarcomas is the mesenchymal stem cell (MSC), the precursor of osteoblasts as has been suggested in mouse models (7). Genome wide expression profiling to identify genes that are involved in response to chemotherapy and survival of osteosarcoma have been reported (8-10). Respectively 104, 44 and 60 differentially expressed genes were reported when comparing good and poor responders to chemotherapy. Remarkably these lists of genes do not overlap by one single gene.

Here we report on a genome wide expression profiling study on a homogeneous series of high-grade central osteosarcomas of patients younger than 40 years of age. Using strict criteria to correct for multiple testing we were not able to identify genes that were significantly different when comparing good and poor responders. Comparing the osteosarcoma expression profiles with the putative progenitor cells of osteosarcoma, i.e. mesenchymal stemcells (MSCs) and the same MSCs differentiated into osteoblasts resulted in the identification of large sets of genes that show very significant differential expression. These genes could be grouped

according to signal transduction pathways in which they function, thereby identifying possible culprit molecular events responsible for osteosarcoma genesis.

MATERIALS & METHODS

Patient material and Mesenchymal stem cells

Patients and their clinical data are listed in Table 1. All patients were treated at Leiden University Medical Center (LUMC). For osteosarcoma patients the difference in response to chemotherapy was stratified as good or poor response, using the Huvos criteria (11). Good response was defined if less than 10% of the tumor cells are viable after pre-operative chemotherapy, poor response if more than 10% of the tumor cells are viable. This response rate has been shown to be the best predictive marker for prognosis (12). Chemotherapy protocols include both pre- and postoperative treatment and were comparable (4). Osteoblastoma patients were treated with surgery only. Difference in survival of osteosarcoma was stratified as good if patients were still alive after 5 years follow-up, whereas poor survivors were patients who died from their disease within this time window. Disease course for osteoblastoma patients was usually without remission, except recurrence in one patient.

RNA was extracted from frozen biopsies, which were obtained before pre-operative chemotherapy was administered. For osteosarcoma core biopsies with at least 70% tumor cells and with non-necrotic tissue were used in this study. For osteoblastoma the resected tumors were used for RNA extraction.

We used human bone-marrow-derived mesenchymal stem cells and osteoblasts derived from the same cells upon osteogenic differentiation. Cells were either isolated from bone marrow samples as previously described (13). MSC1, MSC2 and FMSC1 were obtained from the department of Hematology, Leiden University Medical Center, Leiden, The Netherlands. 220L and 240R were purchased from Tulane University, New Orleans. All cells used were derived from adult patients, except for FMSC1, which was derived from fetal bone marrow and were obtained according to the ethical guidelines of the national organization of scientific societies (FEDERA). All cells were characterized either at passage 2 or passage 3 via FACS analysis as previously described (14). The phenotypes were uniform among all the different cells tested and in agreement with those reported for MSCs: i.e. CD90, CD105, CD166, HLA-A, B,C positive (>95%) and CD34, CD 45, CD31,CD80, HLA-DR negative (<5%). Furthermore all the cells were tested for their ability to be committed, under the proper conditions, towards adipogenesis, chondrogenesis and osteogenesis, as previously described (14). All cells that were induced to osteogenic differentiation showed diffuse positive staining for alkaline phosphatase activity and alizarin red positive calcium depositions, as previously described (14).

All tissue samples were handled in a coded fashion, according to National ethical guidelines ("Code for Proper Secondary Use of Human Tissue in The Netherlands", Dutch Federation of Medical Scientific Societies, http://www.federa.org).

Expression array analysis

RNA was extracted from frozen tissue sections of 20 μm as described previously (15). Generation of cRNA and labeling was performed according to the Affymetrix protocol, briefly, 10 μg RNA was used to generate double-stranded cDNA by an oligo-dT primer and a T7-RNA polymerase promotor. Reverse transcription and subsequent amplification and labeling were done in accordance with protocols recommended by Affymetrix using the BioArray HighYield RNA Transcript Labeling kit (ENZO Life Sciences, Farmingdale, NY). Every step of the reverse transcription and labeling procedure is monitored by gel electrophoresis and spectrophotometry.

Labeled RNA is hybridized with Hu133A GeneChip Arrays (Affymetrix, Santa Clara, CA) according to the manufacturer's protocol (http://www.affymetrix.com/support/technical/manuals.affx) and scanned on a Affymetrix GeneChip scanner.

Quality of the hybridization is assessed by calculating the ratio of the 5' and 3' features for the reference genes GAPDH and actin. When this ratio is greater than 2, this is a measure of poor quality and the chip is discarded.

All expression array data are available at the BJC online supplementary material website.

Data analysis

GeneChip data were normalized using GC-RMA, an algorithm provided by the Bioconductor project (http://www.bioconductor.org/) which looks only at perfect match values (16). The algorithm runs under statistical language R and was shown to give less false positive variance in technical duplicates and has a greater sensitivity and specificity (17) as was recently confirmed in our laboratory (18).

The Spotfire decision site for functional genomics was used to perform unsupervised hierarchical clustering on all genes with a variance of at least 0.5.

In order to select genes that can be used as classifiers for histological response on pre-operative treatment and survival, Limma (linear models for microarray data) package of Bioconductor (http://www.bioconductor.org) was applied to the data set. Limma is a moderated t-statistic that detects differentially expressed genes between groups, given the natural variance within these groups, corrected for the false discovery rate due to multiple testing (19).

For pathway analysis, the array data were mined with GO-Elite, a tool to identify pathways that are most significantly changed between groups (http://www.genmapp.org/go_elite/go_elite.html and PMID: 15961447). To visualize gene expression data in biological pathways GenMAPP was used (20).

Quantitative reverse transcriptase PCR was performed as described previously (21). Primers for control genes and Wnt5a have been submitted to the Real Time PCR Primer and Probe Database (http://medgen.ugent.be/rtprimerdb/).

TABLE 1.
Clinical data

Sample ID	Chip no.	Type	Age	Gender	Subtype[1]	Adj. CT[2]	Chemo Response	Overall Survival	metastasis
L1370	IB10	osteosarcoma	14	male	HG Conv.	PIA	Good	good	lung
L1372	IB12	osteosarcoma	10	male	HG Conv.	AP	Good	good	0
L1382	IB14	osteosarcoma	16	male	Tel.	PIA	Poor	poor	lung
L1385	IB16	osteosarcoma	13	female	Tel.	MA	Poor	poor	lung
L1016	IB19	osteosarcoma	4	male	HG Conv.	AP	Poor	good	0
L2620	IB21	osteosarcoma	16	male	HG Conv.	AP	Poor	poor	lung+bone
L1375	IB22	osteosarcoma	8	male	HG Conv.	AP	Poor	good	local
L428	IB32	osteosarcoma	16	male	HG Conv.	AP	Poor	good	lung
L436	IB33	osteosarcoma	18	male	HG Conv.	MA	Poor	good	0
L432	IB34	osteosarcoma	17	male	HG Conv.	AP	Poor	poor	lung
L361	IB35	osteosarcoma	16	female	HG Conv.	AP	Poor	good	0
L1368	IB36	osteosarcoma	10	female	HG Conv.	PIA	Good	good	0
L1376	IB37	osteosarcoma	9	female	HG Conv.	AP	Good	good	0
L1386	IB38	osteosarcoma	12	female	HG Conv.	AP	Poor	poor	lung
L2702	IB39	osteosarcoma	16	male	HG Conv.	AP	Good	poor	lung
L2302	IB40	osteosarcoma	19	female	HG Conv.	AP	Poor	good	0
L2296	IB41	osteosarcoma	16	male	HG Conv.	AP	Good	poor	lung+else
L2295	IB42	osteosarcoma	40	female	HG Conv.	AP	Poor	good	0
L2611	IB43	osteosarcoma	20	female	HG Conv.	AP	Good	good	0
L2300	IB44	osteosarcoma	13	male	HG Conv.	AP	Good	good	0
L2294	IB45	osteosarcoma	17	female	HG Conv.	AP	Poor	good	0
L2290	IB46	osteosarcoma	36	male	HG Conv.	AP	Poor	poor	local
L2301	IB47	osteosarcoma	25	male	HG Conv.	AP	Poor	poor	lung+else
L2281	IB48	osteosarcoma	17	male	HG Conv.	AP	Poor	poor	lung
L2289	IB54	osteosarcoma	11	male	HG Conv.	AP	Poor	good	0
L578	IB55	osteoblastoma	22	male				relapse	
L579	IB56⋆	osteoblastoma	22	male				relapse	
L580	IB57	osteoblastoma	13	male				remission	
L581	IB58	osteoblastoma	16	male				remission	
L601	IB59	osteoblastoma	44	male				remission	

FMSC-OB-diff	IB49	osteoblasts
MSC1-OB-diff	IB50	osteoblasts
220-OB-diff	IB51	osteoblasts
240-OB-diff	IB52	osteoblasts
MSC2-OB-diff	IB53	osteoblasts
MSC1	IB54	MSC
MSC2	IB61	MSC
C220R	IB62	MSC
C240R	IB63	MSC
FMSC	IB64	MSC

[1] HG = high grade, [2] Adj. CT = adjuvant chemotherapy; PIA = cisplatinum, ifosfamide and adriamycin; AP = adriamycin and cisplatinum; MA = methotrexate and adriamycin; MSC = mesenchymal stem cell; HG conv = high grade conventional, Tel. = Telangiectatic
* IB 56 is the recurrence from IB 55

RESULTS

Comparing expression profiles of osteosarcomas

For 25 pre-operative biopsies from high-grade central osteosarcomas we obtained good quality genome wide expression data. One sample was repeated twice and three were repeated once to test for technical reproducibility. All four samples were most similar to their duplicates as demonstrated by hierarchical clustering, since replicates always clustered together (data not shown). For further analyses we used only one of the replicates. The entire file containing all expression profiling data can be found in supplementary Table 1.

Hierarchical clustering of all osteosarcoma profiles did not result in separation into groups, implying no big differences between possible clinical subsets. Previous publications reported that there are significantly differentially expressed genes when comparing osteosarcomas from patients with good versus poor response to chemotherapy (8, 9). However we could not identify any significantly expressed gene when comparing good and poor responders when applying a moderated T-statistic, that corrects for multiple testing as described in the methods section.

For all patients at least 5 year of follow up data was available. Poor survivors are defined as having less than 5 year survival as compared to good survivors with more than 5 year. The same T-statistic was used for the classification in good and poor survival, however no significantly differentially expressed genes were acknowledged and thereby no prognostic markers identified.

Genes differentially expressed due to comparing cultured cells and primary tissue
In order to identify biological processes involved in osteosarcoma genesis the expression profiles of the 25 osteosarcomas were compared with profiles of the presumed progenitors of this tumor, i.e bone marrow derived mesenchymal stem cells (MSC) (n = 5) and osteoblasts derived from these MSCs (13). Furthermore profiles of five osteoblastomas were included, which are not considered as benign precursors, since these tumors have never been reported to progress to osteosarcoma. Hierarchical clustering clearly distinguished the four groups into separate clusters (Fig. 1). The t-test in Limma assigned many significant differentially expressed genes when doing pair-wise comparisons (table 2).
The GO-Elite program selected the pathways that are most significantly different when comparing groups. GO-Elite ranks pathways with excess of differentially expressed genes. One of the most significant pathways when comparing MSCs with osteosarcoma was the MHC class II receptor activity pathway, which was upregulated in osteosarcoma. It is difficult to understand how the increase of such a pathway could contribute to mesenchymal transformation. We hypothesized that some of the genes identified by the T-test are merely different because cultured cells (MSCs) are compared with primary tissue. The genes that are most likely to belong to this category are those that show similar expression in the cultured MSCs and osteoblasts as well as in primary osteosarcoma and osteoblastoma, but significant difference between the group of cultured cells and the primary tissues. To identify these genes Venn diagrams were made of all differentially expressed genes for all comparisons using the limma package from Bioconductor (http://www.bioconductor.org). A final Venn diagram (Fig. 2) identified 492 genes that are likely to be different because of comparing cultured cells with primary tissue. The overlapping category in Fig 2 consists of all genes that are significantly different when cultured cells are compared with tissue, for both the highly malignant osteosarcomas as well as the benign osteoblastomas. The procedure to construct the VENN diagrams is explained in the legend of Fig 2. GennMAPP analysis was performed on the entire dataset, with the 'culture-tissue' category marked as leading parameter in the expression dataset, marked purple. The group of eight genes in the MHC classII receptor pathway that had a p-value of less than 0.05 appears to consist of seven genes that were assigned to the purple-colored 'culture-tissue' category (Fig 3). This suggests that the approach to filter out the genes that may be the result of comparing cultured cells and tissue is a valid one. However, this approach has its limitations because separate genes can not be validated with a gold standard, nor can be excluded that there are genes in this set that are similarly differentially expressed between MSCs in vivo versus both osteoblastomas and osteosarcomas.

FIGURE 1.
Hierarchical clustering

Hierarchical clustering of expression profiling data clearly shows separate clusters for osteosarcomas, osteoblastomas, MSCs and the same MSSCs differentiated to osteoblasts

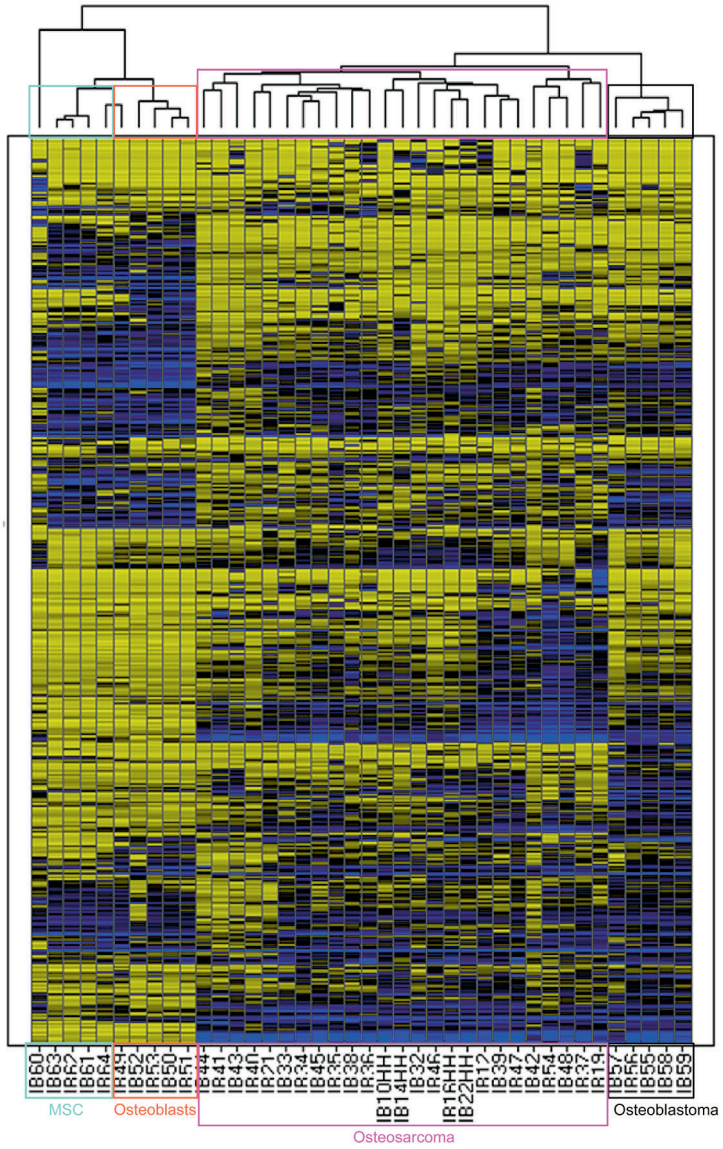


FIGURE 2.
Venn diagram of the 'culture-tissue' gene subset

The circles from these VENN diagram represent the differentially expressed genes when comparing two groups of arrays. The overlap between two circles contains the genes that are the same in both comparisons. OS = osteosarcomas; OB = osteoblastomas; MS = mesenchymal stem cells; DO = MSCs differentiated to osteoblasts. The lower VENN diagram displays the overlap of the 492 differentially expressed genes when comparing expression profiles from primary tissue (OS, osteosarcoma and OB, osteoblastoma) with cultured cells (MS MSCs and DO, differentiated to osteoblasts). The circle OSMS_OBMS contains all genes differentially expressed when comparing osteosarcoma and MSC that overlap with the differentially expressed genes when comparing osteoblastoma and MSC. OSDO_DOOB is the same as OSMS_OBMS, but for MSCs differentiated to osteoblasts.

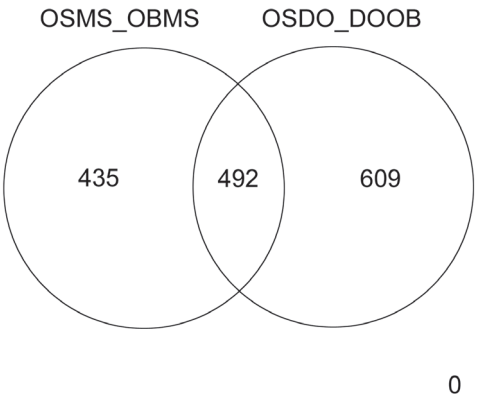


FIGURE 3.
MHC class II normal versus tissue cultrure related

MHC classII receptor activity pathway with genes that are differentially expressed between osteosarcoma and MSCs colored. Green is upregulated in osteosarcoma, purple indicates that a gene belongs to the 492 genes of the culture-tissue set. The left panel was analyzed without taking this set into account, the right set with the 'culture-tissue' gene set as the first parameter

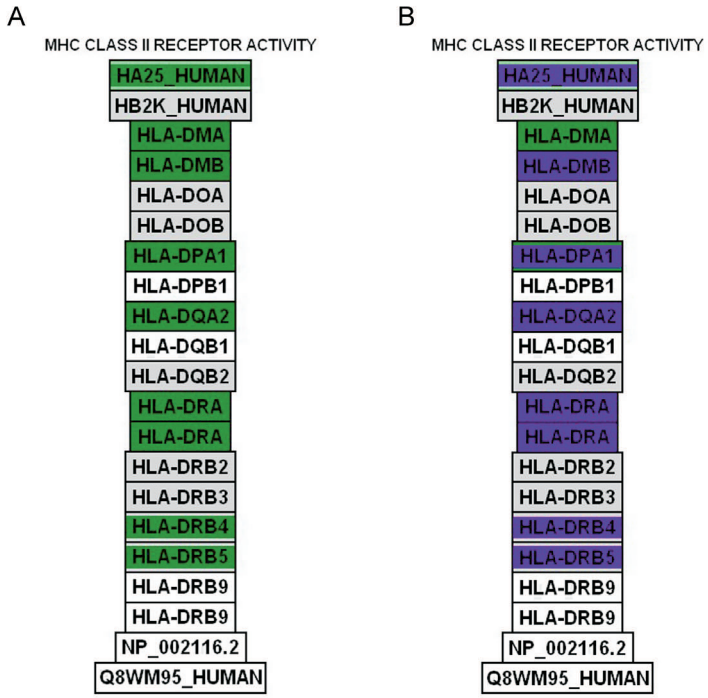


TABLE 2.
Group comparisons and nr of significant genes identified with Benjamini-Hochberg adjusted p-value

Comparison	total p<0.05	up	Down	avg of 100x 5 OS	Presumed process
OS vs MSC	2973	1159	1814	2456	genes that are altered in osteosarcoma (OS) progression from MSC
OS vs DO	3041	1144	1897	2586	genes that are altered in OS progression from differentiated osteoblasts (DO)
OS vs OB	882	225	657	937	genes involved in malignancy of OS compared to benign osteoblastoma (OB)
DO vs MSC	369	175	194		genes involved in MSC differentiation to osteoblasts
OB vs MSC	1245	606	639		genes involved in osteoblastoma progression from MSC
OB vs DO	1573	770	803		genes involved in osteoblastoma progression from osteoblasts

Comparing osteosarcoma with its presumed progenitors

The 25 osteosarcomas as a single group compared with five cultures of undifferentiated mesenchymal stem cells. This resulted in a substantial number of 3300 differentially expressed genes (corrected p-value< 0.01), of which 1302 genes are higher expressed in MSCs than in osteosarcomas and 1998 lower. We furthermore compared osteosarcomas with the same MSC cultures differentiated to osteoblasts. This resulted in 3335 differentially expressed genes ($p<0.01$). Table 2 summarizes the results of all comparisons made. There is a large overlap of 1006 genes in the osteosarcoma versus MSC and the osteosarcoma versus differentiated osteoblasts (DO). One gene that was significantly over-expressed in osteosarcoma was Wnt5a. This gene, involved in non-β-catenin Wnt signaling (22) has been tested with quantitative RT-PCR on the same series of RNA that has been used on the microarrays as an alternative method to verify the array-data. Correlation between qPCR and arraydata was good, i.e. 92% (Fig 4).

Given the high number of significantly differentially expressed genes we did not consider it relevant to make a shortlist of the most significant genes. Instead the program GO-Elite was used to identify pathways with a high number of differentially expressed genes and GENMAPP was used to look specifically at pathways that are known to be involved in normal osteoblast differentiation. For the GO-Elite analysis we removed the 492 'culture-tissue' artifact genes from the significant list.

FIGURE 4.
q-RT-PCR for Wnt5a

Comparison q-RT-PCR and array data for Wnt5a data shows 92% correlation

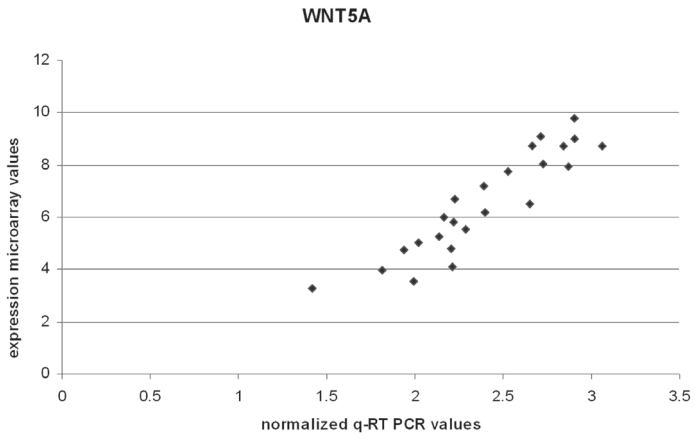


Table 3 lists pathways that contain most differentially expressed genes when comparing MSCs and osteosarcoma. Pathways in this table have an adjusted p-value smaller than 0.05 upon strict statistical criteria i.e. Benjamini Hochberg (23). The significant pathways are associated with DNA replication and mitosis, of which several genes involved in positive regulation are upregulated in osteosarcoma, such as CCNB when compared to MSC. None of the significant genes in these pathways are identified as 'culture-tissue artifacts'.

In order to further mine the data we looked at specific pathways that are known or suspected to be involved in osteosarcoma genesis. Inactivation of the p53 pathway has been reported in osteosarcoma (24) and this is indeed confirmed when comparing expression profiles from osteosarcoma with its presumed progenitor, MSCs and osteoblasts. Fig 5 shows the p53 mediated apoptotic pathway with genes that are downregulated in osteosarcoma ($p<0.05$) in green. Downregulation of p53 mediated signaling is reflected by downregulation of the specific downstream gene BBC3/PUMA.

The Wnt pathway has been shown to play an important role in osteoblast differentiation (25) and therefore here we visualized this pathway with the GenMAPP application using the expression data. Wnt signaling seems downregulated when comparing MSCs or differentiated osteoblasts with osteosarcomas. Fig 6 shows the Wnt pathway when comparing osteosarcoma and MSCs. The picture is similar when comparing with osteoblasts, although less prominent. Both upstream, the Wnt receptors FZD2 and -7 and LRP5 as downstream CCND1 and AXIN are downregulated.

TABLE 3.
Differentially expressed significant pathways

Pathway	Z_score
Comparison OS vs MSC	
macromolecule localization	5.99
mitotic cell cycle checkpoint	5.00
DNA replication	4.57
condensed chromosome, centromeric region	4.04
Comparison OS vs DO	
negative regulation of S phase of mitotic cell cycle	5.34
Comparison OS vs OB	
cell cycle	7.09
spindle	6.34
IgG binding	5.69
cell division	5.43
condensed chromosome\, centromeric region	5.36
proteinaceous extracellular matrix	5.08
chromosome segregation	4.94
DNA replication	4.80
Comparison DO vs MSC	
cadmium ion binding	11.28
trans\-1\,2\-dihydrobenzene\-1\,2\-diol dehydrogenase activity	7.39
acute\-phase response	5.57
steroid biosynthetic process	5.14
sterol metabolic process	5.12
copper ion binding	4.45
Adipogenesis	4.67
Comparison OB vs MSC	
developmental process	7.53
Cholesterol Biosynthesis	7.36
proteinaceous extracellular matrix	5.27
cytokine and chemokine mediated signaling pathway	4.48
Comparison DO vs OB	
negative regulation of transcription\, DNA\-dependent	5.58
amine oxidase activity	4.99
urogenital system development	4.94

Z-score = corrected score as determined by GO-elite. OS = osteosarcoma; MSC = mesenchymal stem cell, DO = differentiated osteoblasts, OB = osteoblastoma

Osteosarcoma versus osteoblastoma

Expression profiles of osteosarcoma were compared with those of five osteoblastomas, a benign bone tumour occurring at a similar site, in the long bones, and in a similar age group as osteosarcoma. The large difference in disease course is reflected by a large set of significantly differentially expressed genes (n = 882) of which 657 are higher in osteoblastoma and 225 higher in osteosarcoma. Comparing osteoblastomas with MSCs/osteoblasts results in less differences (6%/7%) than with osteosarcomas (13%). This may imply that osteoblastomas are more similar to MSCs and osteoblasts than osteosarcoma, thereby reflecting the difference in malignancy. The pathways that are most significantly altered when comparing osteosarcoma and osteoblastoma are the cell cycle, with an upregulation in the malignant tumors and pathways associated with cell division, especially regulation of the mitotic spindle. The significant pathways are listed in Table 3. To determine whether the larger size of the osteosarcoma group (n = 25) underlies this difference in significant genes we repeated the comparisons with only 5 osteosarcomas. Calculations were repeated 100 times for different combinations of 5 osteosarcomas and the results were averaged. The results are shown in Table 2, in the column labeled 'avg of 100 x 5 OS'. This indeed resulted in a reduction of the number of significant genes, but the difference between osteosarcoma versus MSC or osteoblasts was still substantial, i.e. 11 % for MSC and osteoblasts, whereas the comparison for osteoblastoma was only 6 or 7%.

FIGURE 5.
p53 pathway upregulated in osteosarcoma

The apoptosis/p53 pathway components when comparing osteosarcoma with differentiated osteoblasts with genes upregulated in osteosarcoma in green (dark green when p-value <0.01, light green when p<0.05) and genes downregulated in red (red, p-value<0.01, pink p<0.05).

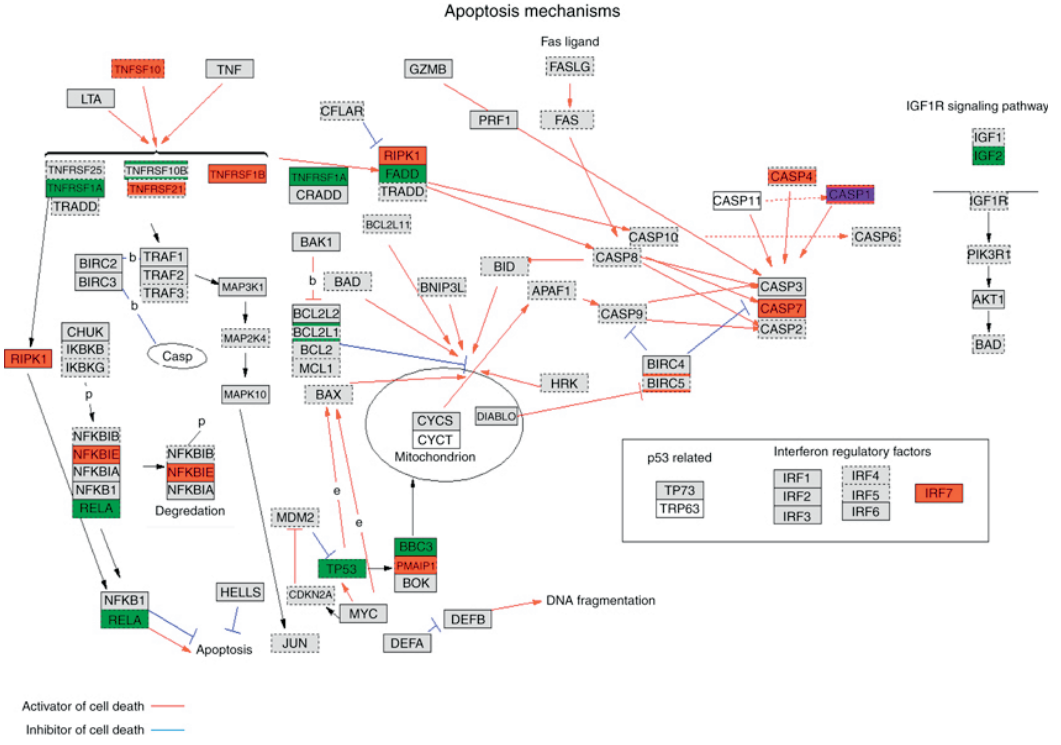


FIGURE 6.
Wnt signalling pathway downregulated in osteosarcoma

The Wnt signalling pathway when comparing osteosarcoma and MSCs, legend is the same as Fig. 5

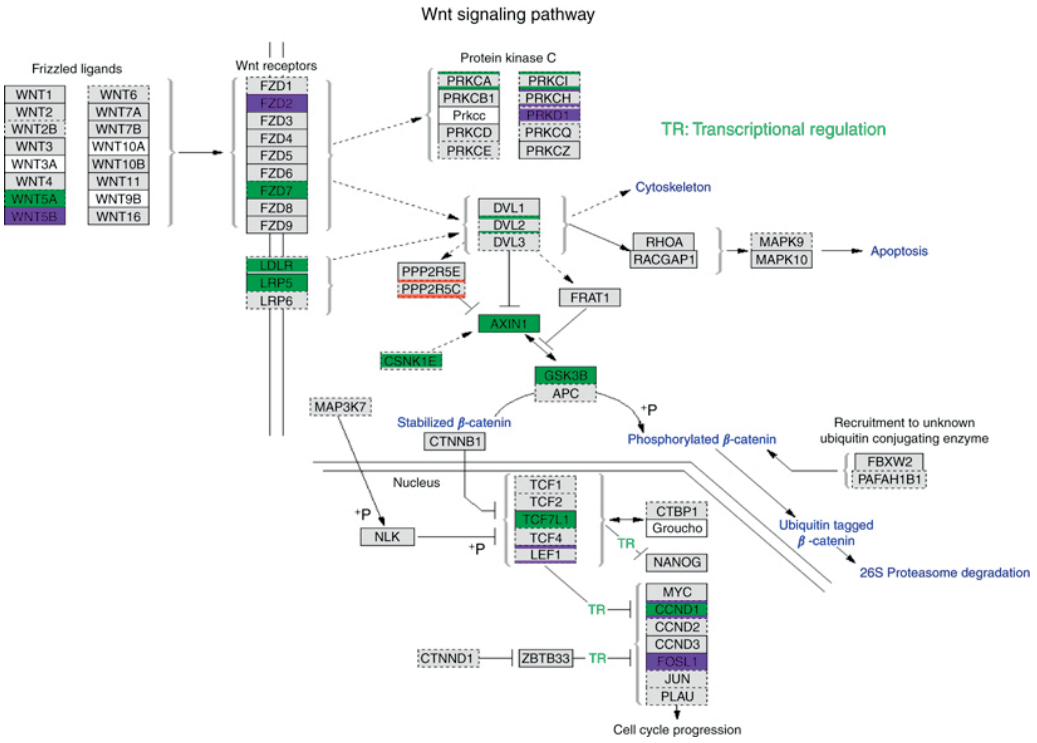
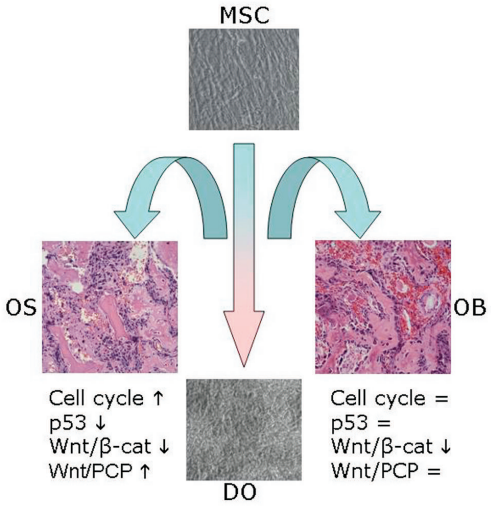


FIGURE 7.
Model for osteosarcoma genesis

Proposed model for osteosarcoma genesis. Osteosarcoma and osteoblastoma originate from mesenchcymal stemcells that are differentiatiting to osteoblasts. Increase in cell cycle activity and overactivity of Wnt/Planar cell polarity signalling and P53 function contribute to malignancy



DISCUSSION

Previous studies on genome wide expression profiling of osteosarcoma have reported lists of genes that were found to be differentially expressed when comparing tumors with a poor histological response to chemotherapy and those with a good response (8-10). Our study, comparing pre-chemotherapy biopsies from 8 good responders with those of 17 poor responding patients did not result in a single significantly differentially expressed gene. Size and homogeneity of the patient cohort, type of expression profiling platform, and statistical analysis may all account for this lack of significant genes. However, patient cohorts did not differ a lot in size, i.e. respectively 30, 28 and 13 cases, compared to 25 in our study, so size appears to be a highly unlikely explanation for this difference. A long follow-up was available for our patient cohort for comparing for outcome of disease, however this did not result in the identification of significantly differentially expressed genes.

Several meta-analysis studies on gene expression profiling provide a clarification for the lack of consistent results between different studies. Ein-Dor et al. report that there are many genes associated with different clinical behavior, but the differences in expression are quite

small and vary with different patient cohorts (26, 27). They conclude that a significant set of genes for predicting survival requires thousands of patient samples. For a relatively rare tumor like osteosarcoma this is obviously not achievable, especially given the variation in clinical presentation and treatment of this tumor.

To identify possible biological characteristics of osteosarcoma, by comparing osteosarcoma expression profiles with profiles from their presumed progenitors, i.e. mesenchymal stem cells (MSCs) and osteoblasts derived from these MSCs by *in vitro* differentiation resulted in a large set of 3300 differentially expressed genes. This result validates our statistical analysis, thereby justifying the negative results obtained with the comparison within the osteosarcoma profiles. However, this set of genes is definitely contaminated with a subset that is the result of the different source of the primary tumor tissues and the *in vitro* cultured MSCs and osteoblasts. Identification of common differentially expressed genes in osteosarcoma and benign osteoblastoma (most probably derived from the same progenitor cells, but with a complete different clinical behavior) as compared to the cultured MSCs and osteoblasts identified pathways that could most probably be attributed to the different sources of RNA. A subset of the 492 genes identified as commonly different in osteoblastoma and osteosarcoma when compared to cultured MSCs and osteoblasts could be assigned to specific pathways, thereby marking these as possible 'culture-tissue artifacts'. Especially the most significant pathway identified by GenMAPP analysis, i.e. up-regulation of the MHC class II pathway in both osteosarcoma and osteoblastoma is the most obvious example, most probably caused by infiltrating cells that contaminate the tumor tissue as has been described (28).

Pathways characterized by an excess of differentially expressed genes between MSCs and osteosarcomas, but lacking the possible 'culture-tissue artifacts' are most likely involved in malignant transformation. The GO-Elite application (http://www.genmapp.org/go_elite/go_elite.html) generates a non-redundant list of significant signal transduction pathways from the Gene Ontology (GO) project from a gene list with specific criteria. The criteria in this study included genes with a significant difference in mRNA expression between osteosarcoma and MSC or MSCs differentiated to osteoblasts. Criteria were strict and corrected for false discovery rate (FDR) due to multiple testing. Upon these restricted p-values the GO-Elite algorithm imposes another FDR correction. Table 3 lists the pathways that survive this double FDR.

The pathways that subsist the FDR correction are involved with cell cycle regulation, mitosis, DNA replication, the ususal suspects when comparing tumors with their progenitor cells. Osteosarcoma is especially characterized by high growth rate and numerous mitotic figures (29) and chemotherapy protocols are aimed at inhibition of the cell cycle. However, the current protocols are not effective in 40% of the cases (4) and this may be due to variable expression of certain cell cycle components.

Of special interest are developmental pathways which are known or suspected to play a role in osteosarcomagenesis. The Wnt signaling pathway shows downregulation when comparing MSCs or osteoblasts with osteosarcoma. Given the crucial role of this pathway in normal osteogenesis (25) and tumorigenesis in general this observation suggests a role for Wnt

signaling that differs from that in colorectal cancer, where upregulation of the pathway is considered as crucial for tumorigenesis (30). Indeed we have recently shown with a functional reporter assay that Wnt/β-catenin signaling seems to be absent in osteosarcoma cell lines (31). In addition we showed absence of nuclear β-catenin staining in primary osteosarcomas, indicative of inactive Wnt/β-catenin signaling. Also osteoblastoma showed a decrease of genes involved in Wnt/β-catenin signaling. The non-canonical Wnt5a ligand, which is involved in Wnt/planar cell polarity (32) was overexpressed in osteosarcoma cells. Both observations in osteosarcoma and osteoblastoma can be clarified from the fact that Wnt/β-catenin signaling is important for maintaining cells in the MSC state (33). Non-canonical Wnt signaling mediated by Wnt5a antagonizes this activity and promotes osteoblastogenesis of MSCs (34). Thus abnormal Wnt5a expression may be a key event in the malignant transformation in osteosarcoma. The findings of this study have led us to propose a model for osteosarcoma genesis, which is shown in Fig 7. Increase of Wnt signaling when comparing DO with MSCs is not observed. Wnt signaling changes during the process of differentiation and at different phases in osteoblastogenesis, different Wnt activities are observed.

The comparison between osteoblastoma and the same presumed progenitor cells MSCs and osteoblasts did not result in pathways associated with cell cycle regulation. The profiles of osteoblastomas have fulfilled a dual purpose in this study, they were instrumental in identifying differentially expressed genes that resulted from a difference in cell culture and primary tissue and they helped to recognize the cell cycle pathway as most important for malignant transformation of osteosarcoma.

From this analysis can be concluded that osteosarcoma differs from its presumed progenitor cells, MSCs and osteoblasts in terms of cell cycle regulation and developmental pathways. Benign osteoblastomas with the same progenitor cells but a much more favorable disease course are not characterized by an increase in cell cycle but by a decrease in components of canonical Wnt signaling.

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4

Testing association of a pathway with survival using gene expression data

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ABSTRACT

Motivation

A recent surge of interest in survival as the primary clinical endpoint of microarray studies has called for an extension of the Global Test methodology to survival.

Result

We present a score test for association of the expression profile of one or more groups of genes with a (possibly censored) survival time. Groups of genes may be pathways, areas of the genome, clusters from a cluster analysis or all genes on a chip. The test allows one to test hypotheses about the influence of these groups of genes on survival directly, without the intermediary of single gene testing. The test is based on the Cox proportional hazards model and is calculated using martingale residuals. It is possible to adjust the test for the presence of covariates. We also present a diagnostic graph to assist in the interpretation of the test result, visualizing the influence of genes. The test is applied to a tumour data set, revealing pathways from the gene ontology database that are associated with survival of patients.

Availability

The global test for survival has been incorporated into the R-package globaltest (from version 3.0), available from http://www.bioconductor.org.

INTRODUCTION

A microarray experiment typically results in many thousands of measurements, each relating to the expression level of a single gene. Single genes, however, are often not the primary theoretical focus of the researcher, who might be more interested in certain pathways or genomic regions that are suspected to be biologically relevant.

For this reason we have introduced the Global Test for groups of genes (1), which allows the unit of analysis of the microarray experiment to be shifted from the single gene level to the pathway level, where a "pathway" may be any set of genes, e.g. chosen using the *Gene Ontology* database or from earlier experiments. For every pathway, the Global Test can test (with a single test) whether the expression profile of that pathway is significantly associated with a clinical variable of interest. This allows researchers to immediately test theoretical hypotheses on the clinical importance of certain pathways. Even when such hypotheses are not directly available from biological theory or past research, the Global Test can significantly reduce the multiple testing problem, because there are typically much fewer pathways than genes.

In the original publication of the Global Test, the clinical variable could be either a continuous measurement or a 0/1 group indicator. Recently, however, there has been a surge of interest in survival time of patients as the primary clinical outcome in a microarray experiment. Many of these studies focus on prediction of survival, e.g. in breast cancer (2-4) and in lung cancer (5, 6). Other studies use multiple testing methods to find genes which are associated with survival (7).

The present paper extends the Global Test methodology to survival outcomes. It allows the researcher to test whether the expression profile of a given set of genes is associated with survival. More precisely it tests whether individuals with a similar gene expression profile tend to have similar survival times. A significant pathway may be a mix of genes which are upregulated for patients with short survival time, genes which are downregulated for the same patients, and other genes that show no association with survival at all.

The test of the present paper is based on the Cox proportional hazards model. Therefore it avoids the requirement of many analysis strategies to choose an arbitrary cut-off (e.g. five years survival), but uses all survival information that is present in the data. Technically, the test is derived from the goodness-of-fit test of (8). The original Global Test was derived in a similar way from a goodness-of-fit test for generalized linear models (9). The two Global Tests are therefore highly comparable and allow quite similar interpretations.

In this paper we also show how the test can be adjusted for the presence of covariates (possible confounders or competing risk factors). This allows better use of the Global Test in observational studies. Furthermore, it allows the researcher to establish that the microarray really adds something to the predictive performance of known risk factors, showing that it is not simply an expensive way to measure risk factors already known. It also allows the test to be used on more complex designs than a simple one-sample follow up study.

The new Global Test method presented in this paper has been incorporated into the R-package *globaltest*, version 3.0, which is available from http://www.bioconductor.org.

The approach will be illustrated on a data set of 17 osteosarcoma patients, testing pathways from the Gene Ontology database.

THE MODEL

The Global Test exploits the duality between association and prediction. By definition, if two things are associated, knowing one improves prediction of the other. Hence, if survival is associated with gene expression profile, this means that knowing the gene expression profile allows a better prediction of survival than not knowing the expression profile.
With this idea in mind we make a prediction model for prediction of survival from the gene expression measurements. The most convenient choice for such a model is the Cox proportional hazards model, which is the most widely used model for survival data in medical research. The Cox model uses the full empirical distribution of the survival times and it can handle censored data, i.e. samples for which the exact survival time is not known, but for which it is only known that the patient is still alive at a certain moment (10). The use of the Cox model requires a true follow-up study design, meaning that patients were not selected on their survival times in any way. If such a patient selection was made, the methods of this paper may not be appropriate: in VantVeer (2), for example, where a selected group of early metastases was compared to a selected group which was at least five years metastasis-free, the original Global Test for a 0/1 outcome is preferable (1).
Suppose the matrix of normalized gene expression measurements for the group of genes of interest is given by the $n \times m$ matrix X with elements x_{ij}, where n is the sample size and m the number of genes in the group. Suppose also that there is a number $p \geq 0$ of covariates for each patient, which we put in an $n \times p$ data matrix Z with elements z_{ij}. It will be assumed that $p < n$, but no such restriction is put on m.
Cox's proportional hazards model (10) (chapter 8) assumes the hazard function at time t for individual i to relate to the covariates as

$$h_i(t) = h(t)e^{c_i + r_i}, \tag{1}$$

where $h(t)$ is an unknown baseline hazard function and $c_i + r_i$ is a linear function of the covariates, which is split up in our case into $r_i = \sum_{k=1}^{m} \beta_k x_{ik}$, relating to the gene expressions, and $c_i = \sum_{l=1}^{p} \gamma_l z_{il}$, relating to the covariates. The hazard function determines the survival function $S_i(t)$, which gives the probability that individual i survives up to time t, through

$$S_i(t) = \mathrm{e}^{-H_i(t)}$$

where $H_i(t) = \int_0^t h_i(s)\,\mathrm{d}s$ is the cumulative hazard up to time t.
In this model showing that the gene expressions are associated with survival is equivalent to rejecting the null hypothesis

$$H_0 : \beta_1 = \cdots = \beta_m = 0$$

that all regression coefficients relating to the gene expressions are zero. If m were always small,

we could test H_0 using classical tests which were developed for the Cox model. These tests do not work for general m, however (for an overview of these classical tests see section 8.2) (10). To obtain a test that works whatever the value of m, we put an extra assumption on the regression coefficients $\beta_1, \ldots, \beta_m$. We assume that the regression coefficients of the genes are random and a priori independent with mean zero and common variance τ^2. The null hypothesis now becomes simply

$$H_0 : \tau^2 = 0,$$

so that the dimension of H_0 does not depend on m anymore. Note that the coefficients $\gamma_1, \ldots, \gamma_p$ of the covariates are not assumed to be random.

The Cox model with random coefficients is an empirical Bayesian model and is closely linked to penalized likelihood methods. It should be noted that we have not assumed a specific distributional form for the regression coefficients; the derivation of our test is invariant to the choice of the shape of this distribution. Choosing a Gaussian distribution results in a Cox ridge regression model (4); choosing a double exponential distribution results in a LASSO model (11). Both models can also be used to predict survival times of patients.

In the context of testing it is most insightful to view the prior distribution of the regression coefficients as the *focus of the power* of the test. The test that will be derived in the next section will be a score test, which has the property that it has optimal power against alternatives with small values of the parameter τ^2. This property stems from the fact that the score test is equivalent to the likelihood ratio test in the limit where the alternative $\tau^2 \to 0$ (12). Alternatives with small values of τ^2 tend to have small values of $\Sigma\beta_i^2$, so that the test can be said to be optimal on average against alternatives with small values of $\Sigma\beta_i^2$. These alternatives are mainly alternatives which have all or most regression coefficients non-zero but small. The test can therefore be said to be optimized against alternatives in which all or most genes have some association with the outcome. This alternative is precisely the situation in which we are interested, because we want to say something about the pathway as a whole.

Alternative tests can easily be derived for regression coefficients with a more complex covariance structure. If the vector $\beta = (\beta_1, \ldots, \beta_m)'$ is assumed a priori to have mean zero and covariance matrix $\tau^2\Sigma$, the resulting test of H_0 would be optimal against alternative with small values of $\beta' \Sigma \beta$. The standard choice of $\Sigma = I_m$ distributes power equally over all directions of β, while a different choice will have more power against deviations from H_0 in directions which correspond to the larger eigenvalues of Σ. This property could be exploited in the derivation of a test for a specific purpose or to incorporate prior knowledge. In this paper we shall restrict ourselves to $\Sigma = I_m$.

DERIVATION OF THE TEST

Testing association of a group of genes with survival can therefore be done by testing H_0 in the empirical Bayesian model (1) with random regression coefficients. In this section we will derive the test statistic for this test. A score test for the same model has also been studied by Verweij (8) in the context of testing the fit of the Cox model. Their derivation was based on the partial likelihood of the Cox model. In this paper we give an alternative derivation based of the full likelihood and a simpler martingale argument.
We derive the test in stages. First suppose that all parameters except τ^2 are known, i.e. the regression coefficients $\gamma_1, \ldots, \gamma_p$ and the baseline hazard function $h(t)$ are known. In this simplified situation it will be relatively easy to derive the score test, which can be generalized to the situation with unknown parameters later in this section.

The basic score test

By definition a score test is based on the derivative of the log-likelihood at the value of the parameter to be tested. Suppose for each individual i we have observed a survival time t_i and a status indicator d_i, where $d_i = 1$ indicates death (the patient died at t_i) and $d_i = 0$ censoring (the patient was lost to follow-up at t_i). The loglikelihood of τ^2 in the model (1) is

$$L(\tau^2) = \log\left\{ \mathrm{E}_{\mathbf{r}}\left[\exp\left(\sum_{i=1}^{n} f_i(r_i) \right) \right] \right\}, \tag{2}$$

where

$$f_i(r_i) = d_i[\log\{h(t_i)\} + c_i + r_i] - H(t_i)\mathrm{e}^{c_i + r_i}$$

is the contribution to the loglikelihood of individual i for fixed r_i, and $H(t) = \int_0^t h(s)\,\mathrm{d}s$ is the cumulative baseline hazard.
From the assumptions on the distribution of $\beta_1, \ldots, \beta_m$ we can derive the distribution of $\mathbf{r} = (r_1, \ldots, r_n)'$, the vector of the linear effects of the gene expressions. This $\mathbf{r}$ has mean zero and covariance matrix $\tau^2 R$, where $R = XX'$. For the general likelihood (2) and an $\mathbf{r}$ of this form, Le Cessie and van Houwelingen (9), have used a Taylor approximation to derive that

$$\frac{\partial L(0)}{\partial \tau^2} = \frac{1}{2}\left(\sum_i R_{ii} \frac{\partial^2 f_i(0)}{(\partial r_i)^2} + \sum_{i,j} R_{ij} \frac{\partial f_i(0)}{\partial r_i} \frac{\partial f_j(0)}{\partial r_j} \right)$$

For the Cox model this becomes

$$\frac{\partial L(0)}{\partial \tau^2} = \frac{1}{2}\left(\sum_{i,j} R_{ij}(d_i - u_i)(d_j - u_j) - \sum_i R_{ii} u_i \right), \tag{3}$$

where $u_i = e^{c_i} H(t_i)$, $i = 1, \ldots, n$, is the hazard incurred by individual i up to time t_i. Note that $d_i - u_i$ is the martingale residual of individual i at time t_i (Klein and Moeschberger, section 11.3) (10).

For known $H(t)$ and known $c_1,\ldots,c_n$, the expression (3) can be standardized to have unit variance and used as the score test statistic. When these parameters are unknown, we must plug in maximum likelihood estimates for them under the null model in which $\tau^2 = 0$. Standardizing the score test is traditionally done using the Fisher Information, calculated from the second derivatives of the loglikelihood. In this case these calculations are very unpleasant, and it turns out to be simpler to standardize using the estimated variance of the test statistic.

Using estimated baseline hazard

We shall first plug in the estimate for the cumulative hazard $H(t)$, but still assume that $\gamma_1,\ldots,\gamma_p$ and hence $c_1,\ldots,c_n$ are known. As the maximum likelihood estimate of $H(t)$ we can take the Breslow estimator (Klein and Moeschberger, section 8.6) (10)

$$\hat{H}(t_i) = \sum_{t_j \le t_i} \frac{d_j}{\sum_{t_k \ge t_j} \mathrm{e}^{c_k}}, \quad i = 1,\ldots,n$$

and write $\hat{u}_i = \mathrm{e}^{c_i}\hat{H}(t_i), i = 1,\ldots,n$.

Using twice the estimated derivative of the log-likelihood (3) as the test statistic and writing it in matrix notation we get the test statistic

$$T = (d - \hat{u})' R (d - \hat{u}) - \mathrm{trace}(R\hat{U}) \tag{4}$$

where $d = (d_1,\ldots,d_n)'$, $\hat{u} = (\hat{u}_1,\ldots,\hat{u}_n)'$ and $\hat{U} = \mathrm{diag}(\hat{u})$, an $n \times n$ diagonal matrix with $\hat{U}_{ii} = \hat{u}_i$.

The derivation of estimates for the mean and variance of T is quite technical and will be given in the separate section 7.5. The estimated mean is

$$\hat{\mathrm{E}}(T) = -\mathrm{trace}(RPP'), \tag{5}$$

where P is an $n \times n$ matrix with i,j-th element

$$p_{ij} = I_{\{t_i \ge t_j\}} \frac{d_j \mathrm{e}^{c_i}}{\sum_k s_{kj} \mathrm{e}^{c_k}}.$$

Each p_{ij} is the increment of the cumulative hazard incurred by individual i at time t_j, so that $\sum_i p_{ij} = d_j$ and $\sum_j p_{ij} = \hat{u}_i$.

The estimated variance of T is

$$\widehat{\mathrm{Var}}(T) = \sum_{j=1}^{n} p_j' \,\mathrm{diag}(t_j t_j'), \tag{6}$$

where p_j is the j-th column of P and $t_j = (I - \mathbf{1}p_j')\,[\mathrm{diag}(R) + 2R(m_j - p_j)]$. The diag of a square matrix is the column vector of its diagonal elements; is an $n \times 1$ vector of ones, and m_j is the j-th column of the matrix $M = (D - P)B$, where $D = \mathrm{diag}(d)$ is a diagonal matrix with $D_{ii} = d_i$ and B is an $n \times n$ matrix with elements $b_{ij} = {}_{\{t_i < t_j\}}$. The elements m_{ij} of M can be interpreted as the estimated martingale residual of individual i just before time t_j.

For purposes of interpretation it is often easier to take

$$T_0 = (d - \hat{u})' R (d - \hat{u})$$

as the unstandardized test statistic. It has $\hat{\mathrm{E}} T_0 = \mathrm{trace}(R\hat{U} - PP')$ and $\widehat{\mathrm{Var}}(T_0) = \widehat{\mathrm{Var}}(T)$, so that it leads to the same standardized test statistic:

$$Q = \frac{T - \hat{\mathrm{E}} T}{\widehat{\mathrm{Var}}(T)} = \frac{T_0 - \hat{\mathrm{E}} T_0}{\widehat{\mathrm{Var}}(T_0)}.$$

Using estimated regression coefficients

In general the regression coefficients $\gamma_1, \ldots, \gamma_p$ of the covariates are not known but must be estimated. Replacing $\gamma_1, \ldots, \gamma_p$ by their maximum likelihood estimates will still give a valid score test for H_0, but with a different distribution of the test statistic. We use the following approximation to this distribution which is derived by Verweij (8).

The estimated martingale residuals $d - \tilde{u}$ based on the estimated $\hat{\gamma}_1, \ldots, \hat{\gamma}_p$ can be approximated in a first order Taylor approximation by

$$d - \tilde{u} \approx (I - V)(d - \hat{u}) \tag{7}$$

with $V = WZ(ZWZ')^{-1}Z'$, $W = \mathrm{diag}(\hat{u}) - PP'$ and Z the $n \times p$ data matrix of the fixed covariates. Therefore the unstandardized test statistic T_0 can be approximated as

$$T_0 \approx (d - \hat{u})' \tilde{R} (d - \hat{u})$$

with $\tilde{R} = (I - V)' R (I - V)$. The expectation of T_0 can be estimated using the formulae in section 7.2. They are approximately

$$\hat{\mathrm{E}} T_0 \approx \mathrm{trace}(\tilde{R} W)$$

and

$$\widehat{\mathrm{Var}}(T_0) \approx \sum_{j=1}^{n} p_j' \, \mathrm{diag}(\tilde{t}_j \tilde{t}_j'),$$

with $\tilde{t}_j = (I - \mathbf{1} p_j')[\mathrm{diag}(\tilde{R}) + 2\tilde{R}(m_j - p_j)]$. To evaluate $\hat{\mathrm{E}} T_0$ and $\widehat{\mathrm{Var}}(T_0)$ we replace the parameter values of $\gamma_1, \ldots, \gamma_p$ by their estimates. Simulations in Verweij (8) show this approximation to be quite accurate.

The distribution of the test statistic

There are two ways to calculate the p-value of the test: by asymptotic theory and by permutation arguments. We outline both options and their advantages.

In equation (7.5) it will be shown that the centered test statistic $T - \hat{\mathrm{E}} T$ can be written as a linear combination of n martingales. Therefore by the martingale central limit theorem (13) the distribution of the standardized Q converges to a standard normal distribution as $n \to \infty$. This fact motivates the use of a normal approximation to the distribution of Q to calculate the one-sided p-value [see also simulation results by Verweij (8). Interesting simulations which give insight in the power of the score test in a random effects survival model are given in Andersen (13).

For small samples the asymptotic distribution may not be reliable enough. An alternative is to calculate Q for all, or a random sample of many (10,000), permutations of the martingale residuals of the n samples. This randomly redistributes the vectors of gene expression measurements over the individuals, while keeping the relationship between the fixed covariates and survival the same. The resulting distribution is another approximation to the null distribution of Q, which can be used to find the p-value. Use of the permutation null distribution requires the assumption that there is no relationship between the gene expressions on the one hand and the covariates and the censoring mechanism on the other hand: permuting destroys these associations. This makes the permutation null distribution less useful when covariates are present.

The main advantage of the permutation-based p-value is that it gives an "exact" p-value, which is guaranteed to keep the alpha level provided enough permutations are used. This is especially useful for smaller sample sizes, where we may not trust the normality of the distribution of Q. The advantage of the asymptotic theory p-value---aside from being much quicker to calculate---is that it has more power: the permutation based p-value does not use the full null distribution, but the null distribution conditional on the set of observed martingale residuals. With this conditioning the test loses some power, as the set of observed residuals is informative for the parameter τ^2.

Counting process calculations

In this technical section we calculate the mean and variance of the test statistic T under the null hypothesis for known $c_1, \ldots, c_n$ but estimated $H(t)$, as given in (5) and (6). For this we will use a counting process notation (13, 14). The strategy we will use is common in martingale theory: we write our test statistic T as the limit of a process $T(t)$ as $t \to \infty$ and decompose $T(t)$ into a martingale and a compensator. The limit of the compensator is the estimator of the mean of T and the limit of the predictable variation process is the estimate of the variance. For an alternative derivation, see Verweij (8).

Let $\mathbf{Y}(t) = (Y_1(t), \ldots, Y_n(t))'$ be the vector of at-risk processes of individuals $1, \ldots, n$ and $\mathbf{N}(t) = (N_1(t), \ldots, N_n(t))'$ the vector of their counting processes. Then $\mathbf{N}$ has intensity process $\mathbf{\Lambda} = C\mathbf{Y}(t)H(t)$, where C is a diagonal matrix with $C_{ii} = e^{c_i}$, $i = 1, \ldots, n$. Write $(1, \ldots, 1)'$, $n \times 1$ and $N(t) = \mathbf{1}'\mathbf{N}(t)$ the total counting process.

In the counting process notation, $d = \mathbf{N}(\infty)$ and $\hat{u} = \hat{\mathbf{\Lambda}}(\infty)$ with $\hat{\mathbf{\Lambda}}(t) = \int_0^t \mathbf{V}(s)\mathbf{1}' \, \mathrm{d}\mathbf{N}(s)$, where $\mathbf{V} = C\mathbf{Y}(\mathbf{1}'C\mathbf{Y})^{-1}$. Wherever possible we will drop the dependence on time for convenience of notation.

Note that the compensator of $\hat{\mathbf{\Lambda}}$ is $\mathbf{\Lambda}$, which is also the compensator of $\mathbf{N}$. Write $\widehat{\mathbf{M}} = \mathbf{N} - \hat{\mathbf{\Lambda}}$. Then $d - \hat{u} = \widehat{\mathbf{M}}(\infty)$ and $\widehat{\mathbf{M}}(t) = \int_0^t (I - \mathbf{V}\mathbf{1}') \, \mathrm{d}\mathbf{N}$ is a martingale vector. Subtracting the intensities and writing $\mathbf{M} = \mathbf{N} - \mathbf{\Lambda}$,

$$\widehat{\mathbf{M}}(t) = \int_0^t (I_n - \mathbf{Y}\mathbf{1}') \, \mathrm{d}\mathbf{M}.$$

The statistic T is $T(\infty)$ with

$$T(t) = \text{trace}[R\widehat{\mathbf{M}}\widehat{\mathbf{M}}' - R\,\text{diag}(\hat{\mathbf{\Lambda}})].$$

From the integration by parts formula [ ][theorem A.1.2]Fleming91 it follows that, almost surely,

$$\widehat{\mathbf{M}}\widehat{\mathbf{M}}' = \int_0^t \widehat{\mathbf{M}}^- \,\mathrm{d}\widehat{\mathbf{M}}' + \int_0^t \mathrm{d}\widehat{\mathbf{M}}\,(\widehat{\mathbf{M}}^-)'$$

$$+ \int_0^t (I - \mathbf{1}\mathbf{V}')\text{diag}(\mathrm{d}\mathbf{N})(I - \mathbf{V}\mathbf{1}'), \qquad (8)$$

where $\widehat{\mathbf{M}}^-(s) = \widehat{\mathbf{M}}(s-)$ is a predictable process. Using (8) and some linear algebra we can say that, almost surely,

$$T(t) = \int_0^t (\text{diag}(R)' + 2(\widehat{\mathbf{M}}^-)'R - \mathbf{V}'R)(I - \mathbf{V}\mathbf{1}')\,\mathrm{d}\mathbf{N}$$

$$- \int_0^t \mathbf{V}'R\,\mathrm{d}\mathbf{N}.$$

Because $\int_0^t (I - \mathbf{V}\mathbf{1}')\,\mathrm{d}\mathbf{N}$ is a martingale and $\text{diag}(R)' + 2(\widehat{\mathbf{M}}^-)'R - \mathbf{V}'R$ is predictable, the compensator of the process T is $-\int_0^t \mathbf{V}'R\,\mathrm{d}\hat{\mathbf{\Lambda}}$, which we can estimate by

$$\hat{\mathrm{E}}T = -\int_0^t \mathbf{V}'R\,\mathrm{d}\hat{\mathbf{\Lambda}} = -\int_0^t \mathbf{V}'R\mathbf{V}\mathbf{1}'\,\mathrm{d}\mathbf{N}.$$

The process $S = T - \hat{\mathrm{E}}T$ is a martingale. It can be written in the following way

$$S = \int_0^t (\text{diag}(R)' + 2(\widehat{\mathbf{M}}^- - \mathbf{V})'R)(I - \mathbf{V}\mathbf{1}')\,\mathrm{d}\mathbf{M} \qquad (9)$$

as the integral of the predictable process vector

$$\mathbf{K} = (\text{diag}(R)' + 2(\widehat{\mathbf{M}}^- - \mathbf{V})'R)(I - \mathbf{V}\mathbf{1}')$$

over the martingale vector $\mathbf{M}$. The predictable variation process of S is therefore $\langle S\rangle = \int_0^t \text{diag}(\mathbf{K}\mathbf{K}')'\,\mathrm{d}\mathbf{\Lambda}$, which we can estimate by

$$\widehat{\text{Var}}(T) = \int_0^t \text{diag}(\mathbf{K}\mathbf{K}')'\,\mathrm{d}\hat{\mathbf{\Lambda}} = \int_0^t \text{diag}(\mathbf{K}\mathbf{K}')'\mathbf{V}\mathbf{1}'\,\mathrm{d}\mathbf{N}$$

To evaluate $\hat{\mathrm{E}}T$ and $\widehat{\text{Var}}(T)$ we use

$$p_{ij} = \int_0^\infty \frac{\mathrm{e}^{c_i}Y_i}{Y}\,\mathrm{d}N_j = \mathbf{1}_{\{t_i \geq t_j\}}\frac{\mathrm{e}^{c_i}d_j}{\sum_{t_k \geq t_j}\mathrm{e}^{c_k}}$$

and

$$m_{ij} = \int_0^\infty \widehat{M}_i^-\,\mathrm{d}N_j = \mathbf{1}_{\{t_i < t_j\}}d_i - \sum_{k=1}^n \mathbf{1}_{\{t_k < t_j\}}p_{ik}.$$

Writing P for the $n \times n$ matrix with elements p_{ij} and M for the $n \times n$ matrix with elements m_{ij}, the results (5) and (6) follow.

INTERPRETATION

When testing a specific pathway for a specific sample of patients, it is usually not satisfactory to only report the resulting p-value. In this section we will discuss some issues related to interpretation of the test result. We show how to calculate and visualize the influence of individual genes on the test result. We also propose an diagnostic which can be used when many genes are associated with survival, to assess whether a gene group is exceptional. We only give the theory here; for an example see section 9.

Interpretation

The test of this paper is derived from the Cox model in the same way as the Global Test in Goeman (1) was derived from the generalized linear model. The functional form of the test statistic is therefore quite similar, the martingale residuals taking the place of the residuals from the generalized linear model in that paper. Much of the interpretation of the test statistic is therefore also quite similar.

Central to all interpretation of the test outcome is the matrix $R = XX'$ which figures prominently in the formula for the test statistic. It is an $n \times n$ matrix which can be seen as describing the similarities in expression profile between the samples. The entry R_{ij} is relatively large if samples i and j have a relatively similar expression profile over the pathway of interest.

To show the role of the matrix R, we can rewrite the unstandardized test statistic T_0 as

$$T_0 = \sum_{i=1}^{n} \sum_{j=1}^{n} R_{ij}(d_i - \hat{u}_i)(d_j - \hat{u}_j),$$

which is the sum over the term-by-term product of the entries of R and the entries of the matrix $(d - \hat{u})(d - \hat{u})'$. The i,j-th entry of the latter matrix is large whenever samples i and j have similar martingale residuals. The test statistic T_0 is therefore relatively large whenever the entries of the matrices R and $(d - \hat{u})(d - \hat{u})'$ are correlated, which is when similarity in gene expressions tends to coincide with similarity in the martingale residual. Hence, the test statistic is large if individuals who die sooner than expected tend to be relatively similar in their gene expression profile and the individuals who live longer than expected also tend to be similar in their gene expression profile.

Gene plot

To investigate the influence of individual genes on the test outcome we can rewrite $R = \sum_{i=1}^{m} x_i x_i'$, where x_i is the i-th column of X $(i = 1, \ldots, m)$, containing the measurements for the i-th gene. The unstandardized test statistic then becomes

$$T_0 = \sum_{i=1}^{m} T_i,$$

where $T_i = (d - \hat{u})' x_i x_i' (d - \hat{u})$ is exactly the unstandardized `global' test statistic for testing whether the `pathway' containing only gene i is associated with survival. The test statistic of a pathway is therefore a weighted average of the test statistics for the m genes in the pathway.

In a plot we can visualize the influence of the individual genes by showing the values $T_i - \hat{\mathrm{E}}T_i$, with their standard deviation under the null hypothesis (calculated using the methods of section 7). An example of such a 'gene plot' is given in figure 1. In this plot, large positive values indicate genes with a large (positive or negative) association with survival and hence genes that make the pathway more significant. As $T_i \propto \|x_i\|^2$, genes with more expression variance tend to carry more weight in the pathway.
Note that the visualized values of the gene influences T_i in the gene plot are essentially univariate: they only depend on the gene i itself. The multivariate nature of the test statistic Q is therefore not visible in the gene plot. It comes in because, although T_0 is the sum of the T_i and $\hat{\mathrm{E}}T_0$ is the sum of the $\hat{\mathrm{E}}T_i$, the variance of T_0 is generally not the sum of the variances of the T_i.

The comparative p

The global test tests the null hypothesis that the pathway is not associated with survival. This null hypothesis only depends on the observed survival and on the genes in the pathway itself: the result is absolute, not relative to the other pathways.
However, there are situations in which one would be more interested in a relative result. If the global test on the set of all genes is very significant, we can usually expect a sizeable proportion of the genes on the array to be associated with survival. In that case we can expect many pathways to show association with survival as well. This will hold especially for the larger pathways, which will often include some of the genes which are associated with survival.
In such situations we propose a diagnostic called "comparative p", which can help interpret the p-value that comes out of the test. The comparative p for a pathway of size m with p-value $\overline{p}$ is defined as the proportion of randomly selected sets of genes of the size m that have an global test p-value smaller or equal to $\overline{p}$. To calculate this comparative p we draw 1,000 or 10,000 random gene sets from the array without replacement.
The comparative p fulfills a role different from the p-value and should only be used alongside it. It is a diagnostic, not a p-value in the statistical sense. It tells whether the p-value of a group of genes is much lower than could be expected from a gene group of its size in this data set.

APPLICATION: OSTEOSARCOMA DATA

We applied the above methodology to a data set of 17 osteosarcoma patients from the Leiden University Medical Center.

Data

A genome wide screen of gene expression in osteosarcoma was done using Hu133a gene expression chips (Affymetrix, Santa Clara, CA). This chip contains 22,283 genes. A successful hybridization was obtained for 17 osteosarcoma biopsies. Three of the samples were amplified,

labelled and hybridized in duplicate, one sample in triplicate. These technical replicates were averaged after gene expression measures were obtained, which was done using *gcrma* (15). No preselection of genes was made.
The 17 patients were followed up to 10 years. Median survival time was 40 months. Available covariates included the presence of metastasis at diagnosis, histology and response to neo-adjuvant chemotherapy. However, as treatment was not uniform over all patients, these covariates were not prognostic and we did not consider them.
Pathway information was obtained from the Gene Ontology (GO) database, using the BioConductor GO package (16). Pathways that were considered of specific interest were cell cycle (GO: 7049), DNA repair (GO: 6281), Angiogenesis (GO: 1525), Skeletal development (GO: 1501) and Apoptosis (GO: 6915).

Analysis

When testing pathways of interest, it is advisable to also test the 'pathway' of all genes on the chip for association with survival. This shows whether the overall gene expression profile is associated with survival. The results for the pathway of all genes and for the five pathways of primary interest are given in table 1. We calculated the p-value using both the asymptotic theory method and the permutation method (using 100,000 permutations).

TABLE 1.
Global Test results for the Osteosarcoma data and the pathways of primary interest. The p-values were calculated using the permutation and asymptotic method. The final column gives the comparative p (see section 8.3).

pathway	genes	Q	perm. p	asym. p	comp. p
All genes	22283	2.446	0.0120	0.0072	---
Cell cycle	1115	2.957	0.0042	0.0016	0.006
DNA rep.	271	3.123	0.0006	0.0009	0.011
Angiogen.	66	0.917	0.1429	0.1795	0.774
Skel. dev.	185	0.002	0.4133	0.4992	0.998
Apoptosis	656	2.533	0.0093	0.0057	0.210

The permutation p-values tend to be somewhat more conservative than the asymptotic p-values, reflecting both the slight loss of power for the permutation test and a deviation from asymptotic normality due to the small number of samples.
In this data set the expression profile over the set of all genes on the chip is significantly associated with survival. Note that this does not mean that every gene on the chip is associated with survival. It means that the patients who die early are relatively similar to each other in

terms of their overall expression profile, while patients who live long are likewise relatively similar. It also means that there is some potential for prediction of survival based on gene expression, even before any pre-selection of genes. The cell cycle, DNA repair and apoptosis pathways are clearly associated with survival, while there is no evidence for this association in angiogenesis and skeletal development.

Because the test for all genes was significant, we expect a sizeable proportion of genes to be associated with survival, so that many pathways will be associated with survival. The comparative p gives a measure whether the p-value found for the pathway is unusually low given that it is a pathway of its size from this data set (see section 8.3). For the results in table 1 10,000 gene sets were sampled for each pathway. We used the asymptotic p-values for the comparative p calculations.

We conclude that cell cycle and DNA repair are more clearly associated than could be expected from a gene set of its size in this data set: only around 60 out of 10,000 random gene sets of size 1,115 have a lower p-value than the cell cycle pathway. The expression profile of the apoptosis pathway is clearly associated with survival, as can be seen from the p-values; however it is not exceptional in that: more than 20% of random gene sets have a lower p-value than apoptosis. The Skeletal development pathway is interesting in its own way: it is clearly not associated with survival ($p = 0.5$) and this is quite exceptional for a pathway of this size in this data set: only around 20 in 10,000 random gene sets had a higher p-value. The skeletal development pathway seems to include uncommonly few genes which are associated with survival.

It can occur in some data sets that the set of all genes is not significant, while some pathways (eg. DNA repair) are significant. This occurs in table 1 for example if we use FDR-adjusted p-values with a threshold of 0.01 (17). The result for all genes can be seen as a false negative test result. However, another valid interpretation is that prediction of survival without biological pre-selection of genes is uncertain, but if it is known a priori that the genes in the DNA repair pathway are likely to be informative, some prediction of survival is possible.

Mining the GO database

If it is not a priori known which pathways are of specific interest, one can also use a data-mining approach, trying to find those pathways which are most significantly associated with survival.

For the osteosarcoma data we explored the Gene Ontology database. Of all GO terms, 4,032 matched at least one gene on the hu133a chip. We excluded all terms which matched only one gene, because the interesting single genes pathways would already have been found in single gene testing. This left 3,080 pathways, which we all tested for association with survival. We used the asymptotic p-value, because due to the randomness in the the permutation p-value it does not give a unique list. Table 2 gives the ten GO-terms with the smallest p-values.

To adjust for multiple testing, one can use the Benjamini and Hochberg FDR (17). All 10 pathways in table 2 are significant on an FDR of 0.05. The p-values of the pathways tend to

have positive correlations because of pathway overlap and pathways being subsets of other pathways. A FDR-controlling procedure that would make use of these dependencies would potentially gain much power in this situation.

TABLE 2.
Global Test results for the Osteosarcoma data on 3,080 Gene Ontology pathways, showing the top 10 FDR-adjusted p-values.

pathway	# genes	Q	FDR-adjusted p
GO:0015630	21	4.306	0.016
GO:0019932	8	4.176	0.016
GO:0045192	2	4.148	0.016
GO:0045595	17	4.060	0.016
GO:0042518	7	4.054	0.017
GO:0000158	8	3.993	0.018
GO:0040008	9	3.944	0.018
GO:0010033	10	3.844	0.023
GO:0006479	13	3.791	0.026
GO:0030111	9	3.766	0.026

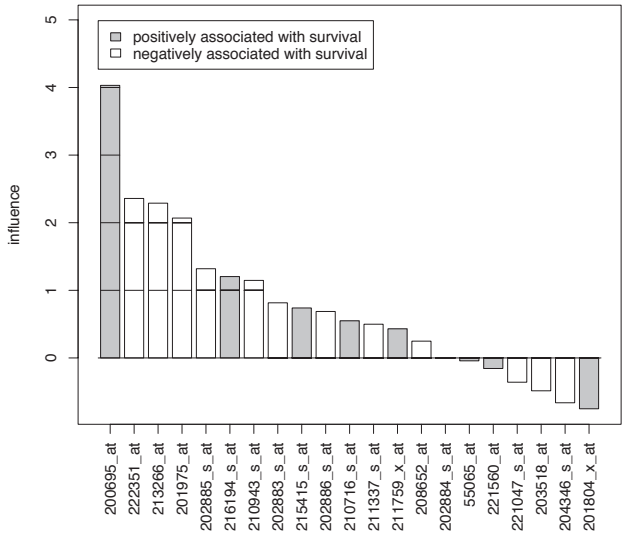
The literature confirmed the importance of many of these GO-terms in tumorigenesis. For example, both microtubule cytoskeleton (GO:0015630) and phosphorylation of Stat3 protein (GO:0042518) are known to be involved in growth and differentiation signaling, processes which are often disturbed in tumors. Second-messenger mediated signaling (GO:0019932) is a superset of the Stat3 pathway. Protein amino acid methylation (GO:0006479) is involved in protein degradation. Alterations in the stability of proteins is often a hallmark of tumors and may affect the aggressiveness of a tumor and thereby the patient's survival.

A diagnostic plot

To learn more about the outcome of the Global Test than just the p-value one can use the diagnostic plot described in section 8. We illustrate the use of this plot on the microtubule cytoskeleton pathway, which emerged on top of table 2.
The gene plot for the 21 genes in this pathway is given in figure 1. Each bar gives the global test statistic for testing whether the gene set containing only that single gene is associated with survival. The test statistic for the whole pathway is a weighted average of the bars of the genes (see section 8.2). The colour of the bars distinguishes between positive and negative association with survival.

FIGURE 1.
Gene plot of microtubule cytoskeleton pathway, showing the sorted global test statistics for testing the 21 single gene pathways which make up the pathway.

Figure 1 shows that at least only four out of 21 genes in the microtubule cytoskeleton pathway show a significant association with survival on their own. Further, the pathway is a mix of genes which are positively and negatively associated with survival. Looking more closely at the gene plot can be a basis for investigating more deeply into the structure of the pathway, perhaps to formulate hypotheses on interesting subpathways.



DISCUSSION

It has often been remarked that the key to successful microarray data analysis lies in an intelligent integration of advanced statistical methods with the vast domain of biological knowledge that is already available. The global test for survival presented in this paper is a step forward in this direction, combining known biological pathway information with the statistical sophistication of the Cox proportional hazards model.
Due to its complexity the Cox model has been slow to find its way to microarray methodology. Most methods require survival to be reduced to a two-valued variable, using an arbitrary cut-off, resulting in unnecessary loss of information. By using the Cox model for survival, gene expression analysis can improve performance and also become better connected to traditional medical statistics.

Pathway information is available from many databases and is essential for the understanding of the outcomes of a microarray experiment. The Global Test methodology allows researchers to look directly for important pathways, without first having to go through single gene testing. This may lead to a better use of pathway information and more directly interpretable results.

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5

Overexpression of the HER-2 oncogene does not play a role in high-grade Osteosarcomas

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ABSTRACT

Purpose

The aim of our study was to determine whether or not expression of the tyrosine kinase receptor HER2 (also known as ErbB2/Her2/*neu*) is overexpressed in human osteosarcomas. We studied 15 biopsy and 18 resection specimens at the mRNA and protein levels.

Patients and methods

The HER2 status in the osteosarcoma specimens was assessed by immunohistochemistry (IHC) and quantitative Real-Time Polymerase Chain Reaction (PCR). In moderately immunopositive cases, fluorescent *in situ* hybridization (FISH) analysis was used in order to identify any possible gene amplification.

Results

Twenty-seven samples were evaluable for IHC, and only one case showed a moderately positive membrane staining. The remaining samples showed no staining or focal cytoplasmatic staining (2 samples). In the moderately positive case, FISH analysis showed no *HER-2* gene amplification. There was also no overexpression of HER2 mRNA, suggesting this sample was a false positive immunostain. HER2 mRNA expression was present in all samples at a similar level to that in the breast cancer cell line MCF7, which does not overexpress HER2 and was used a negative control.

Conclusion

This study shows that HER2 mRNA or membranous HER2 protein overexpression is absent in human osteosarcoma. We noted various inconsistencies in previous published studies, with regard to methodology and the interpretation of the results based on poor methodology. We therefore conclude that the positive data with regard to HER2 overexpression reported in these previous studies are not reliable. Our results suggest that the monoclonal antibody trastuzumab (Herceptin), directed against the HER2-receptor, is not likely to be an effective therapeutic agent in osteosarcoma.

INTRODUCTION

Osteosarcoma (OS) is the most common primary bone tumour, with an incidence of (on average) 6.5 patients per 10^6 children and 2.1 patients per 10^6 per year in adults. The peak incidence is between 10 and 19 years, and when it occurs after 40 years, it is usually associated with a pre-existing condition (1). Metastatic disease, large tumor volume, older age at presentation, axial site of the tumour, histological subtype of OS and histological response on preoperative treatment all have been associated with poor outcome (2-4). However, apart from metastatic disease and axial site, that occur in 10-20% of the cases, none of the other factors have been reliable enough to distinguish between high and low risk groups at diagnosis (2, 3). Chemotherapy induced tumour cell necrosis can be assessed only after surgery. Consequently, there is clearly a need to identify new predictive factors at time of diagnosis (2).

With the recent progress in the understanding of molecular biology of cancer, the cell surface receptor HER2 (also called $p185^{HER2}$) has suggested to be predictive for survival (5-7).

The *HER-2* gene (also known as ERBB2 or *neu* gene), located on chromosome 17q21 (8) encodes for a 185kD transmembrane receptor (9) and belongs to the epidermal growth factor (EGF) tyrosine kinase receptor superfamily (10). *HER-2* is an oncogene, and HER2 overexpression in vitro (11) and in human cancers, particularly in 25-30% of breast cancer patients, has been associated with disease behavior (12).

Overexpression of the normal HER2 receptor at the cellular membrane, above a critical level, results in cellular transformation and malignant cell proliferation in athymic mice (13). This oncogenic effect can be reverted by the use of monoclonal antibodies, directed against the HER2 protein (14). Furthermore, both in vitro and clinical studies have reported increased response rates to chemotherapeutic drugs when these are combined with anti-HER2 antibodies (15). Based on these results, and reports that *HER-2* is overexpressed in osteosarcoma, phase II trials have begun to study the efficacy of Herceptin® (trastuzumab), the commercial designation of humanized HER2 monoclonal antibody, in patients with relapsed or refractory osteosarcoma (6, 16) (www.cancer.gov/clinical_trials: MSKCC-99097/NCI-T98-0083 and COG-AOST0121).

Four studies have suggested that *HER-2* is overexpressed in osteosarcoma, however they report different correlations between *HER-2* overexpression and prognosis (5-7, 17). Furthermore, other investigators have not been able to confirm their conclusions (16, 18, 19). In order to clarify these conflicting results and to investigate whether trastuzumab is a suitable therapy in osteosarcoma, we studied the expression of the *HER-2* gene by assessing gene amplification, mRNA- and protein expressions of HER2 in 30 patients.

PATIENTS AND METHODS

Patients

All patients presented to the Department of Orthopedic Surgery of the Leiden University Medical Center with newly diagnosed high-grade osteosarcoma of the limbs (n=32) and the os ileum (n=1) from 1991 to 1999 (Table 1). Diagnosis was made on routine haematoxilin-eosin (HE) staining in 15 pre-treatment Yamshidi core needle biopsy specimens (group A) and in 18 resection specimens (group B) of the primary (n=12) or relapsed (n=6) tumour. If eligible, patients were offered participation in running European Osteosarcoma Intergroup (EOI) studies, such as Europeran Organization for Research and Treatment of Cancer 80861 (20)and 80871 studies (21), the EOI phase II study of intensive chemotherapy with granulocyte-colony stimulating factor (G-CSF) (22) or the recently closed EORTC 80931 trial (23). Patients who did not enter a trial (either refused or were not eligible) were offered short intensive courses of chemotherapy. One patient did not receive chemotherapy because of advanced age. Another patient was treated with doxorubicin only as palliative therapy. Histological response after pre-operative chemotherapy was determined in the resection specimens by a reference pathologist using a modified Huvos grading system. A good response was defined if less than 10% viable tumour cells were seen in the post chemotherapy specimens, whereas a poor response was present in cases where there were 10% or more viable tumour cells. Only patients with a poor response were selected in group B because HER2 status can only be assessed on viable cells, i.e. chemotherapy-resistant cells, and not on necrotic samples. HER2-status was assessed in the biopsy (group A), or resection (group B) specimens.

RNA extraction

RNA was isolated from 30 sections of 20 μm snap frozen fresh osteosarcoma tissue sections, using Trizol reagent (Invitrogen®) according to the manufacturer's instruction. For isolation of mRNA, only tissue containing more than 50% of tumour cells was selected.

Quantification of HER2 transcripts with TaqMan Real-Time PCR

HER2 expression was determined by quantitative real-time PCR (qPCR) using cDNA, synthesized from 2.5 μg reverse-transcribed total RNA in a 100 μl reaction containing 20 μl first-strand RT-PCR buffer (GIBCO), 10 μl 0.1 M dithiothreitol (DTT), 10 μl 10 mM deoxynucleotide triphosphate (dNTP), 25 μl 50 μM random hexamers (PE/Applied Biosystems), 100 U RNAsin (PE/Applied Biosystems), 500 U Superscript II reverse transcriptase (GIBCO). Incubation was for 10 min at room temperature, 60 min at 42°C and 5 min at 95°C. Porphobilinogen deaminase (PBGD), a housekeeping gene, was used as reference in a parallel reaction to quantify the relative results from real-time PCR for HER2. The primers and probe for PBGD were described previously (24). Primers for *HER-2* amplification, derived from Genbank accession number X03363, were 5'-GGC CTG CGG GAG CTG-3' (forward) and 5'-TCC GCT GGA TCA AGA CCC-3' (reverse) resulting in a product of 67 base pairs, detected by the probe (5'-TCC TTT CAA GAT CTC TGT GAG GCT TCG AAG-3' labelled with FAM and the quencher TAMRA. A PCR reaction consisted of 25 μl and contained 2.5 μl cDNA, 7.5 pMol of forward and reverse primer, 7.5 pMol of

TaqMan probe (PE/Applied Biosystems) and 12.5 µl TaqMan Universal PCR Mastermix (PE/Applied Biosystems). PCR was performed up to 50 cycles of 15 s 95°C and 1 min 60°C on a ABI PRISM® 7700 Sequence Detection System. SKBR3, a breast carcinoma cell line with 4-10 fold *HER-2* gene amplification and 128 fold over-expression of HER2-mRNA (25) was used as reference for HER2 expression. Serial dilutions of cDNA generated from SKBR3 mRNA resulted in a calibration curve for HER2 real time PCR values. Real-time PCR results from PBGD were used to quantify the amount of cDNA in each sample. The cell line MCF7 expresses normal levels of HER2-mRNA (25) and was used as a negative control for HER2 overexpression.

Immunohistochemical analysis

Paraffin-embedded, formalin-fixed tissue samples were used for immunohistochemical (IHC) analysis. These were retrieved from the department of Pathology. Bony specimens, that were resected, were decalcified according to routine laboratory methods, using formic acid. All IHC assays were performed on 5 µm tissue sections, mounted on APES coated slides. Plasma membrane associated staining for HER2 was performed using DAKO HERCEPTEST® (Glostrup, Danmark) according to the manufacturer's instructions. HER2 staining was scored as 0, 1+, 2+ or 3+, according to the scoring system provided with DAKO HERCEPTEST®.

FISH for *HER-2* gene amplification

One of the tumours showed a 2+ staining result for HER2. FISH was performed with a section from this specimen using the Vysis® FISH test kit for the detection of *HER-2* gene amplification, according to the manufacturer's instructions. Using a fluorescence microscope, the HER2 copy numbers and the centromere chromosome 17 copy numbers were counted in the tumour cells.

RESULTS

Patients

Patient clinical characteristics and outcome are listed in Table 1. Biopsy samples of 15 patients (group A) were studied. All 15 samples (ID no 1-15) were from primary tumours, three of which later relapsed (nos. 5, 12 and 15). Samples of group B consisted of 12 post chemotherapy resection of osteosarcomas or specimens of pulmonary (nos. 21 and 29), distant bone (no. 24) or locally (no. 17, 31 and 32) relapsed patients. From the latter 3 patients, biopsy samples at primary diagnosis are in the upper panel (nos. 5,12 and 15, respectively). The mean age of the patients in group A was lower (mean 22 years, range 7-48 year) than those in group B (mean 37 years, range 14-82 year). The localisation of the osteosarcomas was similar in both groups, mainly in the femur (in 80% and 72% in group A and B respectively). Other sites were the tibia in 2 cases in each group and in the humerus, clavicle and pelvis. Histological subtyping was high-grade conventional in all the cases in group A, except one sample that was high-grade osteoblastic. In group B, four samples were of the teleangiectatic, and one had a malignant fibrous histiocytoma (MFH)-like subtype (see table 1).

TABLE 1.
Patients clinical data and results of our study.

Id number	Age at Dx	Sample	Localization Tumour	Histological Subtype	Chemother Treatment	Response	Outcome	HER2 mRNA	IHC	FISH
Group A										
1	16	B	Ti	Co	Yes	PR	surv	0.025	0	
2	20	B	Hu	Ob	Yes	PR	surv	0.003	0	
3	9	B	Fe	Co	Yes	GR	surv	0.013	0	
4	7	B	Ti	Co	Yes	GR	surv	0.033	2+	no ampification
5	48	B	Fe	Co	Yes	PR	surv	0.031	0	
6	28	B	Fe	Co	Yes	PR	DOD	0.004	0	
7	20	B	Fe	Co	Yes	GR	DOD	0.004	0	
8	29	B	Fe	Co	Yes	PR	DOD	0.004	0	
9	20	B	Fe	Co	Yes	PR	DOD	0.034	0	
10	17	B	Fe	Co	Yes	NA	DOD	0.016	0	
11	20	B	Fe	Co	Yes	PR	DOD	0.028	0	
12	20	B	Fe	Co	Yes	PR	DOD	0.034	0	
13	33	B	Fe	Co	Yes	GR	DOD	0.008	NA	
14	19	B	Fe	Co	Yes	PR	DOD	0.002	0	
15	21	B	Fe	Co	Yes	GR	DOD	0.019	0	

Group B									
16	18	R	Fe	Co	Yes	PR	surv	0.001	no tumor
17	48	R	Fe	Co	Yes	PR	surv	0.020	0
18	20	R	Hu	Co	Yes	PR	surv	0.005	0
19	20	R	Fe	Co	Yes	PR	surv	0.029	0
20	14	R	Ti	Co	Yes	PR	surv	0.047	0
21	22	R	Cla	Co	Yes	PR	surv	0.030	no tumor
22	64	R	Fe	Te	Yes	PR	surv	0.011	0
23	82	R	Fe	Te	no	NA	surv	0.016	0
24	12	R	Fe	Co	Yes	NA	DOD	0.013	0
25	42	R	Ti	Co	Yes	PR	DOD	0.022	no tumor
26	26	R	Fe	Te	Yes	PR	DOD	0.013	NA
27	57	R	Fe	MFH	Yes	PR	DOD	0.038	0
28	30	R	Fe	Co	Yes	PR	DOD	0.049	0
29	64	R	Pel	Co	Yes	NA	DOD	0.001	0
30	36	R	Fe	Co	Yes	PR	DOD	0.027	0
31	21	R	Fe	Co	Yes	PR	DOD	0.012	0
32	20	R	Fe	Co	Yes	PR	DOD	0.105	0
33	71	R	Fe	Te	Yes	PR	DOD	0.018	NA
MCF7								0,014	

Abbreviations: B = biopsy, R = resection, Fe = femur, Ti = tibia, Hu = humerus, Cla = clavicle, Pel = pelvis, Co = high grade conventional, Ob = osteoblastic, Te = teleangiectatic, MFH = MFH-like, PR = poor response, GR = good response, Surv = survived disease, DOD = died of disease, NA = not available.

HER2 mRNA expression

HER2 mRNA expression was assessed by real-time PCR, using RNA from the osteosarcoma specimens. The mean absolute value for HER2 mRNA expression in group A is 0.017 (range 0.003 – 0.034) and 0.025 (ranging 0.001 – 0.105) in group B. The values of HER2 expression in both groups were similar to the HER2 expression in the breast cancer cell line MCF7 that has a HER2 expression value of 0.014. None of the tumour samples had values in the same range as the HER2-overexpressing cell line SKBR3, which was set at 1.0 in this study. All values fell within the range of normal HER2 expression, similar to the expression observed in normal breast tissues. Even the highest value of HER2 expression in group B (0.105) can be regarded as not being overexpressed particularly as no protein expression was seen in this sample.

Immunohistochemistry

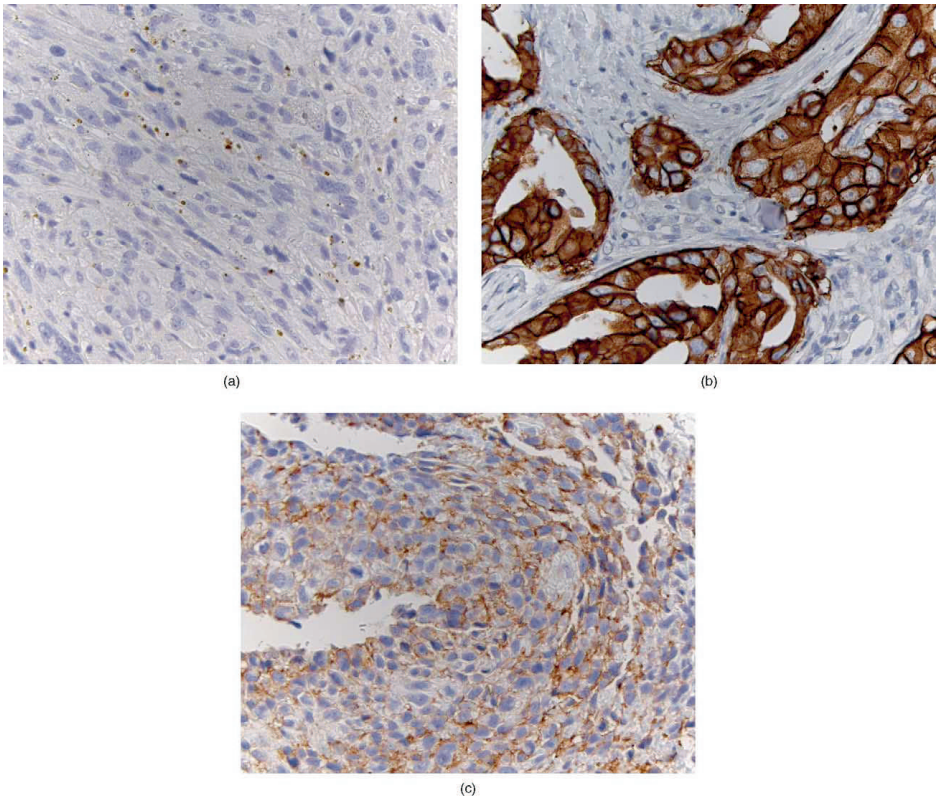
Three of the 33 samples were lost after immunostaining during retrieval procedures. In an additional three samples, no vital tumor was left on the histological section. IHC was not repeated in these samples. Nearly all of the samples showed no HER2 plasma membrane-associated staining. An example of negative immunostaining is shown in Fig. 1(a). Fig. 1(b) shows positive membrane staining in a control breast cancer sample with proven *HER-2* gene amplification. This represents a 3+ score. Only one osteosarcoma sample (patient no. 4) shows moderate positive immunostaining of the membrane, which was scored as 2+ (Fig. 1(c)). Focal cytoplasmatic IHC positivity was seen in two other samples, but as discussed previously, this was not considered as positive for HER2 overexpression.

FISH

Fluorescent *in situ* hybridisation (FISH) was performed in the osteosarcoma sample with 2+ positive membrane staining, and did not show any *HER-2* gene amplification.

FIGURE 1.

Immunohistochemical staining for HER-2 protein expression: (a) osteosarcoma negative for HER-2; (b) breast cancer sample positive for plasma membrane-associated HER-2 expression (score 3+); (c) osteosarcoma from patient no. 4 with moderate positive expression of HER-2 (score 2+).



DISCUSSION.

In this study, a single case of moderately (2+) positive membrane staining was recorded (fig. 1c). However, quantitative RT-PCR or FISH analysis could not confirm HER2-mRNA overexpression or *HER-2* gene amplification, respectively, suggesting this was a false- positive immunostain.

Usually, the HER2 protein is overexpressed as result of *HER-2* gene amplification and concomitant elevated mRNA expression (26). Nevertheless, protein overexpression has been

reported in the absence of gene amplification (12, 27-29). Interestingly, clinical studies suggest that cases with *HER-2* gene amplification have a poor outcome, whereas patients who show protein expression without gene abnormalities do not have an increased risk for a more aggressive disease course and death (30, 31).

HER2 status in osteosarcoma has been investigated in 8 other clinical studies (5-7, 16-19, 32) (Table 2).In five of these studies, HER2 overexpression was reported to occur in 42%-63% of the patients with primary non-metastatic osteosarcoma, and in 10%-58% of the cases that had pulmonary metastases at diagnosis (6, 7) or had relapsed (5, 17, 32).Three studies correlated HER2 overexpression with a poor response to pre-operative chemotherapy and a poor outcome (5, 6, 32). Remarkably, two other studies conclude that HER2 overexpression predicts better survival and is less frequent in metastatic disease (7, 17).

In four remaining osteosarcoma studies, including ours, no HER2 overexpression could be demonstrated (16, 18, 19).These inconsistent findings regarding the HER2 status and its significance in osteosarcoma raise questions about the reliability of some studies and may be explained by methodological differences.

The HER2 status in the published osteosarcoma studies has been assessed mainly by IHC. These studies differ considerably in use of antibody and quality controls, scoring systems, interpretation of positivity of the sample and validation of IHC result. In breast cancer, HER2 testing and standardization of its method has been an important issue, because only patients with HER2 overexpression are eligible for trastuzumab treatment (33).The quality of the antibody used is important, since a high rate (up to 40%) of false positive test results has been reported, due to a highly variable sensitivities (34). False positive cases are particularly noted when moderate (e.g. IHC2+) positivity occurs, and in these cases confirmation of the positive result with other tests is required (35).

Six different scoring systems to assess HER2 positivity have been used in the osteosarcoma studies (5-7, 17, 32). Interpretation of stained samples may have a high inter-observer variability and low rate of reproducibility (36, 37).This particularly occurs when the staining is heterogeneous, weakly positive, in non-malignant cells, cytoplasmatic staining or when retraction artifacts occur (38). Cytoplasmatic immunostaining is considered to be an IHC artefact (19, 39), and only complete membrane staining should be included when interpreting results (38). Only one of the five studies in osteosarcoma that scored membrane staining specifically, reported to have positive results (6). However, a poorly characterized antibody, 5B5, was used in this particular study and this antibody has not been used in other studies. Most of the ostesarcoma studies included the mandatory positive and negative controls for IHC, usually a patient sample with and without known HER2 overexpression. However, our series is the only one to use positive and negative cell lines as a control.

Validation of the IHC HER2 results by the use of other assays was done in four out of eight studies (5, 18, 19, 32).Validation assays included were immunoblotting (IB), single-stranded conformation polymorphism (SSCP) and Southern blotting (SB) (5), RT-PCR (19), and Fluorescent In Situ Hybridization (FISH) (18, 32). Except for one study that used FISH (32), no evidence for HER2 overexpression was found in the other validation analyses.

This confirms the results of our study, that showed no HER2 mRNA overexpression, assessed with a quantitative Real-Time-PCR technique, which is the only method, mentioned above that quantitatively assess HER2 mRNA expression (40).
FISH has proven to be an accurate and reproducible assay to detect *HER-2* gene amplification (41). Zhou and colleagues found *HER-2* gene rearrangement in seven of 12 tested samples, but unusual criteria were used to define the *HER-2* gene amplification (32). Accurate determination of low level *HER-2* gene amplification using FISH, requires assessment of *HER-2* copy number relative to chromosome 17 centromere number to distinguish between HER2 gene amplification and aneusomy of chromosome 17 (41), which frequently occurs in osteosarcoma (42). Furthermore, *HER-2* gene amplification in HER2-overexpressing breast cancers is usually observed in the most of the tumour cells (41).
Thus to conclude, our results show that HER2 does not play a role in the tumour biology of osteosarcoma and that pilot studies, using trastuzumab, as a drug with potential tumour-inhibiting properties, are not likely to benefit to patients with this bone tumuor.

TABLE 2.
Overview of clinical HER2 studies in osteosarcoma

	Number of		Immunohistochemistry				% HER2+ samples		HER2+ IHC samples related to		
Author (ref)	patients	samples	Antibody	Scoring system	Control	Other assays	IHC+	Other assay	PR (%)	EFS	OAS
Onda (5)	26	26	CB11 CBE1	Qualitative (M)	–	IB, SB, SSCP	42	0	67	–	HER+ 14% HER– 84%
Gorlick (6)	53	53	5B5 / Herceptest	Semi-quantitative (M)	+	–	45	ND	57	HER+ 47% HER– 79%	–
Akatsuka (7)	81	81	CB11	Semi-quantitative (M and/or Cy)	+	–	63	ND	58	HER+ 72% HER– 46%	HER+ 82% HER– 57%
Akatsuka (17)	19	38	CB11	Semi-quantitative (M and/or Cy)	–	–	PT 50 PuMet 10	ND	46	–	–
Zhou (32)	25	37	Ab3	Semi-quantitative (M and/or Cy)	+	FISH	PT 44 PuMet 58	67	NS	NS	NA
Kilpatrick (16)	41	41	CB11 Oncor	Semi-quantitative (M and/or Cy)	+	–	0	ND	NA	NA	NA
Maitra (18)	21	21	AO485	Semi-quantitative (M)	+	FISH	0	0	NA	NA	NA
Thomas (19)	33	66	AO485	Semi-quantitative (M)	+	RT-PR	0	0	NA	NA	NA
Present study	33	27	Herceptest	Herceptest guidelines; (M)	+	RT-PCR / FISH	1*	0	NA	NA	NA

M, Membrane staining; Cy, cytoplasmatic staining; IB, immunoblot; SB, Southern blot; SSCP, single stranded confirmation polymorphism; PT, primary tumour; PuMet, pulmonary metastases; PR, poor response to chemotherapy; EFS, event free survival; OAS, overall survival; NA, not applicable; NS, not significant. * Can be regarded as false-positive (see text).

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6

Osteosarcoma of the hands and feet: a distinct clinico-pathological subgroup

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ABSTRACT

Background. Osteosarcomas of hands or feet are rare and seemingly these cases differ in presentation and behavior compared to the usual location.

Methods and materials. Clinico-pathological presentations of patients with osteosarcomas of the hand or foot were studied and compared with published cases.

Results. Forty osteosarcomas were identified among 4.221 cases, representing 0.95% of all osteosarcomas. Thirty of these were well-documented. Mean age at diagnosis was 43 (hands) and 36 (feet) years and male-female ratio was 1.2:1 and 2.0:1 respectively. In the hand 62% of the osteosarcomas presented in the metacarpals, 23% in the phalanges, and only 2 cases occurred in the carpal bones. Distribution in the foot was tarsal bones 56%, metatarsal bones 33% and phalanges 11%. Of the cases in the hand, 54% were of high-grade, and of those in the foot 71%. Survival of osteosarcomas of the hand or foot was 81%. Only patients with high-grade osteosarcoma died of disease. Histological grade was the only significant variable, related to survival. High-grade osteosarcoma of the hand or feet should be treated similar to those in conventional sites.

Conclusion. Osteosarcomas of hands or feet are rare and in a relative high proportion low-grade. Survival in high-grade cases is comparable to conventional sites.

BACKGROUND

Osteosarcoma is rare, accounting for less than 1% of adult malignancies and 3%-6% at the pediatric age, with peak incidences in adolescence and after 60 years of age. Of skeletal tumours in the hand or foot 13%-15% are malignant, most of these are chondrosarcomas. Between 0.3%-2.0% of tumors at these sites are osteosarcoma (1, 2). The incidence of osteosarcoma in the bones of the hand and foot has been reported to be low, around 0.9% (2, 3), and its histology appears to be unusual (4-21).

Against this background, osteosarcoma of the hand or foot is clinically unexpected and the diagnosis is often delayed or initially erroneous (5, 18, 19, 22-28), leading to delayed or inappropriate treatment decisions (18, 27, 29). The rarity of osteosarcoma at this site also has led to a debate about the appropriate treatment of osteosarcoma, which obviously cannot be assessed in large case series. Hand and foot osteosarcomas have been considered prognostic favorable (5, 13, 30), but numerous case reports demonstrated fatal outcome (5, 10, 12, 31-33). To obtain a better insight in hand and foot osteosarcoma, we studied all cases from two large case registries and clinical and pathological data of this cohort were compared with the combined data from the literature. A treatment recommendation is given based on the results of this study.

METHODS

Patient data

Patient cases were retrieved from the Netherlands Nationwide Computerized Archive for Pathology (PALGA) over the period 1984-2010, looking for the search terms "Osteosarcoma" and "Hand" or "Foot". To be included in the data base, a patient had to have been diagnosed with an osteosarcoma based on biopsy or resection specimen, according to the WHO-classification (34) and ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up of bone tumours (35). Staging procedures had been done according to the ESMO guidelines, including chest radiographs, Computed Tomograms (CT) of the lungs and ^{99m}Tc-bone scintigraphy. All cases that were not localized in either the hand or foot were excluded, as well as those with an incorrect histological diagnosis (Supplemental figure). A similar search strategy was executed on the data base of the Netherlands Committee on Bone Tumors (NCBT), containing 1.733 osteosarcomas over the period 1953-2010. Double registries were identified and reduced to a single entry. The clinical data were updated and the radiology and pathology reports of patients were reviewed (JKA, HMJAK, HG).

The clinical and follow-up data, the radiology and pathology reports from the Istituto Ortopedica Rizzoli (IOR), in Bologna, Italy were reviewed (PP, MA, DV). This data base contains well documented files of 2.240 patients with osteosarcoma of all grades, which had been treated in the IOR, as well as and 248 consultation cases covering the period from 1980-2010.

Statistical analysis
A competing risk model was employed to estimate the cumulative incidence of death due to treatment failure (36, 37). Competing risks are applied to situations where more than one competing endpoints are possible. In our situation there are two different endpoints: death due to disease and death due to other causes. The occurrence of death due to other causes prevents the occurrence of the event of interest (death due to disease). We are interested in the probability of death due to disease in the presence of the competing event death.
All analyses concerning competing risk model have been performed using the mstate library (38, 39). SPSS-18 was used for the remaining statistical analysis.

RESULTS

Patient data
The cohort consists of 10 cases from the Dutch data bases, 20 cases from the IOR and 10 cases from the IOR consultation files. This adds up to 40 cases of osteosarcoma in the hand or foot among a total of 4.221 osteosarcoma cases (0.95%), 13 of which in the hand (0.31%) and 27 in the foot (0.64%).

Patient characteristics, clinical and pathologic data
Clinical features (Table 1 and 2)
Gender. The ratio of male (n=25) to female (n=15) patients was 1.67:1. In the hand, an almost equal number of male (n=7) and female patients (n=6) is found (ratio male:female = 1.16:1, Figure 1a). In the foot more patients were male (n=18) than females (n=9, ratio male:female = 2.0:1, Figure 1b).

Age. The age distribution of the patients is shown in Figure 1c. The mean age ± standard deviation (SD) of all patients in this series was 38.2 ± 17.6 years, ranging from 9-74 years. The mean age of patients with osteosarcoma of the hands was higher than that of patients with osteosarcoma of the foot (mean age 42.3 ± 23.6 years [9-74 years] vs. 36.3 ± 16.2 years [17-68 years] respectively).

TABLE 1.
Clinical and radiological data from 30 well document patients. Extracomp = extracompartimental, intracomp = intracompartimental, prox = proximal.

Gender (F/M)	Age (years)	Duration of complaints before diagnosis (months)	Tumor localization	Radiology
Osteosarcoma Hands				
M	39	12	Scaphoid	mixed, extracomp, size 3x3x3 cm
F	47	4	Metacarpus 3	mixed, extracomp, size 3,5x2x1,5 cm
F	36	6	Metacarpus 2	lytic, extracomp, size 3x1,5x1,5 cm
F	63	9	Scaphoid	mixed, intracomp, size 1,5x1,5x1 cm
F	74	12	Metacarpus 3	mixed, cortex destruction, soft tissue mass with calcification, 6x6x7 cm
M	13	2	Phalanx 3, middle	permeative, cortex destruction, soft mass, 1x1x2 cm
F	58	36	Phalanx 4, prox	inhomogeneous sclerotic, well defined, 1.4x1.4x3cm
M	49	NA	Metacarpus 2	2.5x2.5x2.5 cm
M	58	12	Metacarpus 3	sclerotic, cortex destruction, soft tissue mass with calcification, 6.5x2x2 cm
M	61	12	Phalanx 5, prox	lytic/osteoblastic destruction, soft tissue mass with calcification, 2.5x3x2.5 cm
M	9	3	Metacarpus 3	sclerotic, expansive, 4x2.5x2.5 cm
Osteosarcoma Foot				
F	18	16	Calcaneus	sclerotic,extracomp, size 6x4x4 cm
M	37	37	Talus	lytic, extracomp, size 4x4x3 cm
M	17	3	Cuboid	mixed, extracomp, size 8x6x5 cm
M	30	4	Talus	lytic, extracomp, size 4x3,5x3,5 cm
F	47	24	Metatarsus 5	lytic, extracomp, size 3x2x2 cm
F	34	9	Cuboid	mixed, extracomp, size 4x2,5x2,5 cm
M	57	30	Cuboid	mixed, extracomp, size 6x4x3 cm
M	38	6	Cuneiform 2	lytic, extracomp, size 3x2x1,5 cm
F	50	8	Metatarsus 2	mixed, extracomp, size 3x1,5x1,5 cm
M	25	20	Metatarsus 2	mixed, extracomp, size 3x1,5x1 cm
M	20	3	Calcaneus	mixed, intracomp, size 5x3x3 cm
M	53	12	Calcaneus	mixed, extracomp, size 5x4x4 cm
M	28	6	Metatarsus 2	sclerotic, extracomp, size 1,5x1,5x1 cm
M	20	48	Metatarsus 1	lytic, extracomp, size 2x2x2 cm
F	24	12	Calcaneus	lytic, intracomp, size 4x3x2 cm
M	26	3	Metatarsus 2	mixed, intracomp, size 2x1x1 cm
M	37	6	Phalanx 3, prox	permeative, cortex destruction, soft tissue mass, calcification 3x2.5x2.5 cm
M	19	accidentally	Metatarsus 1	lytic, 2x2x4.5 cm
M	18	2	Calcaneus	soft tissue mass, 4.5x3x3 cm

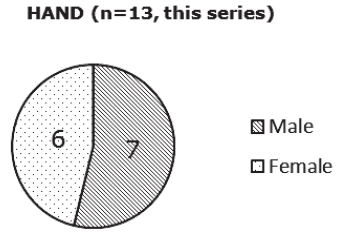
TABLE 2.
Clinical and histologic data of 10 patients with osteosarcoma of hand or foot. Data from the consultation files from IOR/Rizzoli Orthopedic Institute.

Gender (F/M)	Age (years)	Localization tumour	histology
Osteosarcoma Hand			
M	14	Metacarpus 1	Osteoblastic OS
F	39	Metacarpus 2	Peri-osteal OS
Osteosarcoma Foot			
M	68	Phalanx I, Prox	Osteoblastic OS
F	64	Talus	Osteoblastic OS
F	64	Calcaneus	Osteoblastic OS
F	17	Cuboid	Chondroblastic OS
M	54	Calcaneus	LG Central OS
F	46	Metatarsal 2	LG Central OS
M	42	Phalanx I, Prox	LG Central OS
M	26	Metatarsal 1	LG Central OS

LG = Low grade, prox = proximal.

FIGURE 1.

Gender distribution of the study patients with an osteosarcoma of the hands (n=13; *Figure 1a*) or the feet (n=27; *Figure 1b*). *Figure 1c* shows the age distribution of the patients in this study, divided in decades.



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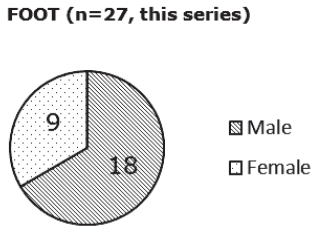

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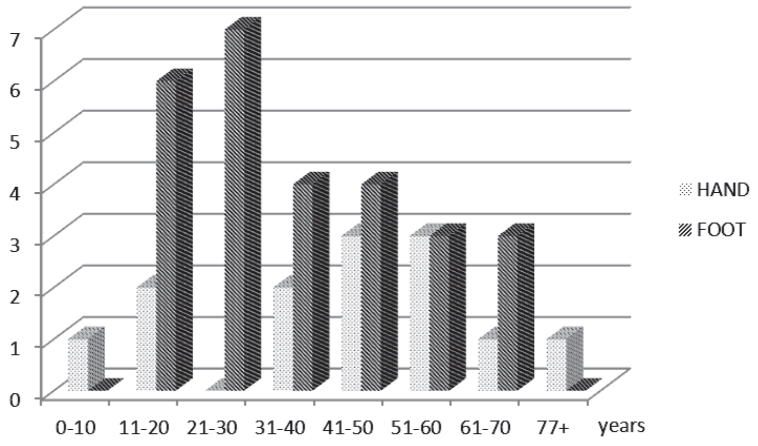


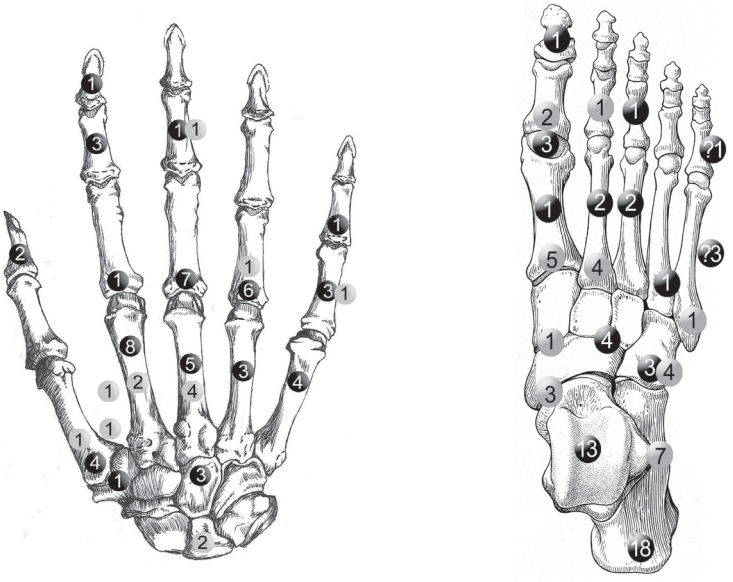
1c

Duration of history of symptoms. All patients complained of pain, either with or without swelling. One case was an incidental finding. For the 30 cases for which this information was available, the average duration of symptoms was 13 months (range 2-48 months), before a diagnosis of osteosarcoma was made, shorter in osteosarcoma of the hand (11 months [2-36 months]) than in those of the foot (14 months [2-48 months]).

Location of the osteosarcoma in the bones of hand and foot. Figure 2a shows the site distribution of our cases of osteosarcoma in the hand compared to the literature. Eight cases (62%) were located in the metacarpals, 3 (23%) in the phalanges and 2 (15%) in the scaphoid bones. In the foot (Figure 2b), 15 cases (54%) were located in the tarsal bones, 10 (36%) in the metatarsals and 3 (11%) in the phalanges.

FIGURE 2.

Distribution of 65 cases of the osteosarcomas of the hand (literature n=52, black balls and this series n=13, gray balls; *Figure 2a*) and 80 cases of osteosarcomas in the foot (literature n=53, black balls, and this series n=27, gray balls; *Figure 2b*). Question marks before a figure means no exact location otherwise than type of bone given.



2a 2b

Localized and metastatic osteosarcoma. Of 29 patients with known disease status, 2 (6.9%) had pulmonary metastases at the time of diagnosis. One case was from a primary grade 2 osteosarcoma of the first metatarsus of the foot, the other was from a high-grade osteoblastic osteosarcoma of the cuboid.

Radiological features (Table 1,Figures 3a-c and Figures 4a-c)

Radiologically, the lesions were mixed lytic and sclerotic or sclerotic on conventional radiographs. Pure radiologically sclerotic lesions (Figure 3a) sometimes preceded a definitive mixed appearance later in the course of the disease (Figures 3b and 3c). In nearly all cases, soft-tissue extension was observed on radiology. In one case, MRI showed minimal cortical destruction, but a large soft tissue mass around the abnormal calcaneus (Figures 4a and 4b). To differentiate between a primary soft tissue process with secondary bone involvement and primary bone disease with secondary soft tissue involvement, a subsequent performed CT-scan showed mineralization in the soft tissue mass (Figure 4c), highly suggestive for osteosarcoma.

Histology (Table 2 and 4, Figures 3d-e and Figures 4d-e)

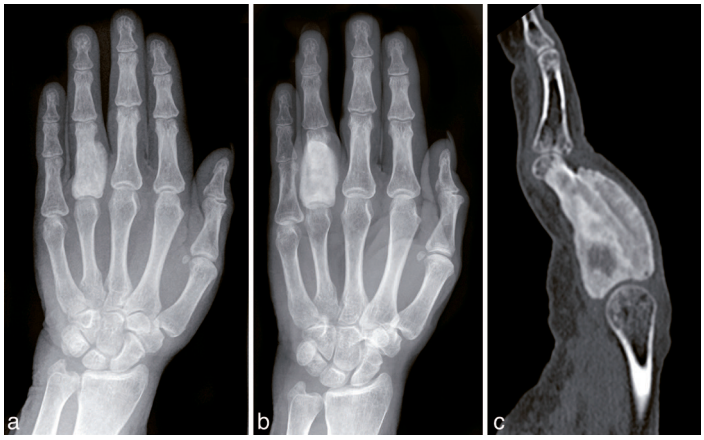
A biopsy was performed in all the patients after radiology indicated malignancy. In 26 cases (65%) a high-grade osteosarcoma was diagnosed, in 2 (5%) an intermediate-grade and in 12 (30%) a low-grade osteosarcoma (Table 3). Of the 13 osteosarcomas of the hand, 7 (54%) were high-grade lesions, 6 of which of the high-grade conventional subtype (osteoblastic (n=4) or chondroblastic (n=2)) and one small cell osteosarcoma. Two periosteal osteosarcomas (intermediate grade) were localized in metacarpal bones and 4 low-grade central osteosarcomas in the carpal (n=1), metacarpal (n=2), or phalangeal (n=1) bones of the hand. Figures 3d and 3e illustrate a low-grade osteosarcoma of the 4th proximal phalanx from the patient with inhomogeneous mineralization of the phalanx.

Of the 27 cases of the foot, 19 (70%) were high-grade, of which 15 high-grade conventional osteosarcomas (osteoblastic (n=12), chondroblastic (n=2), fibroblastic (n=1)) and the 4 others high-grade unconventional osteosarcomas (osteoblastoma-like (n=1), small cell (n=1), and telangiectatic (n=2)). Figures 4d and 4e illustrate an osteoblastic osteosarcoma of the calcaneus. Eight (30%) were low-grade central osteosarcomas, but intermediate-grade osteosarcomas were not found in the feet.

In 4 patients a diagnosis of osteomyelitis, osteoblastoma, enchondroma or dedifferentiated chondrotumour was made prior to the diagnosis of osteosarcoma, suggesting progression from a benign to a malignant lesion.

FIGURE 3A-E.
Osteosarcoma of the hand of a 58 years old female patient.

Figure 3a is the first conventional radiograph in 2006 of a this patient and demonstrates a well-defined, predominantly sclerotic lesion of the proximal phalanx of the 4th finger. *Figure 3b* is the conventional radiograph three years later. The lesion shows considerable growth. *Figure 3c* is the sagitally reformatted CT, 4 months later as *3b*, showing thickening of the phalanx with inhomogeneous mineralization, a solid periosteal reaction but no soft-tissue mass, suggestive of osteosarcoma arising from fibrous dysplasia. *Figure 3d*. Lightmicrograph demonstrating in an overview tumour cells, infiltrating and permeating in the cortex (upper part of the image). The tumour is moderately cellular towards the cortex and the osteoid producing tumour cells contain round to oval nuclei, some of these containing nucleoli. *Figure 3e*. In the center of the specimen the tumour cells intervene with the pre-existent lamellar bone. There is light to moderate cellular pleomorphism. Tor exclude a pre-existing fibrous dysplasia, the characteristic mutation analysis of exon 8 and 9 of the GNAS gene was negative.



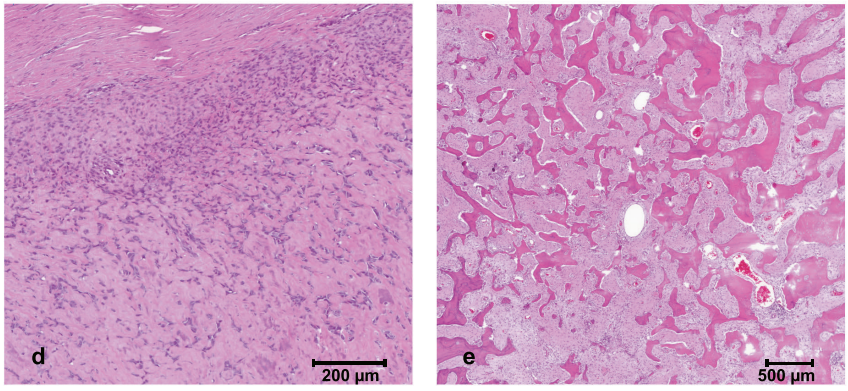


TABLE 3.

Clinico-pathological characteristics of cases with OS of the hand or foot. The data from literature are retrieved from case reports or larger series. For references, see under Discussion. High grade conventional includes osteoblastic, chondroblastic, fibroblastic and not otherwise specified subtype. High grade unconventional includes osteoblastoma resembling, sclerotic, telangiectatic and small cell osteosarcoma. SD = standard deviation.

	HAND		**FOOT**	
	this series (n = 13)	**literature (n = 56)**	**this series (n = 27)**	**literature (n = 53)**
Mean age ± SD (years)	42.3 ± 20.3	43.3 ± 23.6	36.3 ± 16.2	28.2 ± 16.6
Male : Female	1.16 : 1	1.38 : 1	2.00 : 1	1.65 : 1
Duration symptoms (range) months	11(2-36)	21(0.1-96)	14(2-48)	22(0.5-144)
Bones affected (%)				
Carpal/talar	15%	8%	56%	72%
Metacarpal/metatarsal	62%	46%	33%	17%
Phalanges	23%	46%	11%	11%
Histology (number/%)	13	33	27	51
High Grade conventional	6 (46%)	19 (58%)	15 (56%)	33 (64%)
High Grade unconventional	1 (8%)	1 (3%)	4 (15%)	7 (14%)
High Grade surface	-	2 (6%)	-	-
Periosteal OS	2 (15%)	2 (6%)	-	-
Low grade central	4 (31%)	2 (6%)	8 (29%)	7 (14%)
Parosteal	-	7 (21%)	-	4 (8%)

TABLE 4.

Treatment and outcome of 30 cases of OS of hand or foot. LG = low-grade. Dox = doxorubicin, CDP = cisplatin, HD-MTX = high-dose methotrexate, Ifo = ifosfamide, Cycl = cyclophosphamide, ACT-D = actinomycine-D, Adj = adjuvant, Neo-adj = neo-adjuvant, Pre-Op = preoperative, DOD = died of disease, DOC = death other cause, NED = no eveidence of disease, PD = progressive disease. EURAMOS-1 for chemotherapy: see www.euramos.org.

histology OS	surgery	chemotherapy	outcome (NED/DOD)	duration FU (months)
Osteosarcoma Hands				
Chondroblastic	amputation	Dox,CDP HD-MTX-IFO (Adj)	DOD	42
LG Central	marginal resection	no	NED	104
LG Central	wide resection	no	NED	64
LG Central	wide resection	no	NED	16
Chondroblastic	wide excision	no	DOC	64
Small Cell	amputation	Dox,CDP x 4 (Adj)	NED	176
LG Central	wide resection	no	NED	10
Osteoblastic	marginal resection	NA	NA	NA
Peri-osteal	resection	no	DOC	156
Osteoblastic	wide resection	no	DOD	42
Osteoblastic	incomplete (biopsy)	no	DOD	48
Osteosarcoma Foot				
Osteoblastic	amputation	Dox,CDP HD-MTX (Neo-adj)	NED	148
Osteoblastic	amputation	LD-MTX, CDP (Adj)	NED	115
Osteoblastic	amputation	Dox,CDP,IFO (Adj)	NED	248
Telangiectatic	amputation	Dox,BLEO,CYCL,ACT-D (Adj)	DOD	20
Chondroblastic	amputation (2-ray)	Dox, CDP,IFO (pre-Op)	NED	134
Osteoblastic	amputation	Dox,CDP, IFO (Neo-Adj)	DOD	13
Fibroblastic	amputation	Dox,CDP,IFO (Neo-Adj)	DOC	27
Osteoblastic	marginal resection	Dox,CDP,HD-MTX (+Ifo)(Neo-Adj)	NED	34
Osteoblastic	wide resection	Dox,CDP,IFO (Neo-Adj)	NED	24
Osteoblastic	wide resection	no	NED	76
Telangiectatic	wide resection	Dox,CDP,HD-MTX (Neo-Adj)	NED	109
Fibroblastic	amputation	Dox,CDP,IFO (Adj)	NED	171
LG Central	wide resection	no	NED	320
LG Central	marginal resection	Dox,CDP,MTX (Adj)	NED	294
LG Central	curettage	no	NED	86
LG Central	wide resection	no	NED	117
Small Cell OS	wide resection	EURAMOS-1 (Neo-Adj)	NED	37
Osteoblastoma-like	amputation	EURAMOS-1 (Neo-Adj)	PD	85
Osteoblastic	wide resection	EURAMOS-1 (Neo-Adj)	NED	13

Treatment and follow-up of the patients

Radical surgery of the lesions was by amputation in 11 (37%) of 30 cases, all high grade tumors. Wide resection was performed in 12 cases with involvement of osteosarcoma in the small tubular bones (metacarpus/metatarsus or phalanges) and in cases where the calcaneus was involved. In 7 cases limb-salvage surgery was done for high-grade osteosarcoma, and in 5 other cases of low-grade central osteosarcoma. Marginal excision was done in 3 cases with low-grade osteosarcoma of the metatarsus and metacarpus, in one case each of an osteoblastic osteosarcoma in the cuneiforme bone and one extra-osseous osteosarcoma in the hand, and in one case of a periosteal osteosarcoma of the 3rd metacarpal bone in the hand. In 2 cases intralesional surgery was done, curettage in one patient with a low-grade central osteosarcoma in the calcaneus.

Chemotherapy as part of the initial treatment was applied in 17 cases of this cohort (59%), all in high-grade osteosarcoma (n=16) or low-grade osteosarcoma with metastatic disease at diagnosis (n=1). In 4 high-grade osteosarcoma patients no chemotherapy was given while in one patient with high-grade osteosarcoma treatment data was not available.

Disease outcome

Survival of the 29 patients for which this information was available, is summarized in Table 4. Overall, 3 patients (10%) died of non-osteosarcoma related causes. Five (19%) of the remaining 26 patients (7 hand and 19 foot) died of osteosarcoma, one (4%) has progressive, intractable disease, 17 (59%) are in first persistent remission and 3 (12%) are in remission after relapse. Of one patient no follow-up data was available.

The cumulative incidence of death due to treatment failure for all patients (hands and feet) at 5 years was 20% (Figure 5a). The cumulative risk of death was 38% (at 4 years) and 11% (at 2.5 years) ($p=0.57$) for patients with osteosarcoma of the hands or feet respectively (Figure 5b). All patients who died of disease and the one patient with progressive disease, had high-grade osteosarcoma, while no patient with low-grade osteosarcoma died from disease (Figure 5c). Figure 5d shows the cumulative incidence of death of patients treated with versus without chemotherapy, which was 20% at 4 years, equal for the two groups after correction for tumour grade, applying a log-rank type test developed by Gray (40).

Due to the small sample size of the cohort it is not possible to detect any statistical significant difference between the two groups. The Fine and Gray hazards' regression model has been employed to assess the effect of covariates like age, duration of symptoms, histology or type surgery, but the results were not significant.

FIGURE 4A-E.
Example of an osteosarcoma of an 18 years old boy in the calcaneus.

Figure 4a is a coronal T1-weighted MRI of this patient, that demonstrates a lesion with intermediate signal intensity originating in the calcaneus with a considerable soft-tissue mass. The permeative cortical destruction and soft-tissue mass suggests malignancy. *Figure 4b* is a coronally reformatted CT, demonstrating mineralization in the soft-tissue mass indicating bone formation by the tumor. *Figure 4c* is an axial contrast-enhanced T1-weighted image with fat-suppression that shows inhomogeneous enhancement of the mass in and surrounding the calcaneus. The CT demonstrates mineralization more readily than the MR. *Figure 4d*. Low power micrograph shows irregular sized, atypical tumour cells in an ossifying matrix. *Figure 4e*. The more detailed photograph demonstrates clearly the pleomorphic tumor cells with atypical nuclei, and intercellular deposition of osteoid.

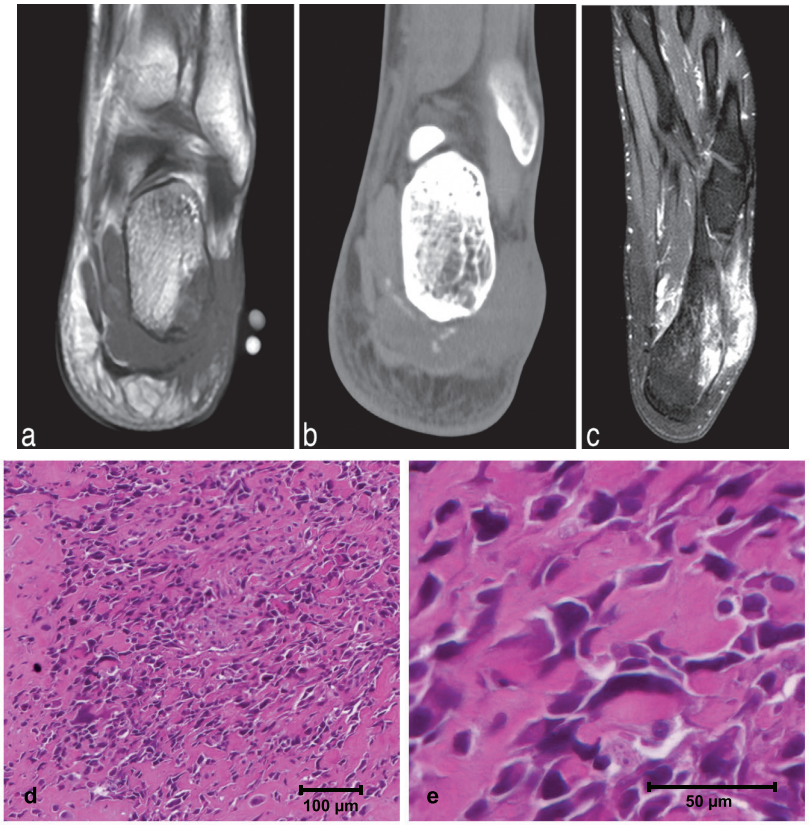
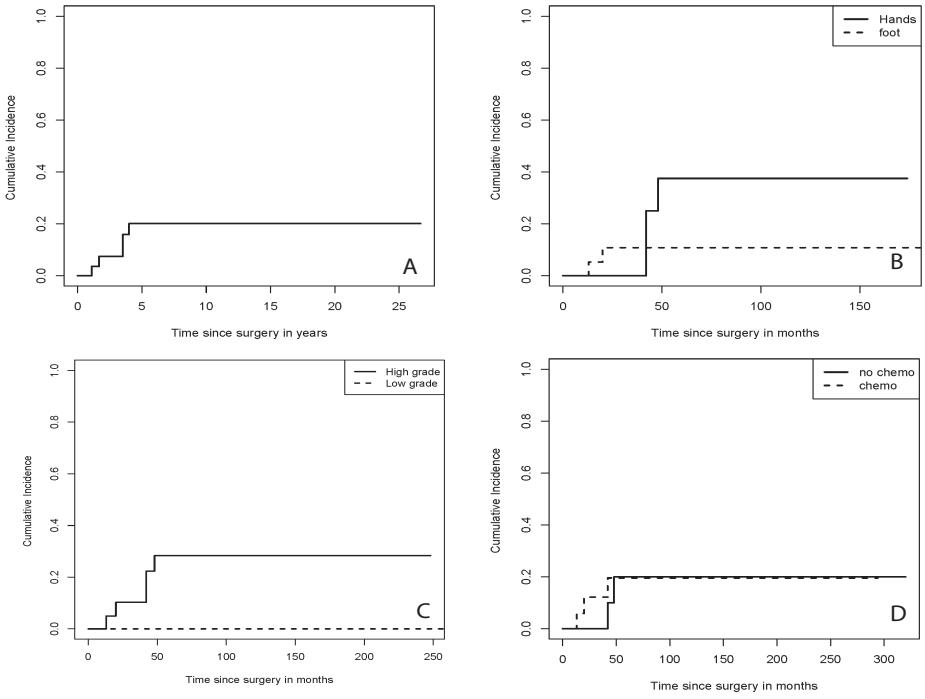


FIGURE. 5.

Outcome of the patients in this study, reflected as cumulative incidence of death for all patients. Cumulative incidence of death is explained under the section Statistical Methods in the main text. *Figure 5a* shows the cumulative incidence of death for the whole group of patients with an osteosarcoma of the hand or foot. *Figure 5b* demonstrates a better outcome for patients with an osteosarcoma in the foot compared to localization in the hand. *Figure 5c*. Only patients with high-grade osteosarcoma died of their disease, none of the patients with low-grade or intermediate grade tumours. *Figure 5d*. The cumulative incidence of death after chemotherapy in patients with high grade tumours was not different from patients who were not treated with chemotherapy.



DISCUSSION

This largest cohort of patients with osteosarcoma in either hand or foot as yet reported describes 40 patients, representing 0.95% of a series of 4.221 osteosarcoma patients, an incidence consistent with the literature (2, 3, 5). Osteosarcoma of the hand amounts to 0.31% of all osteosarcomas, slightly higher than that reported in the literature (0.10%-0.18%) (30, 41). Osteosarcoma of the feet in this series represents 0.64% of all osteosarcomas, lower than the reported range (0.9% - 1.6%) (8, 9), but in a range similar to the Mayo Clinic series (0.71%) (5, 6).

The results of this study demonstrate that patients with osteosarcoma of the distal extremities are older, have a different gender distribution, differ in symptom history and grade of malignancy, consistent with the literature (42-44). Patients with osteosarcoma of the hands presented at any age above the 4th decade (Table 3). Patients with osteosarcoma of the foot were on average 6 years younger than those with osteosarcomas in the hand. Earlier reported patients were younger, but also around the 3rd decade. This age is higher than the adolescent age, when osteosarcomas of long tubular bones have their peak incidence (42-44).

Regarding gender, we found osteosarcomas of the hand to occur more frequently in male patients, with a male:female ratio (2.0:1) lower than reported (Table 3). However, the male:female ratio we found for osteosarcoma of the foot was higher than that in the literature (ratio 1.65:1). This is different from osteosarcoma at conventional sites, which has a male:female ratio of 1.2:1 in the age group of 24-59 years old (42).

Regarding the duration of the symptom history, this generally is one year or even longer, but shorter than reported in the literature, where it is twice as long (Table 3). This might suggest that hand and foot osteosarcomas have a slower growth rate than those in the long tubular bones, but can also be explained by the high proportion of low-grade osteosarcoma. However, the interval between first complaint and diagnosis for our high-grade tumours was still 11.6 (2-37) months, versus 15.1 (3-48) months for the low-grade osteosarcoma patients.

Regarding histology, we found an overrepresentation of low grade (30%) subtypes of osteosarcomas of the distal extremities. In conventional osteosarcoma, the low-grade central subtype comprises around 1% (45, 46) and the parosteal subtype to 4% (47).
Compared to the 33 cases reported in the literature (10, 12, 15, 28, 33), we found fewer high-grade tumours of the hand (Table 3), but a higher proportion of low-grade central osteosarcomas in either hand and foot. We found no high-grade surface or (low-grade) parosteal osteosarcoma of the hands whereas 2 cases of high-grade surface (18, 48) and 7 cases of parosteal osteosarcomas have been reported (7, 10, 11). We found 2 patients with periosteal osteosarcomas of intermediate grade with an equal number in the literature (10, 19).
The 51 published cases in the foot compare well with our 27 cases. As in our series, most

(64% compared with our 56%) were of the high-grade conventional type (5, 6, 22, 26, 27, 31, 32, 49-57), the unconventional high-grade subtypes being infrequent (14% compared to our 15%) (5, 13, 14, 20, 21, 58) but high in comparison with osteosarcoma in conventional sites. Furthermore, a high proportion of the osteosarcomas of the foot were of low grade (22% compared with our 29%) (5, 6, 16, 17, 23-26). We conclude that the clinico-pathological behavior of osteosarcomas of the hand/foot differs from that of osteosarcomas at the conventional sites.

In comparison with the literature (Figure 2) we found osteosarcomas more often in the metacarpal (46% compared to our 62%) and carpal bones (8% compared to our 15%) (Figure 2a). In the foot 1/3 of all osteosarcomas occurred in the metatarsal bones whereas in the literature this was nearly 1/6. We confirm that most osteosarcomas occur in the tarsal bones, the calcaneus (26%), the cuboid (15%) and the talus (11%) being more frequently involved than the metatarsal bones (33%), but less frequently than reported in the literature (calcaneus 34%, talus 24% and cuboid 13% respectively; Figure 2b).

Most patients (85%) complained of pain or a painful swelling, and a minority only had a swelling as the presenting symptom. In contrast to osteosarcoma of conventional sites, where the symptomatic period in 90% of the cases lasts less than 6 months (59), the symptomatic period in our cohort was on average one year. Delay in diagnosis in distal extremity osteosarcoma has been reported (5, 59), and can be explained in part by benign lesions that evolve into osteosarcoma as is in 3 of our cases. Pain, especially during rest or at night with or without swelling, should alert the clinician on the possibility of a sarcoma, despite its rarity at these sites (5, 60).

In contrast to the 12%-16% metastatic rate in osteosarcoma at conventional sites (43, 61), metastatic osteosarcoma was observed only in 2 patients (7%) in this series. We found a higher rate of metastases than the 2.4%-3.8% reported earlier (6, 10). One of our patients with metastatic disease had low-grade osteosarcoma, which is exceptional. However, metastases in low-grade lesions have been described after recurrence, and are frequently the result of incomplete resection of the tumour (45, 62, 63). In 1/3 of the recurrences, dedifferentiation had resulted in a higher-grade osteosarcoma (grade 3 or 4) and poor outcome.

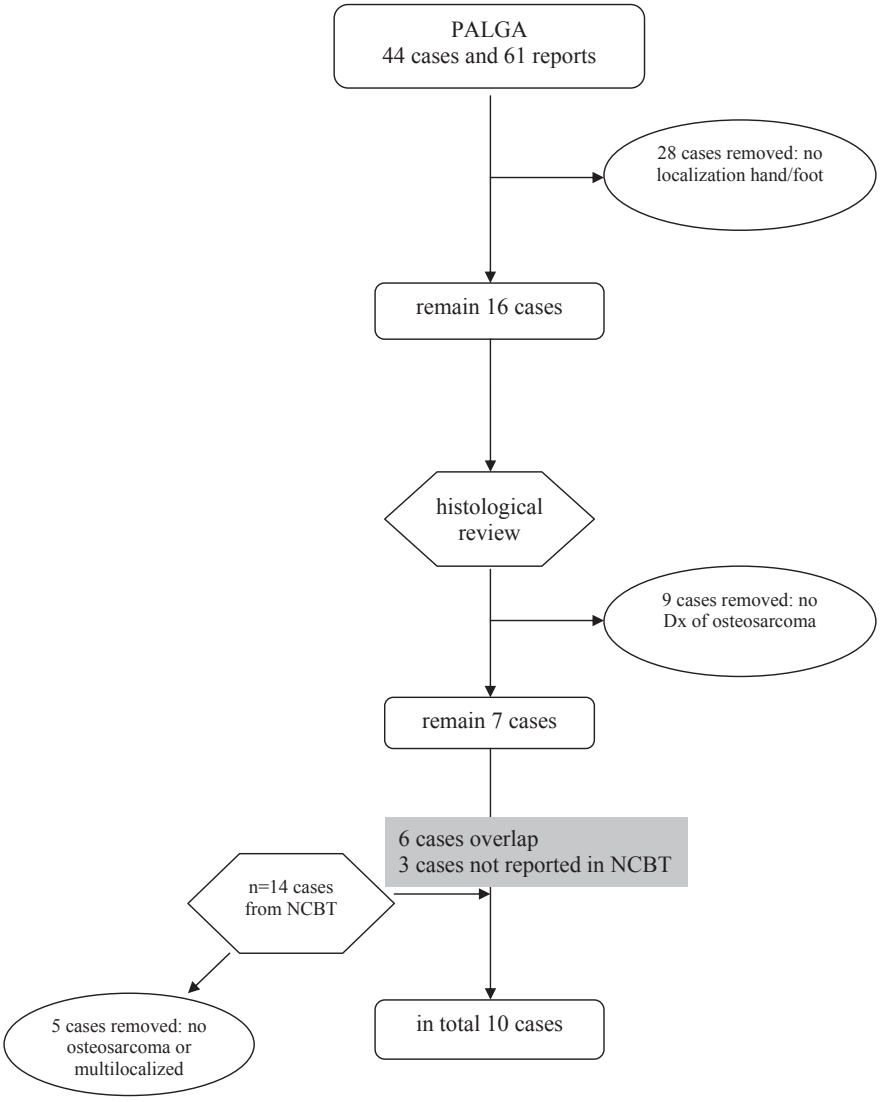
Grade was in this study the only significant co-variable relevant for outcome. In our cohort, all patients who died of disease had high-grade osteosarcoma (Figure 5c). Foot osteosarcomas show a cumulative death rate of 11% at 2 years, while for hand osteosarcomas this is 38% at 4 years.

After correction for grade, there was no significant difference in outcome between patients who did or did not receive chemotherapy ($p=0.33$) (Figure 5d). This might be explained by the low number of patients, but given the rarity of the disease prospective studies assessing the additional value of chemotherapy are problematic. We also conclude that osteosarcoma in the hands has a worse prognosis than those of the foot (Figure 5b), even though high-grade osteosarcoma is more frequent in the foot (71%) than in the hand (54%). We suggest that osteosarcoma of the hand has a prognosis similar to the long tubular bones of the skeleton, justifying treatment with chemotherapy of high-grade lesions of the hand. In the absence of

more direct evidence, we recommend (neo-)adjuvant chemotherapy in high-grade lesions of the hand and foot.
Low-grade lesions might be treated sufficiently by surgery alone. There is a debate about the margin in low-grade central osteosarcoma, because it was shown that all patients with less than wide margins had recurrences (45, 62-64). In our series five of the 9 low-grade tumours were treated with wide excision, 3 had marginal resection and one patient underwent curettage. All of these patients were alive after a median follow-up of 126 (6-320) months. Based on the fact that there were few recurrences in low-grade osteosarcoma, radical surgery without chemotherapy is advised in low-grade osteosarcomas of the hand or foot.
In conclusion, hand and foot osteosarcomas are rare (1% of all osteosarcomas) and have a biologic behavior which is different from osteosarcoma at conventional sites, when not corrected for grade of malignancy. Relatively a high proportion of osteosarcomas in these sites are low-grade, and grade is the only significant prognostic variable for risk of death. Grade 3-4 osteosarcoma should be treated with adequate surgery and neo-adjuvant chemotherapy, whereas grade 1-2 lesions should be approached by wide margin surgery only.

SUPPLEMENTAL FIGURE.

Selection flow diagram of cases from the Dutch data bases PALGA and NCBT. For abbreviations see: "Methods" section.



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7

Prognostic factors in pulmonary metastasized high-grade osteosarcoma

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ABSTRACT

Introduction

Resection of pulmonary metastases has previously been reported to improve outcome in high-grade osteosarcoma patients. Factors influencing survival in osteosarcoma patients with pulmonary metastases are important for clinical decision making.

Methods

All 88 osteosarcoma patients with pulmonary metastases either at diagnosis or during follow-up treated at the Leiden University Medical Center between January 1, 1990 and January 1, 2008 under the age of 40 were included in this study, including 79 cases of conventional, 8 cases of telangiectatic and 1 case of small cell osteosarcoma.

Results

In total, 56 of 88 patients with pulmonary metastases were treated by metastasectomy. Resectability of pulmonary metastases was the main prognostic factor. In patients with primary non-metastatic osteosarcoma, a longer relapse free interval to pulmonary metastases was significantly associated with better survival ($p = 0.02$). Independent risk factors determining worse survival after metastasectomy in multivariate analysis were male sex ($p = 0.05$), higher number of pulmonary nodules ($p = 0.03$), and non-necrotic metastases ($p = 0.04$). Whether surgery for recurrent pulmonary metastases was performed did not influence survival. Histological subtype of the primary tumor, histological response in the primary tumor after neo-adjuvant chemotherapy, occurrence of local relapse, local resection or amputation of the primary tumor and age at diagnosis did not influence outcome.

Conclusion

This cohort of patients with detailed follow-up data enabled us to identify important risk factors determining survival in osteosarcoma patients with pulmonary metastases. We demonstrate that after repeated metastasectomies, a subset of patients can be cured.

INTRODUCTION

High-grade osteosarcoma is a malignant bone tumor mainly affecting adolescents and young adults (1). Since the introduction of (neo-)adjuvant chemotherapy, long-term overall survival has improved to about 60%, with failure of therapy mainly attributed to chemoresistant metastatic disease. At diagnosis 15–25% of patients present with clinically detectable metastatic disease (synchronous pulmonary metastases) and about 40–50% of patients with primary non-metastatic disease experience relapse, mainly to the lungs (metachronous pulmonary metastases) (2-8).

Resection of pulmonary metastases with or without second-line chemotherapy has been reported to improve outcome in osteosarcoma patients with pulmonary metastases and surgery is currently standard treatment in many institutions for patients in whom metastases are deemed resectable. Despite aggressive surgery, however, many patients still relapse. The two largest single-institution studies to date investigating prognostic factors determining survival of osteosarcoma patients with lung metastases undergoing metastasectomy have had conflicting results. Studies conducted at the Rizzoli Institute concluded that higher numbers of pulmonary nodules, bilateral disease, and incomplete resection were independent prognostic factors for poor survival after metastasectomy (9-11). This was confirmed in a large multi-center study and in several smaller studies (12-15). In contrast, several studies concluded that neither number of pulmonary nodules nor other clinical parameters such as disease-free interval, bilateral disease or resection margins significantly affect survival (16-18). In the subgroup of patients with metachronous disease, longer disease-free interval has been associated with a favorable outcome, both in smaller single-center studies and in large multi-center studies (12, 14, 15, 19). The role of second-line chemotherapy is unclear, with only some authors describing a moderate survival benefit when administered in addition to metastasectomy (20).

In the current study, we sought to determine factors determining outcome in a cohort of patients with extensive follow-up data with high-grade osteosarcoma metastasized to the lungs, including 79 cases of conventional osteosarcoma, eight cases of telangiectatic osteosarcoma and one case of small cell osteosarcoma.

PATIENTS AND METHODS

Definition of Cohort

Between January 1990 and January 2008, 197 patients under the age of 40 were treated for high-grade osteosarcoma at the Leiden University Medical Center. Excluded were patients with insufficient follow-up data (n=12) and unresectable primary tumor (n=11). Of the remaining 174 patients, all 88 patients who had pulmonary metastases either at diagnosis or during follow-up were included in this study (Table I). Patients were treated according to one of the consecutive European Osteosarcoma Intergroup (EOI) trials 80861 (8) and

80931 (5) (doxorubicin and cisplatinum, with or without high-dose methotrexate, bleomycin, cyclophosphamide, actinomycin-D, and vincristin) or according to the EURAMOS-1 trial (www.euramos.org) (doxorubicin, cisplatinum, and high-dose methotrexate with or without interferon-alpha or etoposide and ifosfamide).

TABLE I.
Overview of Patients With Pulmonary Metastases of High Grade Osteosarcoma Under Age 40, Treated From 1990 to 2008

	Pulmonary metastases		
	Synchronous (group A)	Metachronous (group B)	Total
No metastasectomy for pulmonary metastases (group 1)	1A, 5 (5.7%)	1B, 27 (30.7%)	32 (36.4%)
Metastasectomy for pulmonary metastases (group 2)	2A, 21 (23.9%)	2B, 35 (39.8%)	56 (63.6%)
Total	26 (29.5%)	62 (70.5%)	88

Imaging Studies

Pulmonary metastases were diagnosed by routine computed tomography (CT) scans of the lungs and additional staging with ^{99m}Tc-bone scintigraphy, and if needed magnetic resonance imaging (MRI). Follow-up included clinical investigations and X-rays of the primary site and lungs. Number and distribution of pulmonary nodules were noted in case of suspected pulmonary metastases.

Histopathology

Primary tumors were histologically classified according to the criteria of the World Health Organization (1). Resected specimens of primary tumors were evaluated for histological response to neo-adjuvant chemotherapy; good response was defined as less than 10% vital tumor tissue. Resected specimens of pulmonary metastases were evaluated for number of resected nodules and viability of resected tumor tissue. In addition, completeness of resection and occurrence of pleural contamination were noted on macroscopic and microscopic examination.

Statistical Analysis

Unpaired *t*-tests, contingency analyses (χ^2 method) and univariate survival analyses (Kaplan–Meier method, log rank test for comparison of survival curves) were performed using GraphPad Prism 5.0. Multivariate survival analyses and survival analyses with continuous input variables were carried out according to the Cox proportional hazards model in SPSS version 16.0. Survival time was calculated from date of diagnosis to date of last follow-up or

death (noted as overall survival) or from date of first metastatic event to date of last follow-up or death (noted as overall survival since metastasis). Two-sided *p*-values lower than 0.05 were determined to be significant; *p*-values between 0.05 and 0.10 were defined to be a trend.

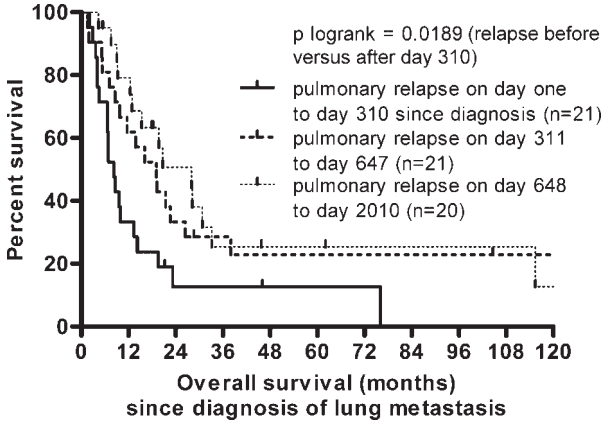
RESULTS

Twenty-six of 88 patients had pulmonary metastases at diagnosis (synchronous pulmonary metastases, group A in Table I) and 62 patients developed pulmonary metastases during follow-up (metachronous pulmonary metastases, group B in Table I). The proportion of high-grade osteosarcoma patients with clinically detectable pulmonary metastases did not change during the study period. About 15% of all high-grade osteosarcoma patients had pulmonary metastases at diagnosis and 35% developed pulmonary metastases during follow-up. There was a male predominance in our cohort (71.6% males vs. 28.4% females) and there was a trend for males to have a worse overall survival (p logrank = 0.08). Most tumours were located in the distal femur (43/88, 48.9%), proximal tibia or fibula (18/88, 20.5%), and proximal humerus (8/88, 9.1%). Less frequently involved were the axial skeleton (5/88, 5.7%) or the other long bones (13/88, 14.8%). One tumour was located in the calcaneus. Histological subtype was conventional osteosarcoma in 79 cases (including 17 chondroblastic, 2 fibroblastic and 6 unusual histological subtypes), telangiectatic osteosarcoma in 8 cases and small cell osteosarcoma in 1 case.

There was no significant difference in overall survival (measured as survival since development of metastases to correct for time dependency of the variable) between patients with synchronous and metachronous pulmonary disease (group A vs. B p = 0.16). When dividing the 62 patients with metachronous lung metastases (group B) into three equal groups of 21 patients each based on the duration of the disease-free interval, longer disease-free interval was associated with better survival, with most deaths occurring in patients in the first tertile (metastasis from day 1 to day 310 since diagnosis, Fig. 1) (p = 0.02). Other factors associated with poor overall survival for patients with pulmonary metastases were higher numbers of pulmonary nodules as determined by CT-scanning (9.3% increase in hazard for each additional pulmonary nodule, p = 0.001) and bilateral involvement (p logrank = 0.008). Patients with bone or other metastases present at the time of diagnosis of the pulmonary metastases had worse overall survival, mainly because in these cases, resection of metastases (pulmonary and others) was often not performed. Histological subtype, histological response to neo-adjuvant chemotherapy in the primary tumour (<10% viable tumour), location of the primary tumour, age at diagnosis, occurrence of local relapse and year of diagnosis did not affect survival in this cohort of patients with pulmonary metastasis.

FIGURE 1.

Kaplan–Meier curve of patients with metachronous pulmonary metastases of osteosarcoma (group B), divided into three equal groups based on the duration of the disease-free interval (tertiles), demonstrating worse overall survival for patients with pulmonary relapse from day 1 to day 310 of diagnosis (solid line, p logrank = 0.02).



Resection of Pulmonary Metastases

The majority of patients with pulmonary metastases were treated surgically for these metastases at least once (56/88, 63.6%). Overall survival of these patients (group 2) was significantly better than of patients ineligible for metastasectomy (group 1, Table I and Fig. 2, $p < 0.0001$). Irresectability of disease as determined in multi-disciplinary meetings including radiologists, pathologists, thoracic and orthopedic surgeons and clinical oncologists was the reason not to perform metastasectomy in most cases (81.3%) (Table II). One patient with radiological evidence of lung metastases who did not undergo metastasectomy is a long-term survivor (duration of follow-up 18 years). In this patient, three pulmonary nodules appeared on CT-scanning 1 year after diagnosis of the primary tumor, ranging in size from 0.5 to 2 cm. The largest nodule had a high density, suggesting calcification. In the following year, lesions progressed in both size and number, after which the lesions stabilized without further treatment. All other patients with clinicoradiological evidence for lung metastases who did not undergo metastasectomy died. In these other cases with unresectable disease, no other curative treatments were available; some patients received palliative chemo- or radiotherapy.

FIGURE 2.

Kaplan–Meier curve comparing overall survival of patients with pulmonary metastases treated surgically (group 1) and non- surgically (group 2). Patients not treated surgically have a significantly worse overall survival (solid line, p logrank < 0.0001).

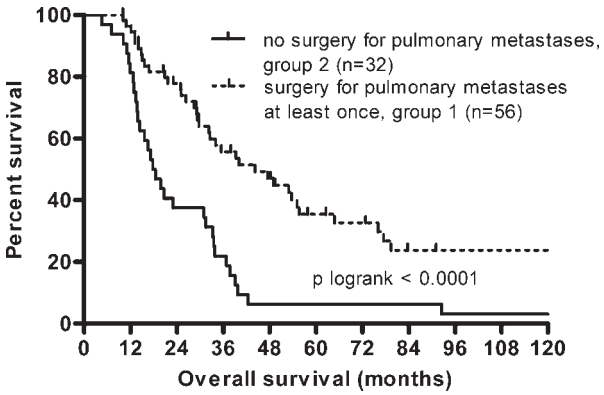


TABLE II.
Reasons Not to Undergo Surgical Removal of Pulmonary Metastases

Number of patients (%)	Reason not to undergo pulmonary metastasectomy
26/32 (81.3)	Unresectable disease (including metastases to other sites (n = 6) and rapidly progressive disease during neo-adjuvant chemotherapy (n = 5))
1/32 (3.1)	Died before metastasectomy could be performed
2/32 (6.3)	Physical or mental condition of the patient did not allow it
1/32 (3.1)	Unknown
2/32 (6.3)	Regression of pulmonary metastasis during (neo-)adjuvant chemotherapy (one patient still alive after 18 years follow-up, one patient had unresectable relapse in other organs 2 years later and died of disease)

Patients not undergoing metastasectomy for pulmonary metastases (group 2) had more nodules (mean of six vs. three, $p = 0.002$), more often had bilateral disease (25% vs. 46%, χ^2 $p = 0.06$) and more often poor histological response to neo-adjuvant chemotherapy in the primary tumor (78% vs. 64%, χ^2 $p = 0.05$) than patients eligible for surgery (group 1). There was no difference in age.

Male Sex, Higher Numbers of Pulmonary Metastases and Viability of Resected Metastases Are Independent Risk Factors After Surgical Treatment of Pulmonary Metastases (Group 2)

Although patients who were selected for pulmonary metastasectomy (group 2) had improved survival (Fig. 2), overall survival was still poor at about 23%. The majority of patients (30/56) underwent thoracotomy just once, but there was no significant survival difference for patients undergoing metastasectomy once or more often ($p = 0.29$). This is also reflected in Figure 3, which demonstrates the possibility of achieving complete remission after repeated metastasectomies. Males had surgery for metastases more often than females (70% vs. 48%, χ^2 $p = 0.05$), but had worse overall survival (Fig. 4, $p = 0.04$). Almost all of the patients with pleural disruption evident on histological examination or incomplete resection of the metastases in at least one of the metastasectomies died of disease, but this failed to reach significance ($p = 0.28$). Twenty-nine patients (51.8%) had chemotherapeutic treatment before at least one of the metastasectomies. This chemotherapeutic treatment was given to patients either as a part of their primary (neo-)adjuvant treatment or to patients presenting with an initially unresectable pulmonary recurrence during follow-up. Nine patients had completely necrotic metastases removed at least once (as determined by histological examination) and there was a trend for better survival in these patients ($p = 0.09$). Whether or not additional treatment was given before metastasectomy, did not influence survival, but there was an association between pre-metastasectomy treatment and subsequent removal of necrotic metastases (χ^2 $p = 0.04$).

As was the case in the entire cohort of patients with pulmonary metastases, higher numbers of metastases visible on CT-scan prior to metastasectomy was associated with worse survival in group 2 patients (who underwent metastasectomy at least once, $p = 0.04$). Similarly, there was a trend for patients with bilateral disease to have worse overall survival ($p = 0.07$). However, 4 of 15 patients with 5 or more nodules on CT-scan did survive after resection of these lesions (follow-up since first metastasis 18–38 months). There was a reasonable correlation between number of metastases as estimated by CT-scan and the number of metastases found at metastasectomy (r2 0.41, slope 0.7, $p < 0.0001$), which did not change during the study period. Factors not associated with outcome were histological subtype of the primary tumor, histological response in the primary tumor to neo-adjuvant chemotherapy, occurrence of local relapse, local resection or amputation of the primary tumor and age at diagnosis. In contrast to analysis performed on the entire cohort of 88 patients, disease-free interval was not associated with prognosis in this subset of 56 patients who underwent surgery for pulmonary metastases. We used multivariate Cox-regression analysis of risk factors in group 2 patients who underwent metastasectomy at least once, entering only variables with a *p*-value lower than 0.10 as determined by univariate analysis. This revealed male sex of the patient, higher numbers of pulmonary metastases and viability of resected metastases to be independent risk factors for worse outcome in pulmonary metastasized osteosarcoma patients treated surgically (Table III).

FIGURE 3.

Flow chart of all patients, demonstrating the possibility of achieving complete remission after repeated surgery for pulmonary metastases (including a patient who received surgery for pulmonary metastases four times and is still in complete remission almost 8 years since the last surgery).

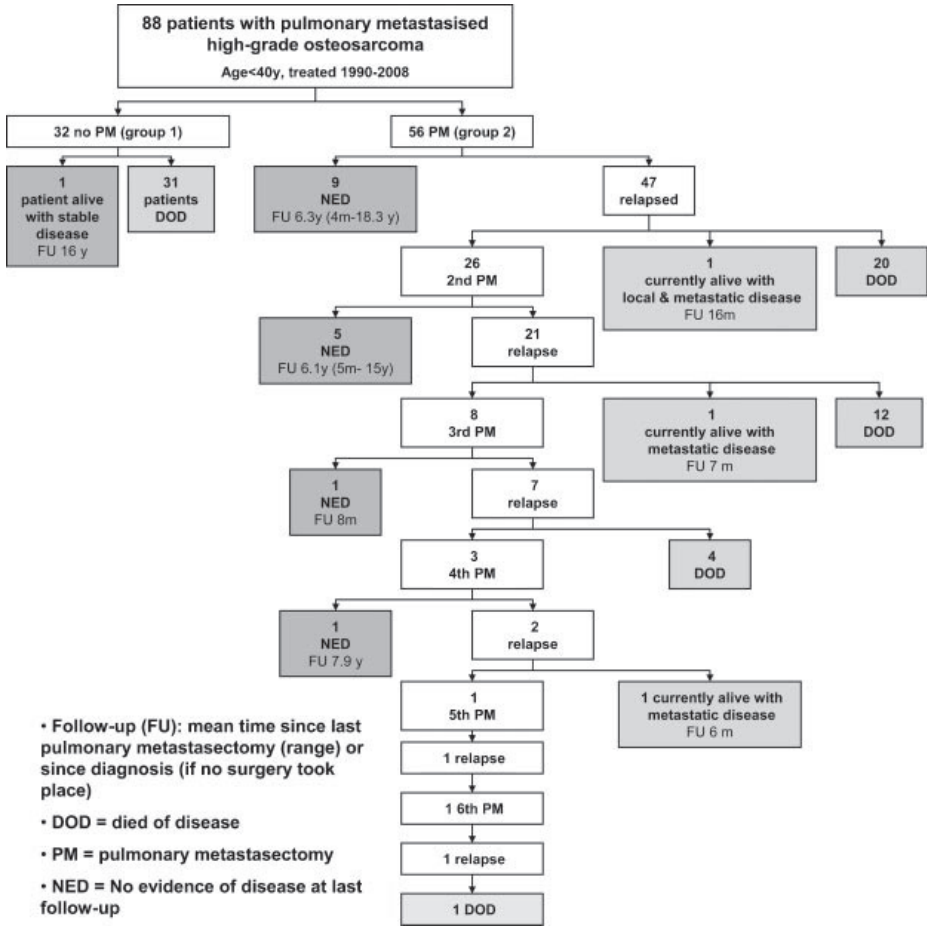
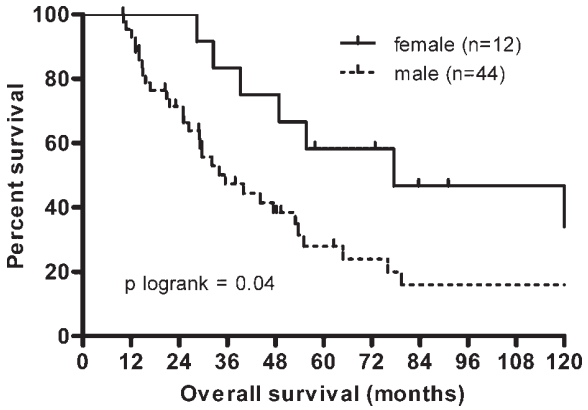


FIGURE 4.

Kaplan–Meier curve of survival after metastasectomy (group 2 patients) for males (dashed line) versus females (solid line). Males had surgery for metastases more often than females (70% vs. 48%, χ^2 p = 0.05), but had worse overall survival (p logrank = 0.04, analysis on subgroup who underwent surgery for pulmonary metastatic disease).



DISCUSSION

In this cohort of patients with pulmonary metastasized high-grade osteosarcoma, 26/88 patients had clinically detectable pulmonary metastases at diagnosis (group A, Table I) and 62/88 relapsed with pulmonary metastases (group B). Metastasectomy was almost invariably required for cure, as has been previously described by others (9, 12, 16). Since pulmonary metastasectomy is a safe and effective treatment, both in pediatric and adult patients, metastasectomy has become standard of care for patients with pulmonary metastasized osteosarcoma when these metastases are deemed resectable and there are no other contra-indications for surgery (including metastasis to other sites) (21, 22). In our well-defined cohort of patients with detailed and extensive follow-up data we were able to confirm important risk factors determining survival in osteosarcoma patients with pulmonary metastases, that is, extent of disease and longer disease-free interval. In addition, we have identified the novel independent risk factors male sex and resection of metastases containing viable tumour cells. The administration of chemotherapeutic agents before metastasectomy is associated with a subsequent higher chance of resecting necrotic metastases, which in turn is associated with better overall survival. This would suggest a potential beneficial role of second-line

chemotherapeutic agents in the treatment of metastasized osteosarcoma, even when these lesions are deemed resectable. However, since we did not find a direct association between chemotherapeutic treatment and overall survival in the group of patients surgically treated for pulmonary metastases, the addition of chemotherapy to the surgical treatment of patients with pulmonary metastases remains unproven. In previously published studies it has also been difficult to establish what the value of second-line chemotherapeutic treatment is to the surgical management of metastasized osteosarcoma (20). This could either mean that the true benefit is very small, or that the treatments employed are too heterogeneous to draw definite conclusions.

As was found in other studies, large metastatic tumor burden (defined as five or more pulmonary nodules or bilateral involvement), has prognostic relevance. It is now well known that spiral CT-scanning (1 mm slices) is more sensitive than conventional CT, allowing the detection of significantly larger number of nodules and also smaller nodules <5 mm in diameter. Even these small pulmonary nodules should be regarded as probable pulmonary metastases when other risk factors for pulmonary nodules, such as smoking history or prior granulomatous disease, are absent. The proportion of patients diagnosed with pulmonary metastases in this study did not change during the study period. Previously, Kayton et al. (23) reported that CT-scanning of the chest underestimates the number of metastatic lesions in osteosarcoma. In our cohort there was a reasonable correlation between number of metastases as predicted by CT-scan and number of metastases as determined at resection. However, we feel that the presence of high numbers of pulmonary nodules should not guide decisions regarding resection if the nodules are resectable; a third of patients with high tumor burden, that is, five or more nodules on CT-scan prior to surgery, survive after resection of the lesions with a median duration of follow-up of 25 months. Similarly, patients experiencing relapse after first metastasectomy can still benefit from repeated metastasectomies if these lesions are resectable and if there is no risk of respiratory compromise.

The reason for the relationship with gender and outcome in our cohort is unclear. There was a male predominance in our cohort (71.6% males vs. 28.4% females). Males had their metastases significantly more often surgically resected than females. However, men had lower overall survival even after resection of metastases. A recently published review of osteosarcoma incidence and survival rates from 1973 to 2004 in the United States noted a worse overall survival for males over all age groups, concordant with previous smaller studies (7, 24, 25). In another study, a strong correlation between male sex and poor histological response to pre-operative chemotherapy was found, although this did not result in worse overall survival (4). Osteosarcoma cells express sex steroid receptors and it has been observed that 2-methoxyestradiol (2-ME), a naturally occurring metabolite of 17beta-estradiol (E2), induces cell death in osteosarcoma cells(26, 27). It remains unclear whether direct effects of sex steroids on neoplastic cells play a role in the observed better outcome for females, or if other mechanisms are underlying this observation.

Patients with osteosarcoma which has a good histological response of the primary tumour to neo-adjuvant chemotherapy have better overall survival (28). In our cohort of patients

with osteosarcoma and metastatic disease (group A and group B), there was no association between response to chemotherapy and survival. Similarly, the previously reported association of chondroblastic tumours with good histological response to chemotherapy and better overall survival, was not present in this cohort of patients with osteosarcoma and lung metastasis. However, when analyzing all patients diagnosed with osteosarcoma in our center, including those that did not develop pulmonary metastases the association between poor histological response and poor survival was also present in our cohort. This suggests that poor histological response to chemotherapy probably determines poor survival through risk of developing clinically detectable, pulmonary metastases. However, once these metastases have developed, the histological response in the primary tumour was no longer relevant in our series.
Analysis of patients with resectable non-metastatic osteosarcoma treated in three consecutive EOI trials demonstrated the association between early recurrence and poor survival (19). In the current study, this association was also present in the subgroup of patients presenting with metachronous pulmonary metastases, but this association disappeared when patients eligible for surgical treatment of the metastases were selected. This indicates that patients presenting with relapse roughly within the first year after diagnosis have a higher chance of presenting with unresectable pulmonary involvement, but treatment of resectable pulmonary disease results in a similar outcome in this group.
In conclusion, this well-defined cohort of osteosarcoma patients with pulmonary metastases treated within a single institution allowed us to establish that higher number of pulmonary nodules, resection of vital metastases and male sex were associated with poor overall survival. In the present study we confirm extent of disease, that is, number of pulmonary nodules, to be an important independent risk factor determining survival in osteosarcoma patients with pulmonary metastases. In addition we demonstrate that female sex and resection of necrotic metastases are associated with better survival after pulmonary metastasectomy. Importantly, we demonstrate that even after repeated metastasectomies, cure can be achieved in a subset of patients.

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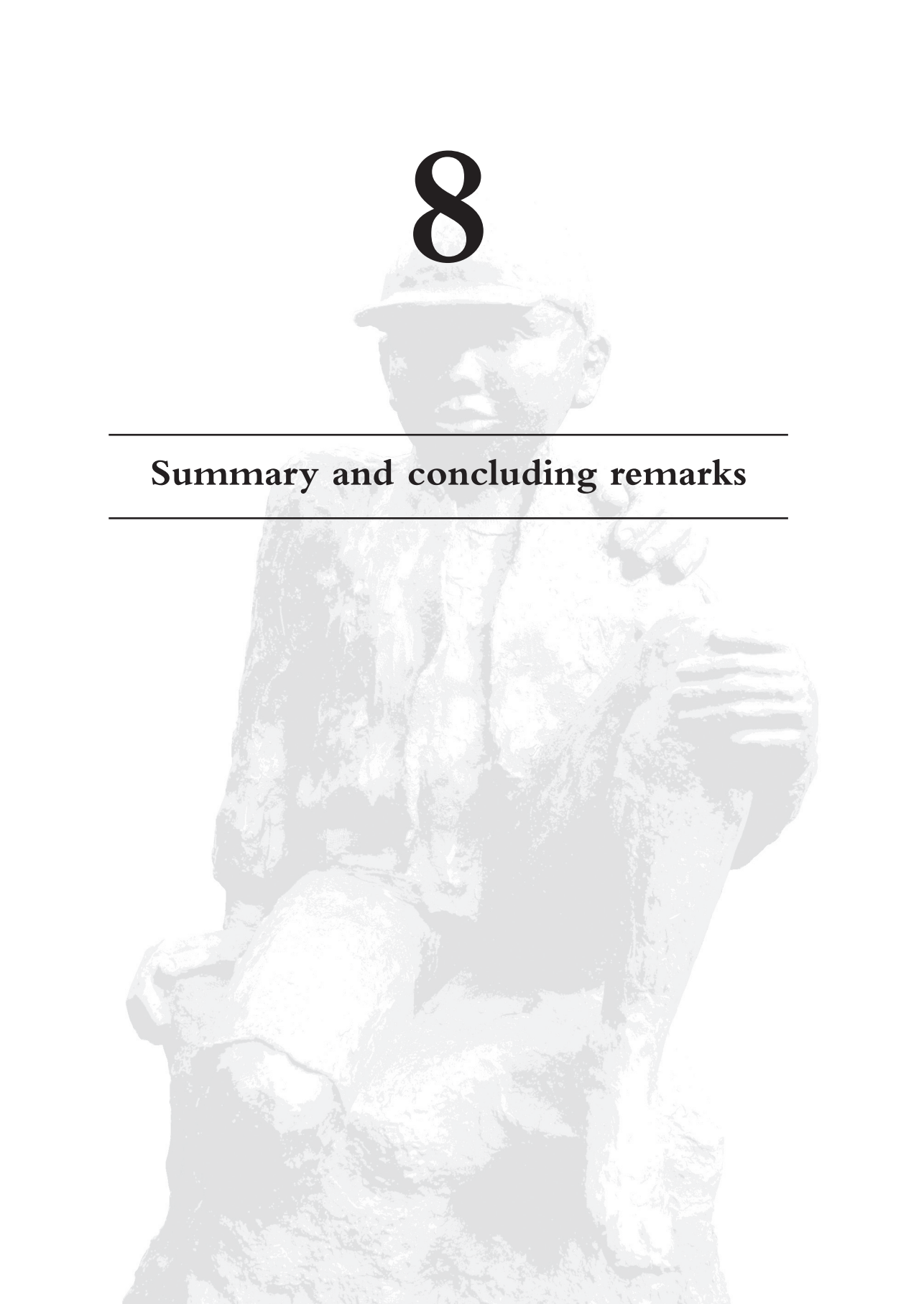
8

Summary and concluding remarks



In this thesis 7 chapters are presented, describing clinical, pathological and molecular studies related to the most common primary bone tumour, osteosarcoma. **Chapter 1**, the **general introduction**, is an overview of epidemiology including incidence, age distribution, localization in the skeleton, risk factors and survival. The objective of this description is to gain more insight in the clinico-pathological behavior of osteosarcoma, based on epidemiologic information (1). The *incidence* pattern is age dependent. Osteosarcoma in children under 5 years of *age* is rare, less than 2% of all osteosarcomas occur in this age group. A steep rise in incidence occurs during puberty, peaking at an incidence of 8.6 cases/10^6 population up to 20 years of age, followed by a low rate of on average 1.7/10^6 population during adulthood (25–59 years of age) (2). A non-unified second peak occurs in people of 60+ years, reaching 4.9/10^6 new cases yearly. Remarkably, this 2nd incidence peak is absent in the Asian people (3). This peak has suggested to be due to secondary osteosarcomas, after radiation or as complication of Paget's disease. The different distribution and histology of osteosarcomas in patients older than 60 years of age suggests indeed a distinctive biological behaviour. An adequate *treatment* is of utmost importance for survival of patients that has not improved the past 2-3 decades. Contemporary treatment consists of pre-and postoperative (neo-adjuvant) chemotherapy and radical surgery. If no clear resection margins can be obtained, the patient has a very high risk of being incurable.

With respect to the *prognosis* of patients with osteosarcoma, the chances for survival after incomplete surgery are less than 15% (4). Hence locations where complete resection is impossible, for example axial or pelvic site, have strong influence on outcome. Axial site is more often present in older patients, therefore age can be biased by the site of the primary tumour with respect to prognosis. For resectable disease, metastases at diagnosis, proximal site and large (≥ 1/3 extremity length) size of the primary tumour are the most important adverse prognostic factors (4-6). Two treatment related factors are also of favourable prognostic importance, these are good histologic response on pre-operative chemotherapy and presence of chemotherapy induced toxicity (7). Other factors, such as pathological fracture at diagnosis, type of surgery, age and gender were of minor prognostic importance. Genetic risk factors, like the Li-Fraumeni syndrome, the (heritable or bilateral) Retinoblastoma, the helicase-mutation syndromes and other diseases in their context to osteosarcoma are discussed in this chapter. The *pathology* of osteosarcoma was discussed, with emphasis on the unconventional subtypes of high-grade osteosarcoma and the low-grade osteosarcoma variants. This was chosen because these variants contribute to only 5% of all osteosarcomas but, were overrepresented in the hands and feet (discussed in chapter 6).

In **chapter 2** the literature of chemotherapeutic treatment of localized, non-metastatic osteosarcoma of the extremities was reviewed. One of the main conclusions was that there are not more than 4 effective cytostatic drugs, where efficacy is defined as an response rate (RR) in phase-II trials of 20% or more. These 4 drugs are doxorubicin (RR 43%), ifosfamide (RR 33%), methotrexate (RR 32%) and cisplatin (RR 26%). Meta-analysis demonstrated that 2-drug regimens (mainly consisting of doxorubicin and cisplatin) are inferior to regimens containing 3 or more drugs. According to this analysis there was no survival benefit of 4-drug regimens compared to 3-drug regimens. Therefore a 3-drug combination such as

methotrexate, doxorubicin (a.k.a. adriamycine) and cisplatin, a regimen referred to as MAP, is considered the best induction regimen and should be used as standard treatment for osteosarcoma in clinical protocols. The debate remains whether adding a high responsive drug, like ifosfamide, to MAP should be reserved for non-responding patients or in cases of progressive disease. Furthermore, it was concluded that investigating more of this type of conventional drug regimens would not be advantageous.

Therefore, we started a study in osteosarcomas to investigate if genome wide gene expression provides a better insight into the biology of this tumour. Gene expression pattern of 25 high-grade osteosarcoma biopsies were correlated to the outcome of disease or response to neo-adjuvant treatment. In addition we investigated if drug targets from such expression data could be determined. The results of this study were presented in **chapter 3** and showed that nearly 3000 genes were significantly differentially expressed in osteosarcoma, compared with non-malignant cells (osteoblastomas, mesenchymal stem cells and mesenchymal stem cells differentiated into osteoblasts). Gene expression profiles could not be correlated to either response to treatment or survival. Analysis at a single gene level proved to be not useful in osteosarcomas, because this tumour has a highly complex karyotype, that diminishes the reliability of single genes to predict the clinical determinants of malignant diseases, unless thousands of samples are used (8). Therefore, pathway analysis was chosen as a method for further analysis of malignant transformation of the mesenchymal stem cell, the presumed precursor of osteosarcoma (9).

At pathway level, we found down-regulation of the Wnt3a/β-catenin signalling (reflected by downregulation of Axin and CCND1), upregulation of the Wnt5a/alternative signalling, overexpression of the cell cycle genes and a disturbed p53/apoptotic pathway (reflected by downregulation of BBC3/PUMA) in osteosarcomas.

The statistical background for the choice for pathways analysis is described in chapter 4. This paper describes the algorithm for the association of the expression of groups of genes with clinical variables. The groups of genes can be clustered based on pathways, as defined for example in the Gene Ontology data base (http://amigo.geneontology.org) (10). The Global Test can test the statistical significance of a certain pathway, attributed to a clinical variable of interest, for instance survival. The test is based on the Cox proportional hazards model, with the possibility to adjust for the presence of co-variables. In this paper, the expression profile of the patients, whose biopsies were analyzed in chapter 3, were tested. Using this model it was found that pathways, involving the cell cycle, DNA repair and apoptosis were associated with survival. It was further concluded that using the Cox model, survival data are not lost and can be adjusted for the presence of co-variates, which allows to improved performance of this test.

In order to establish molecular targets for osteosarcoma treatment, the epidermal growth factor receptor HER2 was mentioned as a candidate and is the subject of research, described in **chapter 5**. Her2 is highly expressed in 25% of the breast cancer patients, and its related tumorigenic effects (11, 12) can be reverted by the monoclonal antibody trastuzumab (Herceptin®) (13). Based on the presumed overexpression of Her2 in osteosarcomas (14-16), a phase-II study with trastuzumab was initiated in osteosarcomas (www.cancer.gov/clinical_trials: MSKCC-99097/NCI-T98-0083 and COG-AOST0121). However, in our study no

membranous (3+) HER2 overexpression was found, which is a prerequisite for tratstuzumab treatment (17, 18). Neither HER2 mRNA was overexpressed at the gene level, nor FISH analysis showed *HER2*-gene amplification in the single sample that stained moderate (2+) positive membrane staining. We concluded that *HER2*-gene amplification or membranous HER2 protein overexpression is absent in human osteosarcoma, and that we cannot support the principle to treat osteosarcomas with Herceptin.

After we had confirmed the complexity of osteosarcoma at molecular level with the gene-expression study, another question was whether there is also clinical evidence for heterogeneity of osteosarcomas. To answer this question, we studied the clinico-pathological features of osteosarcomas with a rare localization, i.e. the small tubular and flat bones of the hands and feet. The results of this study are described in **chapter 6.** In total 40 patients with osteosarcomas of the hands or feet, obtained from the merged Dutch (10/1733) and the Rizzoli Institutional databases (30/2488) were described, representing only 0.95% of all osteosarcomas, present in both databases. Compared with the usual sites (around the knee or humerus), osteosarcomas in hands or feet occurred in older patients (mean age 42 years), with a male predominance (male female ratio=1.7:1), patients had a longer delay before the definitive diagnoses was made, and had a higher proportion of low grade (30%) and intermediate grade (5%) of malignancy compared to osteosarcoma at conventional sites, that show low-grade malignancy in 1%-2% (19). Overall cumulative incidence of death (CID) of the whole group was 80%, however worse in patients with location in the hands (4y CID 38%) than in the feet (2.5 CID 11%), and no deaths were observed in patients with low-or intermediate grade osteosarcomas. It was concluded that high-grade osteosarcoma of the hands or feet are a peculiar subgroup of osteosarcomas, and that high-grade tumours have a similar prognosis as osteosarcoma in the long tubular bones of the skeleton. It is recommended to treat high-grade osteosarcomas of the distal extremities in the same way as those tumours at conventional sites.

The last part of this thesis, **chapter 7**, deals with osteosarcoma as systemic disease, which occurs as synchronous metastases (metastases at diagnosis, in 16% of the newly diagnosed patients (4, 20-24) or as recurrent or relapsed disease (metachronous metastases), which occurs in 45% of all patients treated for localized osteosarcoma (4, 20-26). A study was done to determine prognostic factors in 88 patients with pulmonary (n=26 synchronic, n=62 metachronic) metastases from the Leiden University Medical Center data base. Overall survival of the patients with resectable metastatic osteosarcoma was 23%, not worse for patients with synchronous versus metachronous metastases. Survival was determined only by resectability of the metastases, even if surgery was more often than once required. Poor prognostic factors for survival in patients who underwent surgery were high (5 or more) number of metastases (HR 1.29), whereas favorable prognostic factors were necrotic metastases (HR 0.17) and female gender (HR 0.41). Although it would suggest that pre-operative chemotherapy could induce necrotic metastases, the trend towards better survival for patients who received chemotherapy, found in this study was not significant (χ^2 p=0.04). Overall, it was concluded that cure can be achieved in a subset of patients with (synchronous or metachronous) metastases by aggressive surgical treatment, but the role of chemotherapy remains elusive.

DISCUSSION

From the above chapters it can be concluded that high-grade osteosarcoma cannot be considered as one disease, but is a heterogeneous tumour at clinical, pathological and genomic level. This may be the reason that contributes to the lack of improvement in survival during the past 3 decades. One of the important findings in this thesis was that there are only 4 effective drugs against high-grade osteosarcoma, i.e. doxorubicin, methotrexate, cisplatin and ifosfamide. After relapse, the treatment options become even more limited, because re-using the same drugs questions their efficacy, and adds to the cumulative toxicity of these drugs (27), like cardiac (28), hearing loss (29), renal damage (30, 31), fertility problems (32, 33) or second malignancies (34). Treatment with the monoclonal antibody Herceptin could not be supported by us and others, because there is no membranous HER2-receptor overexpression on osteosarcomas, as is shown by us and others (35-38). Array analysis revealed up-regulation of cell cycle genes and a disturbed Wnt- and p53/apoptotic signalling as most important abnormal pathways in osteosarcomas compared with non-malignant cells. Upregulation of cell cycle genes is not surprising in cancer cells, neither disturbance of the apoptotic pathway. In order to think of the Wnt-signalling as potential therapeutic target for osteosarcoma, the Wnt-pathway in general and as far relevant for osteosarcoma will shortly be discussed shortly in the next paragraph.

Wnt-pathway

The Wnt signalling plays an important role in developmental biology and in cancer (39, 40). Due to the numerous Wnt-ligands (n=19), Wnt-receptors (Frizzled: Fzd's n=10), co-receptors (n=8) and modulators, like Wnt-inhibitors (Dickkopfs, Wnt-inhibiting factors, soluble Fzd-related proteins and proteoglycans) the downstream signals after ligand-receptor binding are pleiotropic and tissue specific, spatial-and time dependent (39, 41, 42). That means that the effect of Wnt signalling in the bone marrow niche (reservoir mesenchymal stem cells (MSC) is different from Wnt signalling in cartilage of tubular bones or in flat bones, or in other tissues. For an extensive discussion about these topics, the reader is referred to some excellent reviews (39, 40, 43, 44).

Modern insights in these complexities have replaced the old distinction of canonical and non-canonical by β-catenin dependent and β-catenin independent respectively, and an overview of both pathways is given in Figure 1. In summary, the β-*catenin dependent (or canonical)* pathway stabilizes cytoplasmatic β-catenin after binding of the Wnt3a (or other "canonical" Wnts) with the Fzd2/7 receptor, by inhibiting the proteosomal degradation of the continuously formed β-catenin (Fig.1 A) (40). Due to the rising cytoplasmatic concentration, β-catenin shuttles to the nucleus, and activates transcription factors for proliferation (de-repression) (45) or induces differentiation (co-activation) of for instance Runx2 (46, 47), a master gene for osteogenesis. The β-catenin independent signalling is activated after binding of Wnt5a with either Fzd2 (Fig.1, B) or Fzd4 (Fig.1 C), with or without the co-receptor ROR2 or with ROR2 as a single receptor (Fig.1 E). The oncogenic transcription factor Jun-N-terminal kinase (JNK)

(48) is activated after Wnt5a-Fzd2 binding mediated by the small GTP-ases Rho and Rac (Fig.1 C), and is called the *Wnt/PCP pathway* (41, 49). The other ß-catenin independent signalling, the *Wnt/Ca^{2+} pathway* (Fig.1 D), activates the transcription factors NEMO or NFAT, which inhibit ß-catenin dependent proliferation (50, 51) and activate osteogenesis (52, 53) respectively. Other modulations of the Wnt5a signalling are shown in Fig.1 by the red circles and are at the level of competitive inhibition of Wnt3a binding with Fzd receptors (Fig.1 F), via the ubiquitin ligase Siah2 (54, 55) or directly via ROR2 activation (56).
Bone development is an complex process, in which Wnt's play an important role in multiple ways (see Fig.2) (for reviews, see (44, 47, 57)). In the early MSC stage Wnt3a/β-catenin is required for lineage fate decision (57-59) and stimulates the proliferation of stem cells to maintain an adequate number of progenitor cells. Furthermore, Wnt3a signalling prevents osteo-chondrogenic progenitors from developing into cartilage and differentiate into osteoblasts (46, 60, 61), but for the definitive differentiation into the osteogenic lineage, Wnt3a needs temporary be downregulated (62, 63), which is mediated via Wnt5a and Dkk1 (62-64). Finally, the β-catenin pathway is required for definitive differentiation of precursor cells into osteocytes (63, 65).

FIGURE 1.
Wnt signalling pathways.

This figure shows a number of different Wnt-signalling pathways, as well as possible cross-talks between the β-catenin dependent ("canonical", column A), and β-catenin independent ("non-canonical", column B-E) pathways. The Wnt3a-activated ("on") β-catenin dependent pathway under column A and the β-catenin repressive ("off") situation in column F. See text for details. The β-catenin independent signalling is divided into the Wnt/PCP/Jnk and Wnt/Ca2+ pathways. Recently, insights have added a Wnt-Ror2 pathway has been added to these pathways, which inhibits the canonical pathway at 4 possible levels. Firstly, by competitive inhibition with the Wnt3a receptor binding (Red circle 1), by increased degradation of β-catenin via ROR2 activated Siah-2 activity (red circle 2), by repression of TCF3 transcription via NEMO (red circle 3), or by directly inhibition of β-catenin-TCF binding (red circle 4). Another cross-talk is β-catenin activation, when Wnt5a binds to the Frizzled4 (Fzd4) receptor. The different and complex outcomes of the various Wnt signalling pathways are the result of binding of "canonical" (represented by Wnt3a) or "non-canonical" (represented by Wnt5a) Wnt-ligands to multiple receptors (Fzd's), either with or without different co-receptors (Lrp's, ROR's or Ryk's), modulated by inhibitors, such as the Dickkopf's (Dkk's), the Wnt–inhibiting factors (WIF's), the soluble Frizzled related proteins (sFRP's), or Sclerostin (SOST). LRP5,6 lipoprotein related protein 5,6; Dvl Dishevelled; APC Adenomatous Polyposis Coli; GSK-3β Glycogen Synthase Kinase-3β; CK1 Casein Kinase-1α; CamKII Calcium/Calmoduline dependent Kinase II; JNK c-Jun N-terminal Kinase; Rock2 Rho-associated Kinase; β-TrCP is a ubiquitin ligase; TCF1/3 T-cell factor 1/3; Lef1 Lymphoid Enhancer Factor 1; Gro Groucho.

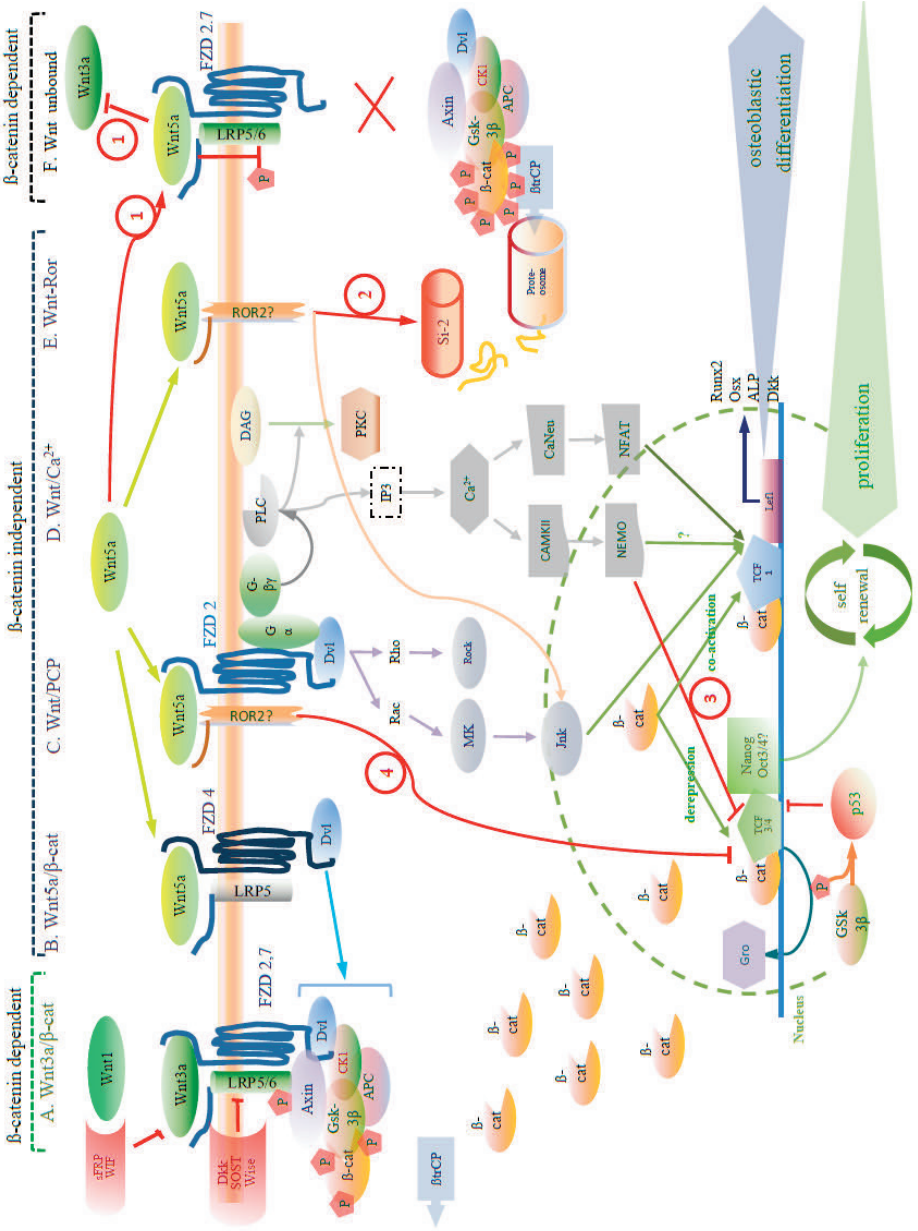
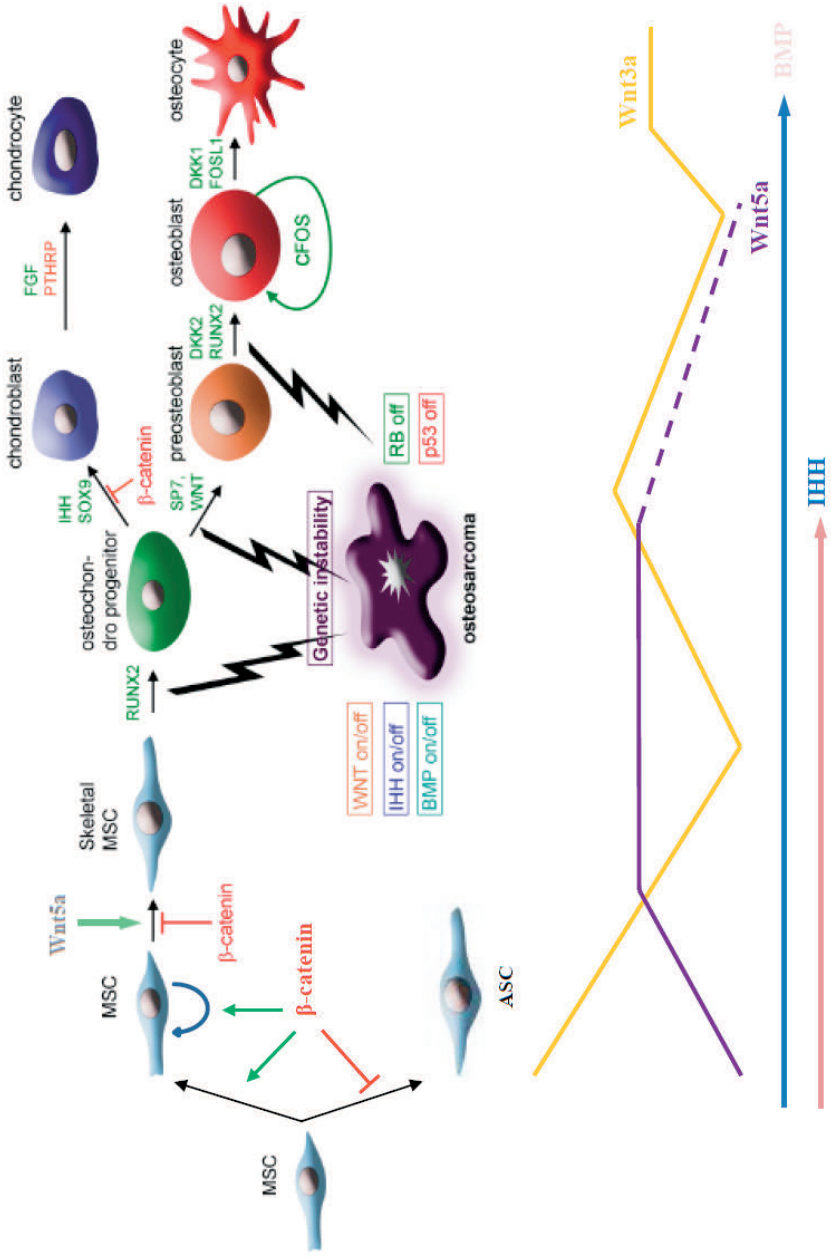

β-catenin dependent
A. Wnt3a/β-cat
B. Wnt5a/β-cat
β-catenin independent
C. Wnt/PCP
D. Wnt/Ca2+
E. Wnt-Ror
β-catenin dependent
F. Wnt unbound
Wnt1
Wnt3a
Wnt5a
sFRP
WIF
Dkk
SOST
Wise
LRP5/6
LRP5
FZD 2,7
FZD 4
FZD 2
ROR2?
Dvl
Axin
Gsk-3β
β-cat
CK1
APC
βtrCP
DAG
PLC
PKC
IP3
Ca2+
CaNeu
NFAT
CAMKII
NEMO
Si-2
Proteosome
Rho
Rac
Rock
MK
Jnk
β-cat
co-activation
derepression
TCF 1
TCF 3/4
Lef1
Nanog
Oct3/4?
Gro
p53
GSk 3β
Nucleus
Runx2
Osx
ALP
Dkk
osteoblastic differentiation
proliferation
self renewal

FIGURE 2.
Wnt-signaling in stages of osteoblastic development.

The two Wnt-signalling pathways are represented by Wnt3a as *Wnt3a/β-catenin dependent* and Wnt5a as *Wnt/β-catenin independent pathways*. The orange and purple line at the bottom of figure 2 are the hypothetical levels of each of the Wnt-pathways, showing a inhibition of the β-catenin pathway, by Wnt5a and probably Dkk1. In the early mesenchymal stem cell (MSC) stage, β-catenin stimulates the proliferative activity of the cell, and induces the MSC to the osteo-chondrogenic lineage, thereby suppressing the adipogenic lineage (ASC = adipogenic stem cell). In a later stage of the osteochodrogenic progenitor cell, Wnt3a stimulates proliferation of the progenitor cells to ensure a pool of these cells, inhibits chondrogenic differentiation (by suppressing SOX9 and IHH) and starts the induction of the osteogenesis by activating the transcriptionfactor Runx2. To continue osteoblastogenesis, Wnt3a needs to be downregulated, which is done by Wnt5a, and probably Dkk1. For the terminal differentiation of the osteoblasts into osteocytes, Wnt3a has to be upregulated again, in order to activate transcription factors is necessary for differentiation to osteocytes. Disruption of this tightly regulated signalling in normal bone development leads to hyperproliferation, defective differentiation commitment control, resulting in genetic instability and osteosarcomagenesis.
In normal osteogenesis, other signalling systems, such as bone morphogenetic proteins (BMP) and Hedgehog (IHH) are required but are not further discussed here.


Wnt5a
MSC
β-catenin
Skeletal MSC
RUNX2
osteochon-dro progenitor
IHH SOX9
β-catenin
chondroblast
FGF PTHRP
chondrocyte
SP7, WNT
preosteoblast
DKK2 RUNX2
osteoblast
DKK1 FOSL1
osteocyte
CFOS
Genetic instability
RB off
p53 off
osteosarcoma
WNT on/off
IHH on/off
BMP on/off
β-catenin
MSC
ASC
Wnt3a
Wnt5a
BMP
IHH

Wnt-signalling, cancer and osteosarcoma

In our array study we found evidence for down-regulation of the Wnt3a/β-catenin pathway and up-regulation of the alternative, Wnt5a pathway. This is in contrast to the activating β-catenin deregulation, which is the driving force for tumourgenesis in most types of epithelial cancers, for example in colon cancer (66), ovarian cancer (67), prostate cancer (68) or lung cancer (69). Wnt3a/β-catenin overexpression has been reported in osteosarcoma, either directly (70, 71) or indirectly by inhibition of the Wnt ligand (72, 73) or due to overexpression of the co-receptor LRP5 (74). However, overexpression of β-catenin, as seen in the Gardner syndrome, did not result in an increased incidence of osteosarcomas (75), whereas in the benign osteoblastomas clear expression of β-catenin was observed (76).

Absent nuclear β-catenin staining was observed in only one other study of osteosarcoma (77). Recently, Mathushansky reported that inactivation of the β-catenin dependent Wnt pathway was tumorigenic in the high-grade undifferentiated pleomorphic sarcoma (78). It was shown that the mesenchymal stem cell was the progenitor of the undifferentiated sarcoma and that down-regulation of the Wnt/β-catenin dependent pathway failed to commit the stem cell to differentiation into mature connective tissue lineages. In addition Wnt5a was defective in regulating a commitment-viability checkpoint, as is known that this non-canonical pathway mediates anti-apoptotic signalling (79). In another study the Wnt/β-catenin signalling was downregulated in Rhabdomyosarcoma cell line, blocking the normal myogenic differentiation and increasing resistance to apoptosis (80). Restoration of the Wnt3a activation resulted in myogenic differentiation.

Another example of the contribution of an inactive Wnt3a/β-catenin signalling is reported in Retinoblastoma's (81). Wnt signalling re-activation significantly decreased the viability of the retinoblastoma cells by p53-induced cell cycle arrest. The authors concluded that the Wnt-pathway acted as a tumour suppressor in the retinoblastoma cells lines, and that loss of Wnt signalling contributed to the tumorigenesis in the retina.

Inactivity of the Wnt3a/β-catenin signalling in our study has been confirmed by Cai et al. (76). Restoration of the Wnt3a/β-catenin pathway by inhibition of GSK-3β, that phosphorylates β-catenin, demonstrated differentiation into bone of 2 of 4 osteosarcoma cell lines.

What exactly the role of the downregulation of the Wnt3a/β-catenin pathway in the tumorgenesis of osteosarcoma is, remains difficult to explain. The hypothesis is that, similar as in undifferentiated sarcomas and rhabdomyosarcomas, bone-progenitor cells will not be able to complete osteogenesis and remain in continuous proliferative state (as was shown by the upregulated cell cycle genes). The overexpressed Wnt5a signalling on the other hand drives the osteo-progenitor cells into the direction of osteogenesis (82). However, due to the disturbed apoptotic regulation these cells lack a differentiation commitment check, that results in progressive genomic instability, which is the hallmark of osteosarcoma (83, 84). However, this is still hypothesis, and it would be a challenge to study Wnt-signalling in the normal osteogenesis and in the disturbed osteogenesis, such as in osteosarcoma, or in other pathologic conditions.

Wnt signalling and potential therapies

Given the observations that the Wnt3a/β-catenin pathway was inactive in osteosarcomas and that 2 of 4 osteosarcoma cell lines differentiated into normal bone after inhibition of GSK3β, it could be argued that therapy, aiming to inhibit proteosomal degradation of β-catenin might be of advantage in patients with the Wnt-pathway in the off-state. One of the most promising compounds to interfere with the proteosomal activity is bortezomib (85, 86). This drug has shown to restore normal bone development in Multiple Myeloma patients (87, 88), irrespective the response on treatment (89). Although the mechanism is not completely resolved, it has been suggested in these patients that bortezomib inhibits the Wnt3a antagonist Dkk1 (87), induced differentiation of osteoblasts via stabilization of β-catenin (86), or via bortezomib induced apoptosis of the tumour cells (90). In mice that were treated with bortezomib, inhibition of cell proliferation and increased apoptosis of the osteosarcoma cells was obsesrved, resulting in regression of the tumour (85). Bortezomib has been used in clinical phase-1 (91), phase-II (92) or phase-III studies (93), is tolerated well with few side effects. Even in combination with other drugs, that might be used in (refractory) osteosarcoma, or in older patients, bortezomib can be used safely (94-96). Therefore, bortezomib might one of the few agents worth for future evaluation in osteosarcoma therapy, preferably in a window phase in patients with absent Wnt3a/β-catenin dependent signalling.

CONCLUDING REMARKS

It can be concluded from this thesis that high-grade osteosarcoma is at clinical, pathological and molecular level a heterogeneous disease. To treat high-grade osteosarcoma adequately, neo-adjuvant chemotherapy should be combined with radical surgery, irrespective the localization of the tumour. An adequate chemotherapy regimen for osteosarcoma consists at least of 3 out of 4 effective cytostatic agents, i.e. methotrexate, doxorubicin and cis-platin. A fourth active agent, ifosfamide, should possibly be reserved for patients with refractory disease or patients with relapse. Patients with metastatic pulmonary osteosarcoma should receive surgery in case of resectable disease, whereas the use of chemotherapy is these patients can be considered, but is not of proven value. Patients with irresectable metastatic osteosarcoma should be offered phase-I and phase-II studies, because no response can be expected from other conventional cytostatic drug combinations. With respect to new drug developments, the use of the monoclonal antibody trastuzumab against HER2 is not supported by us, because we were not able to demonstrate overexpression of the HER2 receptor on osteosarcoma cells. At molecular level, a disturbed Wnt signalling was found in addition to abnormal cell cycle regulation and a disturbed p53/apoptotic pathway. This combination of these pathway abnormalities might be oncogenic. Failure of the mesenchymal stem cell to differentiate into the osteoblastic lineage, due to abnormal proliferation and lack of differentiation commitment results in chromosomal instability, which is the hallmark of osteosarcoma. In patients with an inactive Wnt3a/β-catenin signalling the proteasome inhibitor bortezomib might be a candidate drug, to explore its suggested differentiation inducing properties. More research should be directed to study Wnt signalling in normal and disturbed osteogenesis, in order to clarify the mechanisms by which Wnt3a has its effects in osteosarcoma.

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9

Nederlandse samenvatting

In dit proefschrift worden 7 studies beschreven op het gebied van de meest voorkomende maligne primaire bottumor, het osteosarcoom. In **hoofdstuk 1** wordt een overzicht gegeven over de epidemiologie van het osteosarcoom, zoals de incidentie, leeftijdsverdeling, lokalisatie in het skelet, risicofactoren waaronder genetische syndromen, en de overleving. Deze uitvoerige beschrijving op basis van epidemiologische studies is bedoeld om meer inzicht te krijgen in de oorzaak en het klinisch-biologisch gedrag van het osteosarcoom.
De *incidentie* is *leeftijdsafhankelijk*. Bij jonge kinderen is osteosarcoom zeldzaam, minder dan 2% van de osteosarcomen komt in die leeftijdsgroep voor. Een eerste piek in incidentie (8.6 nieuwe patiënten/1 miljoen bevolking) wordt gezien bij pubers en jong volwassenen, gevolgd door een lage incidentie van gemiddeld $1.7/10^6$ in de leeftijdsgroep van 25-59 jaar. Er is een 2e incidentie piek (tot $4.9/10^6$) bij mensen van 60 jaar en ouder, die merkwaardig genoeg bij mensen van Aziatische origine ontbreekt. Men schrijft deze 2e incidentie piek toe aan secundaire osteosarcomen. Het verschil in man-vrouw verdeling en in histologische subtypen tussen oudere en jongere patiënten suggereert een verschillend biologisch gedrag van osteosarcomen.
Een *adequate behandeling* van osteosarcomen is van het allergrootste belang voor de overleving van de patient, die echter sinds de laatste 20-30 jaar niet veel verbeterd is. De huidige therapie bestaat uit pre-operatieve chemotherapie, radicale chirurgie, en chemotherapie na de operatie (neo-adjuvante chemotherapie) waarmee een langdurige overleving van gemiddeld 60% bereikt wordt. *Prognostische factoren* voor osteosarcoom kan men verdelen in factoren die bij de diagnose aanwezig zijn (uitbreiding van het osteosarcoom, zowel systemisch (gemetastaseerde ziekte) als lokaal, en proximale lokalisatie in het bot, metastase bij diagnose) en factoren die gerelateerd kunnen worden aan de behandeling (chemotherapie geïnduceerde tumorcelnecrose en chemotherapie geïnduceerde toxiciteit). Genetische risicofactoren met betrekking tot osteosarcoom worden besproken, zoals het Li-Fraumeni syndroom, het (erfelijke/bilaterale) Retinoblastoom, de helicase-mutatie syndromen en enkele andere zeldzame aandoeningen. Tevens wordt in de inleiding dieper ingegaan op de *onconventionele pathologische subtypes* van het osteosarcoom, omdat deze subtypes vaker bij osteosarcomen van handen en voeten voorkomen (zie hoofdstuk 6).
In **hoofdstuk 2** wordt de literatuur over de chemotherapeutische behandeling van gelokaliseerd osteosarcoom van de extremiteiten gereviewd. Uit fase-2 studies blijkt dat er maar 4 cytostatische middelen zijn met voldoende effectiviteit, gemeten als response rate (RR) van ≥ 20%, te weten doxorubicine (RR 43%), ifosfamide (RR 33%), methotrexaat (RR 32%) en cisplatin (RR 26%). Een meta-analyse toonde dat schema's met 2 middelen een significant slechtere overleving hadden (5-jaar event free survival [EFS] 48%, 5-jaar overall survival [OS] 62%) dan wanneer 3 of 4 cytostatische middelen gebruikt worden (EFS 58%, OS 70%). Maar er bleek geen verschil in overleving te zijn tussen schema's waarbij 3 of 4 middelen gebruikt worden. Daarom concluderen we dat de combinatie MAP optimale resultaten oplevert en als standaard behandeling voor het osteosarcoom in hedendaagse protocollen beschouwd moet worden. Een andere conclusie van deze studie was dat verder klinisch onderzoek met dergelijke combinaties cytostatica geen aanvullende waarde heeft, en dat de ontwikkeling van

nieuwe therapie gericht tegen het osteosarcoom gebaseerd moet zijn op een beter inzicht in het ontstaan en klinisch-biologisch gedrag van deze maligne bottumor.
Mede om deze reden zijn we een studie begonnen naar de gen expressie in osteosarcomen. Het doel van deze studie was om moleculaire mechanismen van deze tumor beter te kunnen begrijpen en de gen-expressieprofielen te koppelen aan overleving en histologische response op chemotherapie. Gen-expressieprofielen van diagnostische biopten van 25 hooggradige osteosarcomen werden onderling vergeleken en met niet-maligne cellen, namelijk mesenchymale stamcellen die beschouwd worden als cellen waar het osteosarcoom uit ontstaat, osteoblasten en (benigne) osteoblastomen. De resultaten van deze studie worden beschreven in **hoofdstuk 3**. Het bleek niet mogelijk om de genexpressie profielen te relateren aan de overleving of histologische response. Er waren ongeveer 3000 genen, die significant verschillend tot expressie kwamen in osteosarcomen vergeleken met de mesenchymale stamcellen of met osteoblasten. Op nivo van een signaalcascade (pathway) vonden we dat genen die bij de cel cyclus en de Wnt-signalering betrokken waren, significant verschillen in osteosarcomen. Zo bleek bij osteosarcomen de Wnt3a/β-catenine (canonical) signalering inactief (indirect bewijs: downregulatie van Axin en CCDN1) te zijn, de alternatieve, Wnt5a signalering is overactief evenals genen die betrokken zijn bij de (regulatie van de) celcyclus en de p53/apoptose pathway is afunctioneel (indirect bewijs: downregulatie BBC3/PUMA). Wat deze bevindingen betekenen wordt in de paragraaf "Discussie" besproken.
Hoofdstuk 4 gaat verder in op de statistische achtergrond van de zojuist genoemde array analyse. Dit hoofdstuk beschrijft een algoritme voor de analyse op genoom nivo, dat de Global test genoemd wordt. Bij de Global Test worden klinische variabelen, zoals histologische respons of overleving gerelateerd aan verschillende expressiepatronen van genen, die gegroepeerd worden in signaal transductie pathways. Van het verschil in expressie van de signaal pathways van osteosarcomen en niet kwaadaardige cellen wordt vervolgens de statistische significantie bepaald. De Global test is gebaseerd op het Cox-proportional hazard model en wordt gecorrigeerd voor co-variabelen. De analyse van de osteosarcomen samples lieten zien dat celcyclus genen, genen betrokken bij de DNA-repair en bij de apoptose geassocieerd waren met de overleving van de patiënten. Tenslotte kon ook nog geconcludeerd worden dat met behulp van de Global test gen expressie studies betere statistische resultaten lieten zien.
In **hoofdstuk 5** wordt een onderzoek naar de aanwezigheid van de epidermale groeifactor HER2 beschreven. Aanleiding hiertoe was een fase-2 onderzoek naar de waarde van trastuzumab (Herceptin) bij osteosarcomen (www.cancer.gov/clinicaltrials: MSKCC-99097 en COG-AOST 0121). Deze studie was gestart op grond van enkele publicaties welke HER2 overexpressie bij het osteosarcoom rapporteerden en daarmee suggereerden behandelen is met het monoclonale antilichaam trastuzumab, naar analogie van HER2 overexpressie bij mammacarcinoom. In de door ons uitgevoerde studie kon echter immunohistochemisch geen membraan overexpressie van de HER2 receptor aangetoond worden. Evenmin was er aanwijzing voor mRNA overexpressie (RT-PCR) of amplificatie (FISH) van het *HER2*-gen. Op basis van deze resultaten is er volgens ons geen rationale voor het gebruik van

trastuzumab. Tot dusver zijn er geen resultaten van de genoemde fase-2 studie gepubliceerd. Nadat met de micro-array studies de moleculaire complexiteit bij osteosarcomen was aangetoond vroegen we ons af of er op het klinische vlak ook nog aanwijzingen waren voor heterogeniteit van osteosarcomen. Om die vraag te beantwoorden wordt in **hoofdstuk 6** een studie beschreven naar de klinische en pathologische kenmerken van osteosarcomen van handen en voeten, een uitzonderlijke lokalisatie. In deze studie worden 40 patiënten geëvalueerd, waaruit blijkt dat deze lokalisatie slechts in 0.95% van alle osteosarcomen voorkomt. Bijzonder was dat deze patiënten ouder zijn bij diagnose (gemiddelde leeftijd van 42 jaar), vaker voorkomt bij mannen dan bij vrouwen (ratio 1.7:1) en een langere periode hebben, voorafgaande aan de definitieve diagnose. Ook is het osteosarcoom van handen en voeten in ongeveer 30% van de gevallen laaggradig, terwijl dit in 1-2% van de gevallen zo is bij osteosarcomen van de knie. De kans op overlijden na 5-jaar follow-up (FU) bij 30 goed gedocumenteerde patiënten was 20%, bij patienten met osteosarcoom van de hand lager (38% na 4 jaar FU) dan bij osteosarcomen in de voeten (11% na 2.5 jaar FU). Er waren geen patienten overleden in de groep osteosarcomen van lage of intermediaire maligniteitsgraad. De enige factor van significant prognostisch belang bleek de maligniteitsgraad te zijn. De conclusie was dan ook dat hooggradige osteosarcomen van handen en voeten dezelfde prognose hebben als osteosarcomen van de knie of schouder en als zodanig behandeld moeten worden.

Hoofdstuk 7 van dit proefschrift gaat over osteosarcoom met pulmonale metastasen. Bij diagnose (synchrone metastasen) worden in 16% van de gevallen pulmonale metastasen gezien; een recidief (metachrone metastasen) komt vrijwel altijd als eerste in de longen voor. De overall survival van patienten die behandeld waren, was 23%. Het bleek dat overleving van de patiënten uitsluitend bepaald werd door de operabiliteit van de metastasen, zelfs als dit meerdere malen noodzakelijk is. Er bleek geen significant verschil in overleving te zijn tussen patiënten met synchrone of metachrone metastasen. Prognostische factoren zijn het aantal metastasen (minder dan 5 of 5 en meer), mate van necrose van de metastasen en vrouwelijk geslacht. Bij patiënten met metachrone metastasen was het ziektevrij interval nog van prognostisch belang. Chemotherapie bleek geen significante prognostische factor te zijn, hoewel een trend voor betere overleving na chemotherapie gezien werd. De eindconclusie van deze studie is dat genezing bij patiënten met pulmonale metastasen bereikt kan worden met herhaalde resectie, en dat de rol van conventionele chemotherapie hierbij nog onvoldoende aangetoond is.

DISCUSSIE

De conclusie die uit de besproken hoofdstukken getrokken kan worden is dat het osteosarcoom biologisch een heterogene en complexe tumor is. Doordat er geen eenduidige moleculair-biologisch kenmerk is van het osteosarcoom stagneert de ontwikkeling van nieuwe medicijnen tegen deze ziekte. Ook het klinisch gebruik van geneesmiddelen bij osteosarcoom is gelimiteerd, zoals we hebben gezien, waarbij het repertoire aan chemotherapeutische mogelijkheden vooral bij de behandeling van een recidief osteosarcoom ernstig beperkt is. Dit wordt mede in de hand gewerkt doordat bij de primaire behandeling alle effectieve middelen al gebruikt zijn, en bij opnieuw gebruiken cumulatieve toxiciteit een belangrijke rol speelt, zoals cardiale toxiciteit, gehoor- en nierschade en fertiliteitsproblemen. Wij hebben geen HER2 overexpressie op osteosarcoomcellen kunnen aantonen, wat noodzakelijk is voor de behandeling met de monoclonale antistof trastuzumab (Herceptin). Uit de array studie kwamen als moleculaire aangrijpingspunten de cel cyclus regulatie, de p53/apoptose signalering en de complexe Wnt-signalering als belangrijkste naar voren. Om iets te begrijpen van de rol de deze deregulatie van de Wnt signalering bij osteosarcomen speelt, volgt er een korte samenvatting van wat er nu bekend is over Wnt-signalering in mesenchymale stamcellen, de voorloper cellen van osteoblasten en osteocyten en bij maligniteiten in het algemeen, en osteosaroom in het bijzonder.

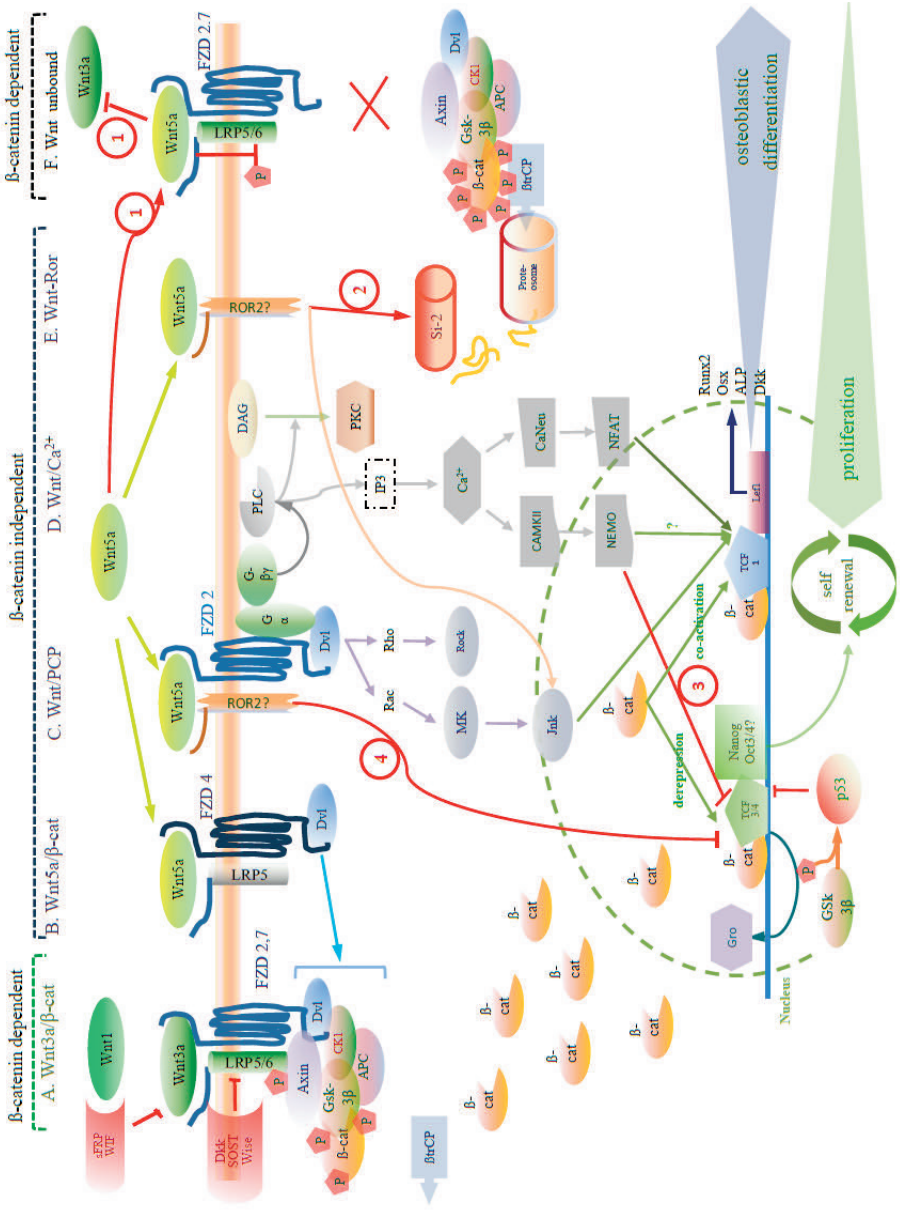
De Wnt-signalering

Wnts (afkorting van Wingless in Drosophila en hetzelfde gen Int1 in muizen) vormen een belangrijk signaleringssysteem die een belangrijke rol in de ontwikkeling, differentiatie en weefselherstel hebben (zie voor uitgebreide reviews de referenties in de Engelse versie samenvatting). Door het groot aantal liganden (signaaleiwitten, n=19), de verscheidenheid aan mogelijke receptoren (Frizzled's (Fzd), n=10) receptoren en co-receptoren (Lrp's, Ror en Ryk, n=8) zijn er talloze reacties mogelijk na ligand-receptor binding. Daar komt nog dat Wnt remmers, zoals Dickkopf's (Dkk), Wnt-inhibiting factors (WIF's), soluble Frizzled related proteins (sFrp's) en extracellulaire eiwitten zoals proteoglycanen de respons kunnen moduleren afhankelijk van het type weefselcel en het stadium van ontwikkeling (contex-dependent). Dat betekent dat het effect van Wnt-signalering in de beenmergniche (reservoir mesenchymale stamcellen) anders is dan dezelfde signalering in kraakbeen, bijvoorbeeld van de metafyse van lange pijpbeenderen (endochondrale botvorming), of de kraakbeenkern van platte beenderen (membraneuze botvorming). Door nieuwere inzichten in de Wnt-signalering is de oudere indeling in canonical versus non-canonical pathway vervangen door respectievelijk het β-catenine afhankelijk en β-catenine onafhankelijk of alternatief systeem. Figuur 1 geeft een overzicht van beide signaleringssystemen en enkele onderlinge verbanden. In het kort komt het erop neer dat het Wnt3a/catenine afhankelijke systeem na Wnt-reptor binding het intracellulaire β-catenine stabiliseert, doordat proteosomale afbraak ervan (Fig.1 F) verhinderd wordt (Fig.1 A). Door de stijgende cytoplasma concentratie komt het β-catenine in de kern, waar het of proliferatie aanzet (de-repression) of differentiatie induceert, door

bijvoorbeeld co-activation van de transcriptiefactor Runx2, wat noodzakelijk is voor de osteogenese.
In het β-catenine onafhankelijke (alternatieve) systeem wordt via het ligand Wnt5a de transcriptiefactor Jun-N-terminal Kinase (Jnk) geactiveerd, dat een rol speelt bij de groei van tumoren (*Wnt/PCP pathway*). Een tweede *activatie* route maakt intracellulair calcium (Ca^{2+}) vrij, wat vervolgens Ca^{2+}-gemedieerde transcriptie activatie geeft, via NEMO of NFAT (*Wnt/Ca2+ pathway*). De alternatieve signalering via Wnt5a kan het catenine afhankelijke systeem op een aantal manieren remmen (zie rode cirkels).
Wnt signalering speelt een grote rol bij de normale botvorming, maar is nog niet tot in alle details opgehelderd. De Wnt-signalering heeft een stadium afhankelijke functie in de mesenchymale stamcel (MSC), die tegengestelde effecten hebben (Figuur 2). In de MSC is de aanwezigheid van β-catenine bepalend voor de daarop volgende richting van de cellijn-ontwikkeling (fate-decision). Het stimuleert de richting naar bot-/kraakbeen, maar het remt de vetcelontwikkeling. Tijdens het daaropvolgende stadium van de osteo-chondrogene voorlopercel remt de aanwezigheid van β-catenine de ontwikkeling van de kraakbeenlijn, en stimuleert de aanzet tot differentiatie naar osteoblast en osteocyt. Maar voordat deze differentiatie definitief kan doorzetten, moet de Wnt3a/β-catenine signalering uitgezet worden, wat via de Wnt-antagonist Dkk1 gebeurt. Het Wnt3a/β-catenine heeft dus 3 functies bij de botvorming: 1. keuze voor de (osteo-chondrale) cellijn ontwikkeling, 2. proliferatie van osteo-chondrale voorlopercellen en 3. definitieve differentiatie inductie tot bot en remming van kraakbeenvorming.
Wnt5a speelt hierbij een rol doordat het de Wnt3a/β-catenine signalering kan remmen, maar zowel de proliferatie als differentiatie van osteoblasten stimuleert.

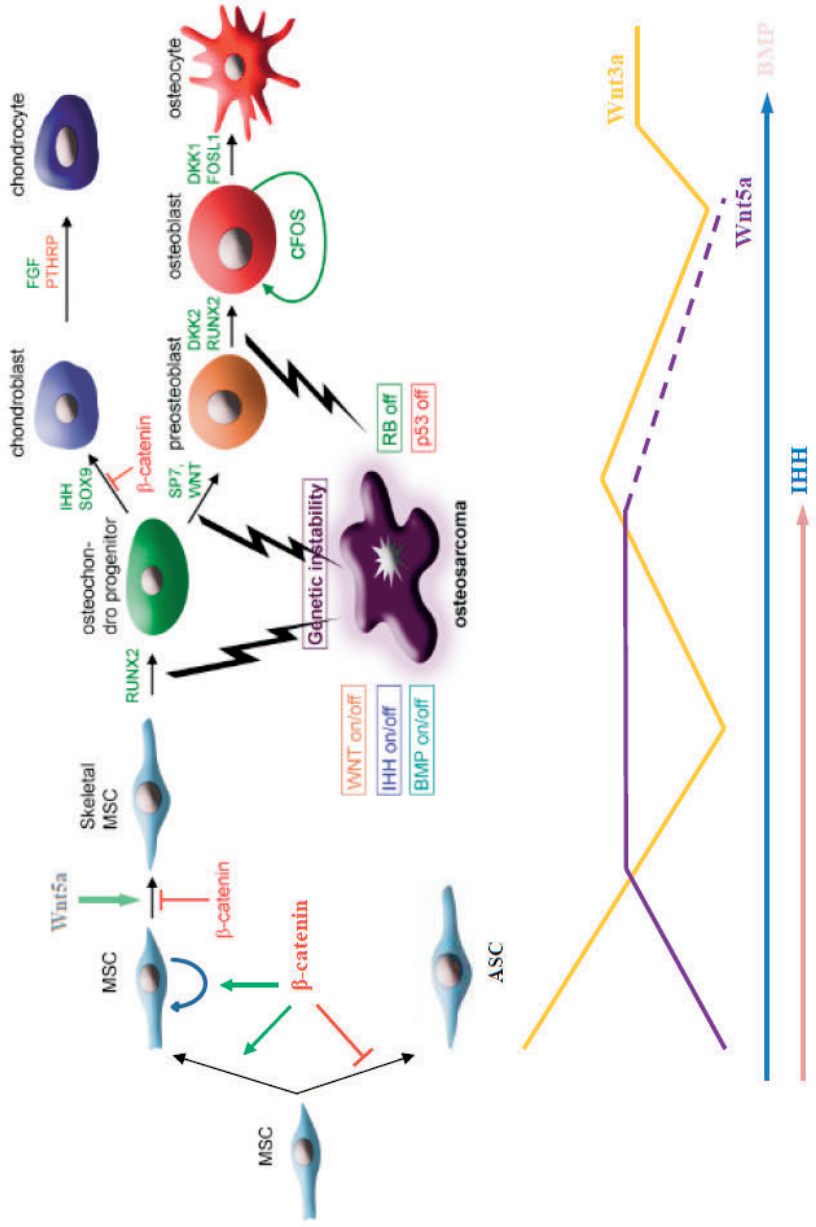
FIGUUR 1.
Wnt signalering

In deze figuur worden een aantal Wnt signalering pathways getoond en cross-talks tussen het β-catenine afhankelijke ("canonical", kolom A) systeem en het β-catenin onafhankelijke of alternatieve ("non-canonical", kolom B-E) systeem. Het geactiveerde Wnt3a/β-catenine systeem ("on") in kolom A en de β-catenin repressive ("off") situation staat in kolom F weergegeven.Voor uitgebreide uitleg en referenties wordt naar de tekst verwezen. Recent is het Wnt-Ror2 pathway toegevoegd aan de 2 andere alternatieve pathways: het Wnt/PCP/Jnk en Wnt/Ca2+ pathway. Het Wnt-Ror2 pathway remt het canonical pathway op 3 nivo's: door competitive remming met de Wnt3a receptor binding (rode cirkel 1), door toegenomen, Siah-2 gemedieerde β-catenine afbraak (rode cirkel 2), door onderdrukking van TCF3/LEF transcriptie complex via NEMO (rode cirkel 3) of rechtstreekse β-catenine/TCF remming. Een andere modulatie van beide Wnt-signaleringsroutes is de activatie van β-catenin via het non-canonical systeem. Als Wnt5a bindt met de Frizzled4 (Fzd4) receptor kan signaaltransductie via β-catenin verlopen (kolom B). De complexe uitkomst van de Wnt-signalering wordt dus bepaald door de binding van diverse liganden (Wnt3a/canonical Wnts's en Wnt5a/non-canonical Wnts) met de verschillende receptoren (Fzd's) en de modulatie hiervan door de aanwezigheid van co-receptoren (LRP's en ROR's), remmers (Dickkopf [Dkk], Sclerostin [SOST], Wnt-Inhibiting Factor [WIF] en Soluble Wnt Related Proteins [sFrp's] en extracellulaire matrix.
LRP5,6 lipoprotein related protein 5,6; Dvl Dishevelled; APC Adenomatous Polyposis Coli; GSK-3β Glycogen Synthase Kinase-3β; CK1 Casein Kinase-1α;CamKII Calcium/Calmoduline dependent Kinase II; JNK c-Jun N-terminal Kinase; Rock2 Rho-associated Kinase; β-TrCP is a ubiquitin ligase; TCF1/3 T-cell factor 1/3; Lef1 Lymphoid Enhancer Factor 1; Gro Groucho.


β-catenin dependent
A. Wnt3a/β-cat
B. Wnt5a/β-cat
β-catenin independent
C. Wnt/PCP
D. Wnt/Ca2+
E. Wnt-Ror
β-catenin dependent
F. Wnt unbound
Wnt1
Wnt3a
sFRP
WIF
Dkk-
SOST
Wise
LRP5/6
FZD 2,7
Axin
Gsk-
3β
β-cat
CK1
APC
βtrCP
Wnt5a
FZD 4
LRP5
Dvl
FZD 2
ROR2?
G
α
G-
βγ
PLC
DAG
PKC
IP3
Ca2+
CaNeu
NFAT
CAMKII
NEMO
Rho
Rac
Rock
MK
Jnk
Sir-2
Proteo-
osome
β-
cat
co-activation
derepression
Nanog
Oct3/4?
TCF
3/4
TCF
1
Lef1
Runx2
Osx
ALP
Dkk
osteoblastic
differentiation
proliferation
self
renewal
Gro
p53
GSk
3β
Nucleus
1
2
3
4

FIGUUR 2.
Wnt-signaling in diverse stadia van de osteoblasten ontwikkeling.

De hypothetische expressie van het Wnt3a/β-catenine (canonical, oranje) systeem wordt weergegeven door Wnt3a, en het Wnt/β-catenine onafhankleijk systeem (non-canonical, paars) door Wnt5a. Het Wnt3a/β-catenin is noodzakelijk voor vroege inductie van de mesenchymale stamcel (MSC) richting osteochondrale cellijn, waarbij de vetcelontwikkeling (adipogene stem cell = ASC) geremd wordt. Tevens is het Wnt3a/β-catenine systeem noodzakelijk voor proliferatie van de osteochondrale progenitors, remming van de kraakbeen (chondrogene) ontwikkeling en activatie van de Runx2-transcriptiefactor, master gene voor de definitieve osteogene ontwikkeling. Voor de voortgang van de osteogene ontwikkeling moet Wnt3a/β-catenine geremd worden, waarschijnlijk gemedieerd via Wnt5a en Dkk1, die het β-catenine systeem kunnen remmen. Voor de terminale ontwikkeling van de osteoblast tot osteocyt moet het Wnt3a/β-catenine systeem weer tot geactiveerd zijn. Verstoring van deze onderlinge samenwerking in de normale osteogenese kan aanleiding zijn voor ongecontroleerde proliferatie en ontbreken van controle (differentiation commitment check) op differentiatie, waardoor toenemende chromosomale instabiliteit ontstaat, een kenmerk van osteosarcoom. Bij de normale botontwikkeling spelen andere signalerings systemen, zoals het Indian Hedgehog (IHH) en de Bone Morphogenetic Proteins (BMP) spelen bij de osteogenese een belangrijke rol, die echter nog onvolledig opgehelderd zijn.


Wnt5a
MSC
β-catenin
β-catenin
Skeletal MSC
MSC
ASC
RUNX2
osteochon-dro progenitor
IHH SOX9
β-catenin
chondroblast
FGF PTHRP
chondrocyte
SP7, WNT
preosteoblast
DKK2 RUNX2
osteoblast
DKK1 FOSL1
osteocyte
cFOS
Genetic instability
RB off
p53 off
osteosarcoma
WNT on/off
IHH on/off
BMP on/off
Wnt3a
Wnt5a
BMP
IHH

Wnt signalering, maligniteit en osteosarcoom

In onze array studie vonden we aanwijzingen voor een ontregeld Wnt signaleringssysteem, waarbij de Wnt3a/β-catenine signalering in osteosarcomen verlaagd tot expressie kwam, en het alternatieve, Wnt5a/β-catenine onafhankelijk systeem juist tot overactief was, vergeleken met mesenchymale stamcellen of benigne bottumoren, osteoblastomen. Dit is precies tegenovergesteld dan de activerende, oncogene rol, die β-catenine speelt bij epitheliale tumoren, zoals colon carcinoom, ovarium carcinoom, prostaat-of longkanker. Maar ook bij osteosarcomen heeft men gevonden dat er β-catenine overactiviteit is, en dus een oncogene rol heeft. Men kan zich wel afvragen hoe in andere situaties waarbij wel overactiviteit van nucleair β-catenine aanwezig is, zoals het Gardner syndroom of bij osteoblastomen, geen osteosarcoom optreedt, als het Wnt3a/β-catenine als een oncogeen gezien moet worden.
Er is maar 1 studie bij osteosarcoom bekend, waarbij geen nucleair β-catenine gevonden werd, dus het Wnt3a/β-catenine inactief is, net als in onze studie. Er zijn andere maligniteiten waarbij een inactief β-catenine systeem gecorreleerd wordt aan de pathogenese van kanker. Bij een hooggradige ongedifferentieerd pleomorfe sarcomen werd aangetoond dat de mesenchymale stamcel de voorlopercel was voor het ongedifferentieerd sarcoom, en dat door uitschakeling van het Wnt/β-catenine systeem de voorlopercel niet verder kon differentiëren tot bindweefsel. Ook was de Wnt5a isgnalering uitgeschakeld in deze tumoren, waardoor een commitment-viability checkpunt uitviel, waardoor cellen ongecontroleerde, ongedifferentieerde groei vertonen, wat leidt tot de vorming van het sarcoom. Bij cellijnen van het embryonale rhabdomyosarcoom werd gevonden dat de Wnt/β-catenine verlaagd tot expressie kwam, en dat re-activatie hiervan tot spiercel differentiatie leidde. Tevens vonden de onderzoekers dat de verlaagde Wnt/β-catenine signalering gepaard ging met resistentie tegen apoptose, wat de differentiatie tot spiercellen blokkeerde. Een laatste voorbeeld van Wnt/β-catenine inactivatie is beschreven bij het retinoblastoom en na reactivatie zag men een p53 gemedieerde proliferatie stop van de retinoblastoomcellen.
Inactiviteit van het β-catenine systeem werd door Cai et al. van ons lab bevestigd, doordat geen kernaankleuring van het β-catenine gezien werd en de Wnt-luciferase reporter studies van de β-catenine responsieve genen in de osteosarcoom cellijnen negatief waren.
Als argument dat inactivatie van de Wnt3a/β-catenine signalering bijdraagt aan de ontwikkeling van het osteosarcoom, kan aangevoerd worden dat remming van GSK3β, dat het β-catenine fosforyleert, waardoor het afgebroken kan worden, in 2 van de 4 osteosarcoma cellijnen differentiatie laat zien tot bot.
Wat dan precies de rol is van de disregulatie van de Wnt-signalering bij het ontstaan van osteosarcoom is niet makkelijk te begrijpen. De hypothese is dat door uitschakeling van de Wnt3a/catenine signalering geen differentiatie meer geïnduceerd kan worden en de voorloper cellen in een permanente proliferatie blijven. Het overactieve Wnt5a leidt tot activatie van de osteogenese, die niet tijds-gesynchroniseerd is met de (gestoorde) differentiatie. Omdat hierbij geen goede differentiatie commitment controle meer is en de apoptose a-functioneel is (zoals uit de pathway analyse is gebleken) ontstaat er in toenemende mate instabiliteit van het genoom, wat het kenmerk is van osteosarcoom (zie figuur 2). Bij 75% van de osteosarcomen is

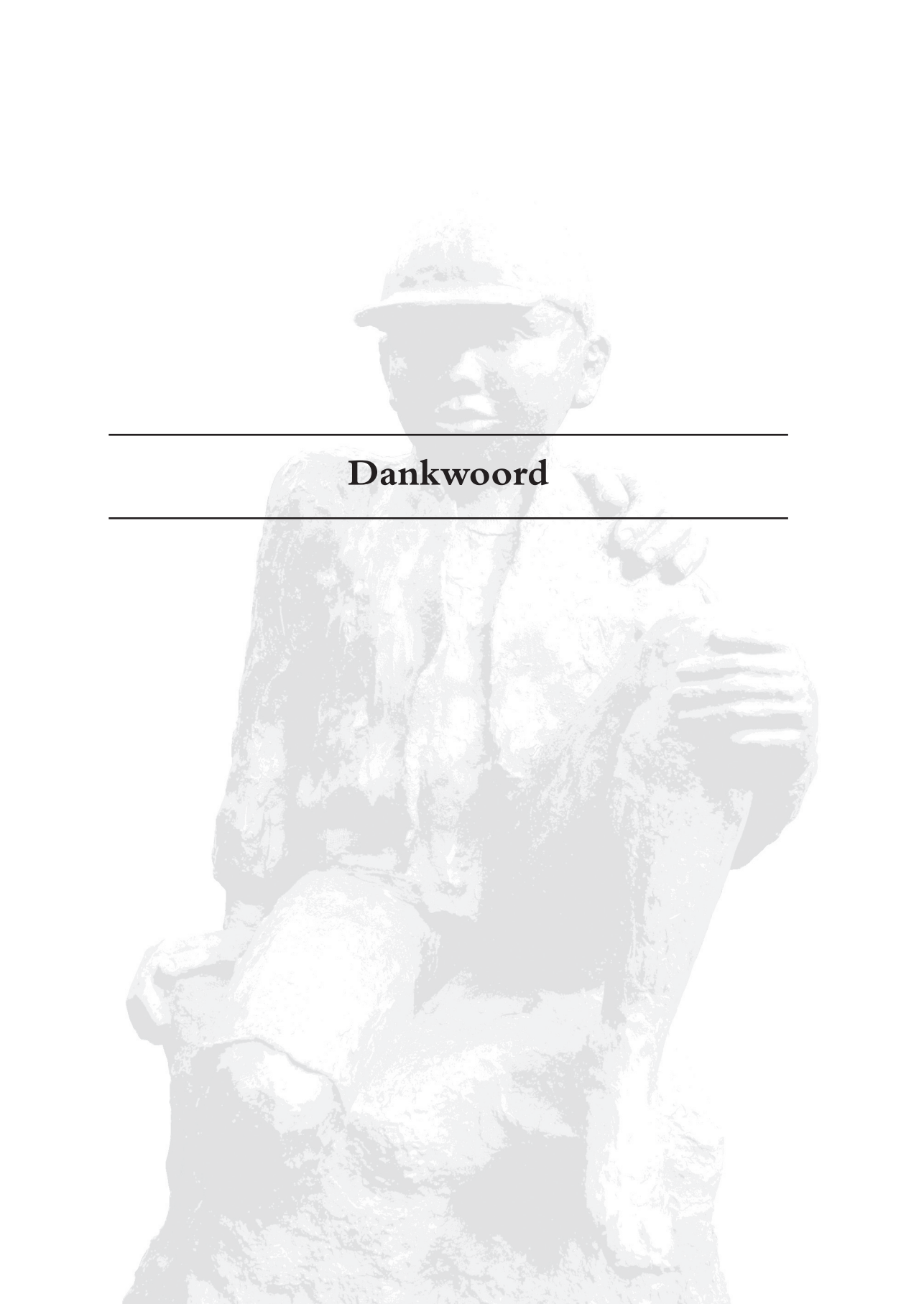
overexpressie van het Wnt5a-ROR2 systeem gevonden, die gecorreleerd was met prognostisch slechtere ziekte. In cellijnen werd Wnt5a overexpressie gecorreleerd met meer invasieve groei. Concluderend zijn er aanwijzingen dat de Wnt3a/β-catenine en het alternatieve Wnt5a-systeem een belangrijke rol spelen bij de tumorgenese van het osteosarcoom. Deze hypothetische voorstelling van de tumorgenese van het osteosarcoom moeten nog wel door studies bevestigd worden. Hierbij zouden de Wnt-pathways en andere signaleringssystemen bij de normale en abnormale osteogenese, zoals bijvoorbeeld het osteosarcoom, naast elkaar bestudeerd moeten worden. De inzichten die hierbij verkregen worden kunnen ons verder kunnen helpen om een exacte beschrijving te kunnen geven van de aard van de disregulatie van deze systemen bij osteosarcoom. De verwachting is dat hierdoor nieuwe targets voor de behandeling van osteosarcoom gevonden worden.

Wnt signalering en potentiele therapie

Gezien de inactivering van het Wnt3a/β-catenine systeem en de bevinding dat bij re-activering differentiatie gezien werd in osteosarcoma cellijnen, kan opgevoerd worden dat medicamenteuze therapie, gericht op remmen van de proteosoom gemedieerde degradatie van β-catenine een effect zou kunnen hebben op patienten met een osteosarcoom, waarbij de Wnt signalering uitstaat. Een van de middelen die hiervoor in aanmerking zou komen is de proteosoom remmer bortezomib. Dit middel heeft bij multiple myeloom patiënten aangetoond herstel van de normale botaanmaak, ongeacht de respons op het myeloom. Hoewel het mechanisme achter het botherstel niet geheel opgehelderd is, denkt men dat bortezomib de Wnt3a antagonist Dkk1 remt, of differentiatie inductie via stabilisatie van β-catenine veroorzaakt, of de tumor cellen in apoptose brengt. Een onderzoeker vond regressie van het osteosarcoom in een muizen model, dat behandeld was met bortezomib, waarbij de proliferatie van de cellen geremd werd, en de maligne cellen toegenomen apoptose vertoonden. bortezomib was veilig in fase-1, fase-2 en fase-3 studies, werd goed verdragen en was weinig toxisch. Zelfs in combinatie met andere cytostatische middelen is bortezomib veilig gegeven. Daarom zou bortezomib een van de weinige middelen zijn die in de toekomst geëvalueerd zouden kunnen worden bij het osteosarcoom met inactieve Wnt/β-catenine signalering.

Samenvattend kan worden geconcludeerd dat hoog-gradig osteosarcoom op klinisch en moleculair nivo een heterogene ziekte is. Dit is mede oorzaak dat er de laatste 3 decennia geen vooruitgang geboekt is op het gebied van de behandeling. Om deze ziekte goed te behandelen is een combinatie van tenminste 3 middelen in de vorm van neo-adjuvante chemotherapie noodzakelijk samen met radicale chirurgie. Dit geldt voor alle osteosarcomen, ongeacht de lokalisatie in het skelet en of er sprake is van primair gemetastaseerde ziekte. Patiënten met een operabel recidief dienen chirurgische resectie als primaire therapie te krijgen, waarbij de toegevoegde waarde van neo-adjuvante chemotherapie niet bewezen is. In dit proefschrift is aangetoond dat er geen nieuwe ontwikkeling op gebied van conventionele cytostatische medicamenten verwacht kan worden, en moet men patiënten met refractaire

ziekte fase-I en fase-II studies aanbieden. Patiënten met een hooggradig osteosarcoom van de handen of voeten dienen overeenkomstig hooggradige osteosarcomen elders in het lichaam behandeld te worden. Bij niet-hooggradige osteosarcomen van deze lokaties kan alleen chirurgische resectie overwogen worden. Het gebruik van monoclonale antilichamen tegen de HER2 receptor kan op grond van onze onderzoekresultaten niet ondersteund worden. Bij de verdere ontwikkeling van nieuwe geneesmiddelen tegen het osteosarcoom neemt het onderzoek naar signaleringssystemen zowel bij de normale als verstoorde osteogene differentiatie een belangrijke plaats in, zoals bijvoorbeeld de Wnt-signalering. Beter inzicht in deze regulatie geeft ook de mogelijkheid osteosarcomen te behandelen op grond van deze inzichten, zoals bijvoorbeeld proteosoom remmers.



Dankwoord

Voordat ik me tot mijn promotoren en co-promotor richt, die ieder hun specifieke bijdrage hebben geleverd aan het tot stand komen van dit proefschrift, wil ik degenen bedanken die mij op een heel speciale manier hebben bijgestaan.

Als eerste wil ik Saskia bedanken voor haar rotsvast geloof in een goede afloop en het verdragen van mijn buien, als het even niet mee zat. Je bent er altijd geweest om mij te ondersteunen, uit te dagen, mijn werk te spiegelen, mijn taal aan te passen en te zorgen dat ik verder ging met schrijven. Ik hoop nog lang met jou door te gaan.

Een bijzondere dank wil ik uitspreken aan alle kinderen met een osteosarcoom, die mij hun leven hebben toevertrouwd. Die mij hebben leren luisteren naar hun verhaal, over hun ziekte, hobbies, sport, scholing en hun angst. Vaak was er hoop, soms blijdschap, maar ook verdriet, boosheid, wanhoop en berusting dat hun leven en toekomst niet eindigde, zoals ze gewild hadden. Juist dat verhaal is voor mij de bron geweest om nieuwsgierig te onderzoeken wat wij als team hen meer konden bieden. Hun verhalen laten mij niet meer los, geven zin aan al mijn stappen in de medische wereld.

Professor dr. Egeler. Beste Maarten, je hebt me binnengehaald in het LUMC en ik wil je vooral bedanken voor de kansen die je me hebt gegeven. Het zal je niet meegevallen zijn om mijn ongebreidelde zucht naar allerlei andere interessante onderwerpen in te dammen en me te helpen focussen op het schrijven. Je hebt me de wegen laten zien die ik moest bewandelen om samen te werken aan een gemeenschappelijk doel, dat vind je zo belangrijk in ons vak.

Professor dr. Hogendoorn. Beste Pancras, ook jou wil ik hartelijk danken voor het vertrouwen dat je mij gegeven hebt, ook gedurende een periode, waarin ik veel met andere zaken bezig was. Ik ben je zeer erkentelijk voor de wijze waarop je je voor mij hebt ingezet, vooral de laatste tijd. Je bent nog steeds een voorbeeld voor mij om het belang te benadrukken van een verbintenis tussen kliniek, radiologie, pathologie en degenen, die zich bezighouden met wetenschappelijk onderzoek. Dat zal ik niet vergeten.

Dr. Cleton-Jansen. Beste Anne-Marie, je zult je vaak afgevraagd hebben waarom die artsen zich niet gewoon alleen maar met mensen bezighouden in plaats van ook zich te mengen in moleculair biologische zaken, waarvoor ze helemaal niet in de wieg gelegd zijn. Je hebt mij geleerd eerst goed te kijken, te vragen wat je niet weet en dan met gepast enthousiasme conclusies te trekken. Ik hoop nog lang met je te kunnen nadenken over bottumoren.

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Mijn dierbare vader, dat je dit nog mee mag maken vind ik een groot voorrecht. Ik deed niet altijd wat jij wilde, maar wanneer ik eenmaal mijn weg gekozen had, dan voelde ik me gesteund door je. Natuurlijk ook mijn zus en zwager, die vaak extra inzet hebben getoond op de momenten dat ik mijzelf weer terugtrok, en dat zonder mopperen.

Ook wil ik nog een aantal mensen kort bedanken die een speciale bijdrage hebben geleverd aan mijn persoonlijke groei en carriere op de afdeling IHOBA.

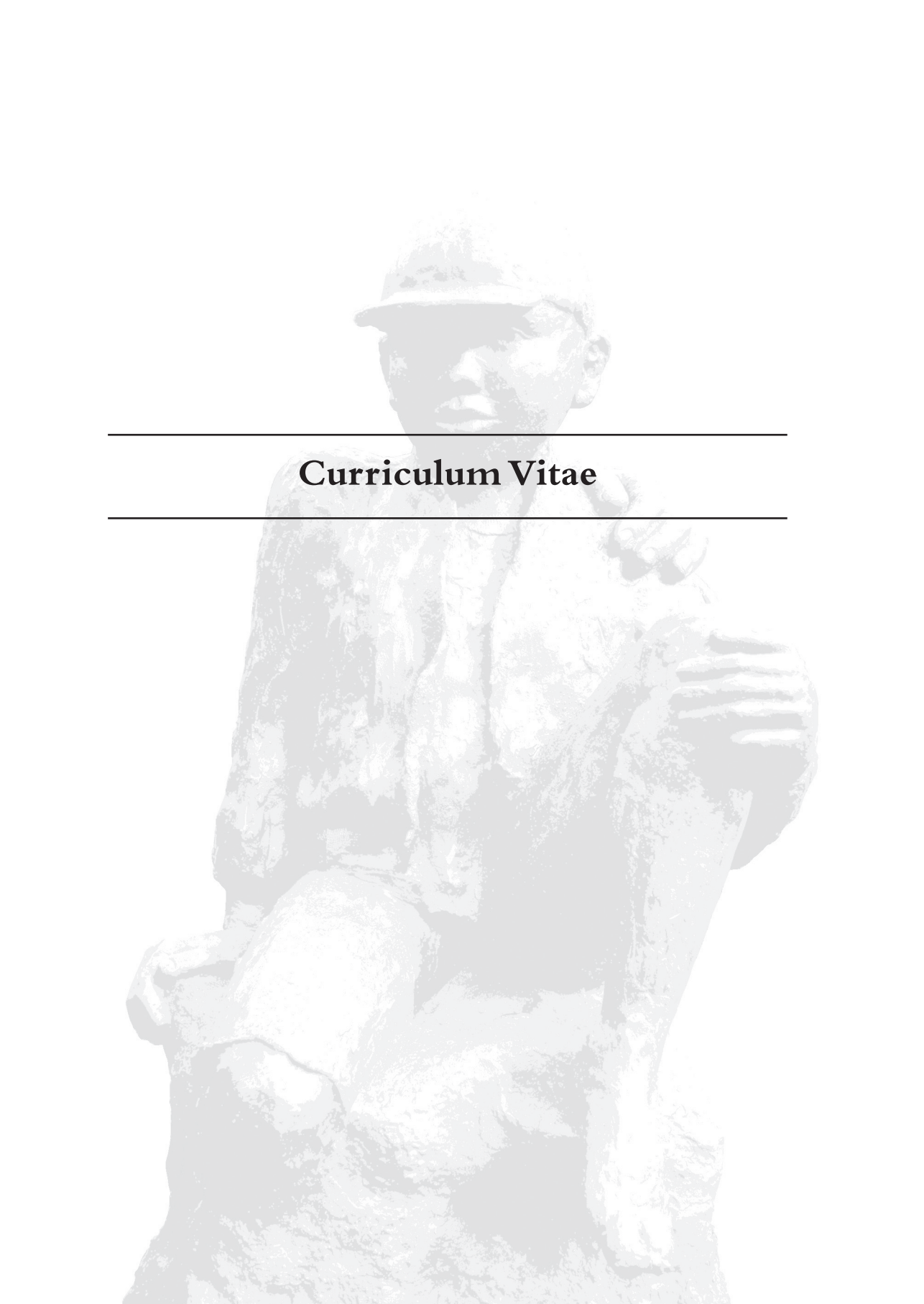
Dr. Frans Smiers, mijn kamergenoot en paranimf. Jouw betrokkenheid bij ons vak, bij mijn privé leven, je soms overdonderende kritiek, maar ook je waanzinnig enthousiasme over elke volgende hobbel die weer genomen was, zijn een warme deken geweest en behulpzaam om dit proefschrift te schrijven. Onze discussies waren pittig, maar je oprechtheid blijft voor mij een voorbeeld.

Dr. Wouter Kollen, mijn overbuurman en paranimf. Jouw onverstoorbaarheid en visie op mijn handelen hebben ertoe geleid dat je een fijne sparring-partner voor me bent. Je laat me andere mogelijkheden zien, bent altijd ondersteunend, stimulerend en je waardeert me zoals ik ben.

Professor dr. Antoni Taminiau, je hebt me geleerd dat werken in de orthopedische oncologie teamwork is. Jij vertelde vaak de diagnose aan de patiënten, en daarna vertrouwde je mij de rest toe. Jij hebt me ook laten zien dat je door je menselijke benadering altijd contact kunt krijgen met de patiënten, hoe jong ook of hoe ongunstig jouw boodschap soms moest zijn. Je bent voor mij het levend bewijs dat je meesterlijk vakmanschap, enthousiasme voor je werk en bemoedigende menselijkheid kunt uitdragen.

Dr. Peter Bekkering, jouw onvermoeibaar enthousiasme voor de kinderen die je geholpen hebt met revalideren is meer dan bemoedigend geweest. De mogelijkheden die je bedenkt om hen weer letterlijk op de been te krijgen dwingen mijn respect af.

Verder wil ik iedereen bedanken die niet bij name genoemd is, maar direkt of indirekt een bijdrage geleverd heeft door steeds weer belangstelling te tonen, te vragen en uit te dagen. Door talloze gesprekken met vrienden, familie, mijn collega's, verpleegkundigen, ouders van patiënten en anderen op de afdeling kinderoncologie,heb ik mijn passie kunnen voeden.



Curriculum Vitae

Jakob Klaas Anninga werd op 1 april 1955 in Holwierde, Groningen, geboren. Hij ging op het Na het behalen van het HBS-B diploma aan het Christelijk Lyceum te Apeldoorn in 1973 begon hij aan de opleiding fysiotherapie in Deventer, die in 1978 succesvol afgerond werd. Na 2 jaar werken als fysiotherapeut begon hij in 1980 aan de studie geneeskunde aan de Vrije Universiteit te Amsterdam, waar het doctoraalexamen in 1986 behaald werd, en succesvol afgesloten met het artsexamen in 1988. Daarna ging hij onderzoek doen op de afdeling nucleaire geneeskunde van het Nederlands Kanker Instituut/Antoni van Leeuwenhoekhuis (NKI/AvL) te Amsterdam (Prof.Dr. C.A. Hoefnagel) en kwam hij in contact met Prof.Dr. P.A. Voûte, in verband met kinderen met neuroblastoom, die aldaar behandeld werden met 131I-Meta-iodo-benzylguanidine (131I-MIBG). Na een half jaar werken met deze therapie in het Southampton General Hospital in Engeland (prof.Dr. D. Ackery), begon hij in september 1989 aan de opleiding algemene kindergeneeskunde in het AMC/EKZ te Amsterdam, opleider Prof.Dr. C. de Groot en Prof.Dr. P.A. Voûte. Na het behalen van de specialisatie algemene kindergeneeskunde in september 1994 begon hij in maart 1995 aan een fellowship kinderoncologie aan de Katholieke Universiteit Nijmegen/het Radboud ziekenhuis. Dit fellowship werd in december 1997 afgesloten met een Locum Consultancy post gedurende 8 maanden in het Yorkshire Regional Center for Paediatric Haematology and Oncology te Leeds, Engeland, onder leiding van Prof.Dr. I. Lewis. Na een periode werkzaam geweest te zijn als algemeen kinderarts ging hij in maart 2001 werken als kinderoncologische in het Leids Universitair Medisch Centrum (LUMC). Behalve betrokken te zijn bij de allogene beenmergtransplantaties, was zijn belangstelling vooral gericht was op solide kindertumoren, in het bijzonder Beentumoren. Hij was als Principal Investigator voor Nederland betrokken bij de introductie en uitvoering van de EURAMOS-1 studie, een multinationale klinische trial voor osteosarcomen. Deze belangstelling voor het osteosarcoom was de basis tot het tot stand komen van dit proefschrift.