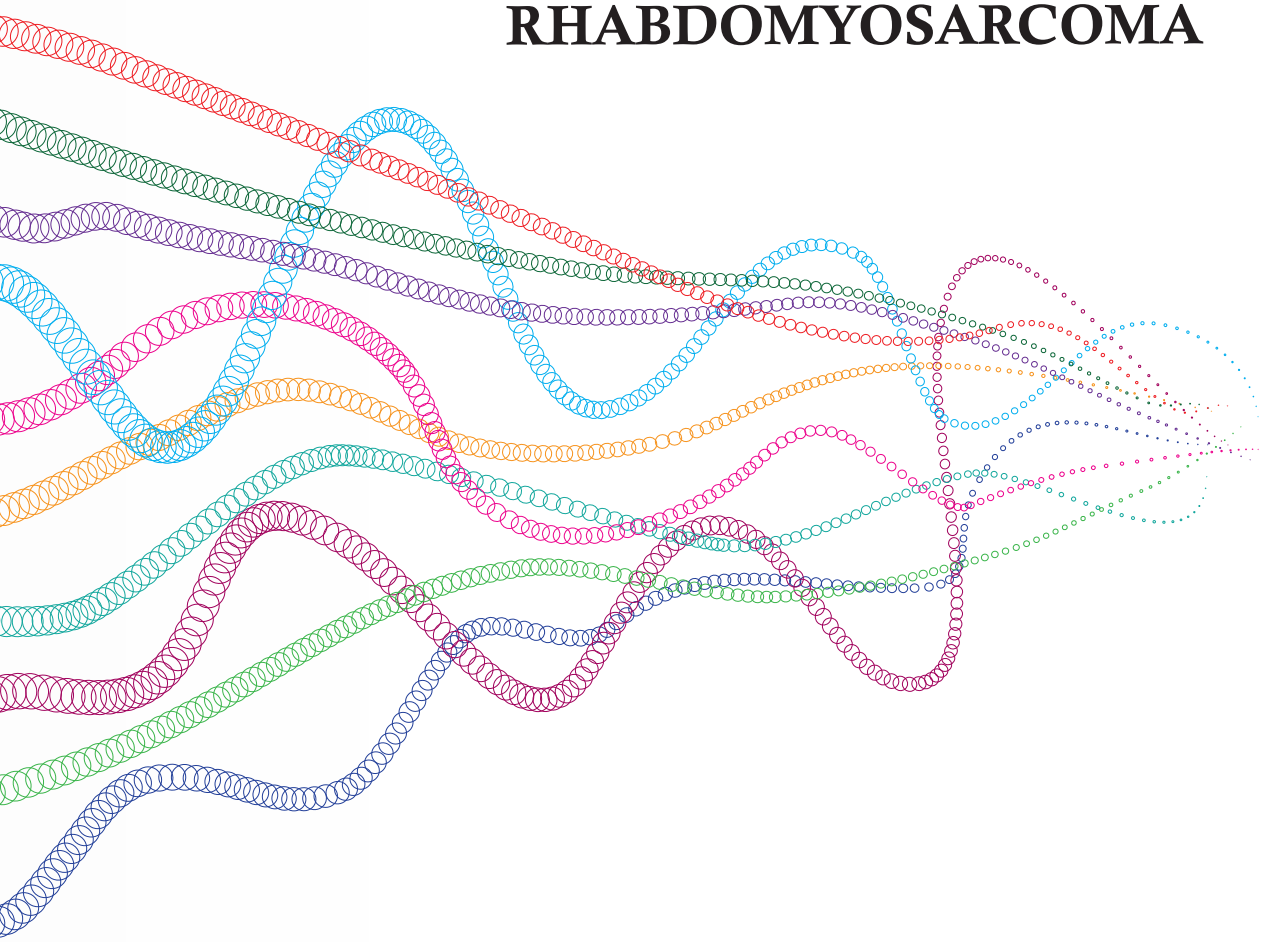
PATHWAYS AND CROSSROADS IN ADOLESCENT AND YOUNG ADULT ("AYA") CANCER, WITH EMPHASIS ON RHABDOMYOSARCOMA



Jorieke Carlijn van Gaal

PATHWAYS AND CROSSROADS IN ADOLESCENT AND YOUNG ADULT ("AYA") CANCER, WITH EMPHASIS ON RHABDOMYOSARCOMA

Carlijn van Gaal

Pathways and crossroads in adolescent and young adult ("AYA") cancer, with emphasis on rhabdomyosarcoma

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Aan mijn ouders,

"True colors are beautiful"

Phil Collins

Paranimfen:
Margot Geerdink
Melissa Roeffen

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PREFACE

The world-wide incidence of cancer increases, and this also holds for the incidence in adolescents and young adults (AYA). For the entire cancer population, much attention is paid to logistics and infrastructure of treatment of cancer patients, while the model of centralization of pediatric oncology has been active since decades. In contrast, the knowledge and awareness of the "AYA" cancer population was very limited up to recently, which was referred to as the AYA gap in cancer care. A few world-wide and local initiatives (e.g. development of Children's Oncology Group AYA committee, opening of a number of AYA oncology units) have been undertaken to increase the awareness and facilitate centralization of care for this particular population of cancer patients with their specific needs.

Two population-based epidemiological studies focusing on the AYA cancer population in the Netherlands are described to provide the fundamentals for the optimization of cancer care for this population in the Netherlands. Furthermore, this knowledge on incidence and survival is useful in the development of international initiatives, such as the design of collaborative studies. It furthermore provides essential population-based data on the incidence of second tumours in this cohort. These studies were conducted in collaboration with the Comprehensive Cancer Centre North-Netherlands (CCCN, E. Bastiaannet), and the Netherlands Cancer Registry (NCR, K. Aben).

The following chapters of this thesis focus on of this thesis focuses on rhabdomyosarcoma (RMS) as an ultimate example of a tumour type overlapping the pediatric and AYA population. The first study provides a multi-centre retrospective study (1977-2009) which adds data to the negative prognostic effect of age on outcome in embryonal and alveolar RMS. This study was conducted in a collaboration between three medical centres in the Netherlands; the department of pediatric oncology (E. de Bont) and medical oncology (W. van der Graaf) at the University Medical Center Groningen (UMCG), the department of medical oncology (S. Sleijfer) at the Erasmus Medical Center Rotterdam, and the department of pediatric oncology (J. Loeffen) and medical oncology (Q. van Hoesel, W. van der Graaf) at the Radboud University Nijmegen Medical Centre (RUNMC).

As survival rates for older patients as well as for particular high risk RMS subgroups remain poor and the aggressive multi-modality treatment regimens induce substantial late effects, we subsequently investigated novel potential treatment targets (ALK and IGF-1R) in RMS patient tumour samples and *in vitro* models. Tumour samples were collected from the previously described cohort, with additional samples collected via PALGA, the nationwide network and registry of histo- and cytopathology in the Netherlands. Diagnoses of all samples were confirmed by U. Flucke (department of Pathology, RUNMC) and all investigations were conducted by the preclinical sarcoma research group ((Y. Versleijen-Jonkers, W. van der Graaf) of the department of medical oncology, which is embedded in the department of Pathology of the RUNMC, head: J. van Krieken).

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CHAPTER 1

General Introduction

GENERAL INTRODUCTION

Although cancer in children, adolescents and young adults (AYA) is relatively rare in comparison to the overall burden of cancer incidence, a world-wide increase in incidence of cancer and cancer-related deaths in young people is emerging (1). This is reflected by the fact that in children 1-4 years, cancer represents the fourth most common cause of death, in children 4-15 years it is the second most common cause of death (2) and in adolescents 15-19 years, again, it is the fourth most common cause of death in developed countries (3).

The development of a worldwide infrastructure for treatment of pediatric cancer patients in large comprehensive clinical trials and standardization of treatment took place over the past three decades. This resulted in a dramatic increase in survival rates among patients from 1 to 15 years of age; the 5-year survival for all cancer combined has increased from approximately 60% in 1975–1978 to 80.6% in 1999–2002, as estimated by the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute in the United States (4;5).

Despite a higher incidence of cancer in the adolescent and young adult (AYA) population compared to children, there has been a lack of awareness and centralization of patients in this age category over time (6). This is reflected by the relatively low accrual and participation of this subpopulation in clinical trials, which is held as one of the factors responsible for a consequent lack in survival benefit when compared to their younger counterparts (7-9). Importantly, the AYA cancer population is unique in ways of a typical tumour type distribution, overlapping both child- and adulthood cancers (1). Furthermore, the awareness of the AYA population being distinct in terms of specific needs and concerns (e.g. fertility, insurance, employment, and psychosocial issues) is rising (6).

During the last years it has been recognized that cancer patients at the age of adolescence or young adulthood deserve more age specific attention resulting in initiatives that have been undertaken world-wide to improve care for these patients (10). In the United Kingdom, the teenager and adolescent cancer population (12-25 years) has been resurrected and it has a very active government funded national teenage cancer trust program with the first AYA cancer unit founded in 1990 in London. Now, there are eight comprehensive AYA centers, either under the guidance of pre-existing medical oncology or pediatric oncology units. This government-funded AYA cancer program has also been an example for Australian (15-25 years) and Canadian AYA cancer care programs (15-29 years, translated from the SEER database). Also, numerous European countries including Italy, Denmark and the Netherlands have followed the UK example by setting up tailored AYA programs/units (mostly patients 15-24 years). The United States' National Cancer Institute (NCI) –(NCI) -funded the Children's Oncology Group AYA Committee formed an AYA Committee in 2000 (15-29 years), focusing on improving outcomes, access to care, and accrual to clinical trials, and subsequently the Adolescent and Young Adult Oncology Progress Review Group (AYAO PRG, 2006), extending the upper age limit to 39 years. To date, there are no dedicated physical AYA units in the United States, but several "virtual" AYA programs have been developed from 1999 up to now.

The basis to increase awareness and facilitate the development of tailored treatment and care of this young cancer population is a throughout description of the epidemiological features (e.g. incidence, distribution, survival) of cancers affecting this population. Another important feature to consider in both children and AYA with cancer is their generally long life-expectancy after surviving cancer, accompanied by an increased risk of development of long-term cancer related issues, including long term morbidity/mortality and second malignancies, due to previous anti-cancer treatment and/or genetic susceptibility (11-20).

In **Chapter 2**, a population-based study concerning AYA patients (12-24 years) diagnosed in the Northern Netherlands and registered by the Comprehensive Cancer Centre North-Netherlands (CCCN) over the period 1989-2003 (N=1,118 patients) is reported. Cancer incidence and survival was the primary focus to get more insight in the age distribution of malignancies in this group with a special focus on the risk of second malignancies.

In **Chapter 3**, an extended AYA cancer study is reported, concerning the Dutch nationwide population-based data of AYA cancer incidence (N= 23,161; 15-29 years) by the Netherlands Cancer Registry (NCR) over the period 1989-2009.

Rhabdomyosarcoma (RMS) is a rare type of soft tissue sarcoma that represents an ultimate example of a typical childhood malignancy which also occurs across the AYA age-spectrum and shows inferior survival rates in the older population (21-23). There are two main histological variants which can be distinguished; embryonal rhabdomyosarcoma (eRMS, 60-70% of RMS) and alveolar rhabdomyosarcoma (aRMS, 30% of RMS). These subtypes show unique age-related patterns of incidence. Although eRMS typically occurs in young children as 70% of the patients are diagnosed within the first decade of life, it shows a broad age range as it might occur in adults up to 70 years of age. In contrast, aRMS rarely occurs in young children, but shows an incidence peak in adolescents and young adults (24).

The prognosis in children with RMS has improved dramatically during the last decades because of the introduction of multi-agent chemotherapy in consecutive multidisciplinary clinical trials and treatment in a centralized setting (25-29). The Intergroup Rhabdomyosarcoma Study Group (IRSG) publications demonstrated successive increases in five-year survival rates between 1972 and 1997; from 55% on IRS-I (27) to 74% on IRS-IV protocols (29). In contrast, data on adults with RMS are scarce and in general show a worse outcome compared to children (30;31). Additionally, a recent Surveillance, Epidemiology and End Results (SEER) report of 2,600 RMS patients confirms that no survival improvements in adults have been made over the past decades (32). Also within the pediatric RMS population, survival was previously reported to be worse in older children when compared to younger children (with the exception of children <1 year of age) (32-35). The factors that are held responsible for this adverse effect of age on outcome of RMS can be divided in two theories; nature (tumour biology) and nurture (treatment). Concerning nature, RMS at the adolescent and adult age seems to act biologically more aggressive, shows more often the adverse alveolar subtype and tends to metastasize more early (30;36;37). With regard

to nurture, there is a lack of availability, accrual and participation in large international clinical trials in older patients. Furthermore, the tolerability of intensive chemotherapy schedules might differ between children and AYA patients and consequently there is a different opinion on the impact of these treatment regimens on patients by pediatric oncologists and adult oncologists. Unfortunately, reliable data on efficacy and toxicity of similar treatment schedules are hardly available in literature.

In **Chapter 4**, a multi-center study concerning the effect of age on clinical presentation and outcome in eRMS and aRMS is reported. This effect of age and other prognosticators (including treatment modalities) was investigated in a multivariate survival analysis in a cohort of 169 patients 0-73 years of age with complete data on follow-up.

Despite the successive survival rates reported in children with RMS, the survival in particular subgroups of patients (e.g. those primarily diagnosed with alveolar histology, distant and/or lymph node metastases at diagnosis, diagnosed at older age, or with refractory/recurrent disease) remains disappointing (32;38-43). Survival in these subgroups is not exceeding 50%, which is particularly low compared to the overall survival of RMS exceeding 70-90% in one of the latest completely reported COG studies (IRS IV, conducted between 1991-1997) (29). Moreover, the high risk of treatment related/induced morbidity and mortality after aggressive chemotherapeutic/ radiotherapeutic/surgical treatment modalities as currently used in RMS (44), urges the need for new therapeutic options in RMS.

'Targeted treatment' is an upcoming strategy that has made significant progress in many types of cancer over the past decade, including sarcomas. An important group of targets within this topic are 'receptor tyrosine kinases' (RTKs), a group of transmembranous signaling proteins, which can be activated by either ligand-binding or self-activation. Consequently, multiple intracellular downstream effector proteins are activated by phosphorylation processes including 1) the MAP kinase pathway (including the downstream signaling molecule ERK), and 2) the PIK3CA pathway (including downstream signaling molecules AKT and mTOR). These downstream effectors play an important role in cell cycle progression, proliferation and regulation of apoptosis. Deregulation of the receptors has been implicated in the oncogenic development of many types of cancer, including sarcomas (45). In contrast to the currently available cytotoxic strategies (inducing non-selective destruction of rapidly dividing cells including normal cells), these 'targeted' agents aim at specific tumour cell death by interfering with specific molecules involved in tumour growth and progression, - ideally- without causing harmful effects to healthy tissues.

A receptor tyrosine kinase which represents a potential treatment target in RMS is Anaplastic Lymphoma Kinase (ALK) (46). In an attempt to identify genome-wide key players in oncogenesis as well as potential molecular targets for therapy, gene expression profiling studies recently identified the presence of distinct molecular signatures based on the presence or absence of the specific translocations in *(PAX3/PAX7-FKHR)* RMS (47-50). In these studies, translocation-positive aRMS was repeatedly associated with *ALK* overexpression (47-49). Moreover, whole genome analysis for PAX3-FOXO1 protein binding sites recently showed a very high affinity for binding to

ALK's 3rd intron yielding increased ALK transcription. In the same study, introduction of lentiviral shRNA against *PAX3-FOXO1* into a *PAX3-FOXO1* positive cell line consequently downregulated *ALK* mRNA levels *in vitro* (51).

ALK overexpression and genetic alterations of the *ALK* gene have been previously identified in multiple malignancies including for example anaplastic large cell lymphomas (ALCL), non small cell lung cancer (NSCLC) (52;53), inflammatory myofibroblastic tumours (IMT)(54;55), neuroblastoma (56-59), as well as small subsets of rhabdomyosaroma (60). Differential underlying mechanisms are responsible for altered ALK expression in these tumours, including translocations (ALCL/NSCLC) (61-63), germline or somatic mutations (neuroblastoma) (56) and genomic gains or amplifications (neuroblastoma, NSCLC) (53;56). The exact underlying mechanism of ALK expression in RMS and its clinical and prognostic implications, however, are largely unknown.

In **chapter 5**, we focus on ALK expression and the underlying mechanism responsible for ALK (over)expression in RMS with special attention to clinical and prognostic implications. We analysed ALK protein expression by immunohistochemical analysis in 189 tissue samples of 145 patients. Furthermore, *in situ* hybridization was performed to analyse *ALK* gene (2p23) copy number alterations and the presence of translocations. Additionally, mRNA sequencing of the *ALK* RTK domain was performed to detect genetic alterations.

The insulin-like growth factor (IGF) system is a RTK signaling pathway previously implicated in RMS. The IGF system (which consists of the ligands IGFI and IGFII, the insulin-like growth factor-1 receptor (IGF-1R), and 6 IGF binding proteins) was already identified in tumour samples and cell lines of RMS. Inhibition by using siRNA, monoclonal antibodies and receptor tyrosine kinase inhibitors resulted in diminished growth in multiple preclinical studies (64). However, clinical trials with monoclonal IGF-1R inhibitors show disappointing clinical response rates and only a temporarily anti-tumour effect (65). These disappointing results reflect the major pitfalls of these targeted therapies. First, the type of inhibitory agent determines its spectrum of cellular activity (e.g. on the cell membrane, intracellular, intra-nuclear, or a combination of these). Monoclonal antibodies are not capable of migrating over the cell membrane, however, small molecule inhibitors (generally less specific) have a broader area of activity as they are also capable of inhibiting intracellular/intranuclear molecules. Second, tumour heterogeneity (within the tumour or between primary tumour and metastatic lesions) might result in only a partial effect, which makes *in vitro* investigations less predictable for clinical effects. Third, recent investigations point out that certain tumour cells are capable to activate escape mechanisms in response to inhibition of a cell-surface receptor by the upregulation of other RTKs (66-69). A potential strategy to overcome this escape mechanism is to block multiple receptors simultaneously.

In **Chapter 6**, we investigate the (co-)expression of the IGF-1R and ALK receptor signaling pathway, as well as the activation of downstream signaling by immunohistochemistry on tissue micro-arrays of 112 paraffin-embedded RMS primary tumour samples (86 eRMS, 26 aRMS). Furthermore we investigate the potential (synergistic) effect of ALK (NVP-TAE684) and IGF-1R (R1507) directed therapies in four RMS cell lines *in vitro*.

Finally, **Chapter 7** is a comprehensive review which highlights the similarities and differences between RMS in children and adults with regard to epidemiology, tumour biology, diagnosis, treatment approach, and accrual to clinical trials with new agents. It concludes with a concept to build a bridge between the two worlds (pediatric and adult oncology) in order to improve care for children, adolescents and (young) adults with RMS.

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CHAPTER 2

Cancer in adolescents and young adults (AYA) in North Netherlands (1989-2003): increased incidence, stable survival and high incidence of second primary tumours

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ABSTRACT

Background: Lack of survival improvement in Adolescents and Young Adults (AYA) with cancer has led to increased awareness of this young population.

Design: We performed a population-based study of incidence and survival of primary tumours and second primary tumours in patients aged 12-24 in North-Netherlands. Age-specific incidence rates per 100,000 and three-year moving means were calculated. Factors associated with incidence and survival were assessed using a Poisson model, log-rank test and multivariate Cox proportional hazard analysis.

Results: From 1989-2003 1118 patients were diagnosed. The total age-specific incidence rates per 100,000 were: Males: 13.4 (12-15 yrs), 26.9 (16-19 yrs), and 27.5 (20-24 yrs) and Females: 13.9, 20.7 and 20.7. M/F ratio was 1.32. The overall estimated annual percent change (EAPC) in incidence was 2.15% ($p<0.01$). Five-year-survival was 80.8%, and did not improve during the study period. With median follow-up of 5.5 years (range 0.0-16.0) in our cohort the Standardized Incidence Ratio (SIR) of second primary tumours was 30.55 (95% CI 19.96-44-76, $p<0.05$)

Conclusion(s): The total incidence of cancer in AYA increased (EAPC 2.15%). Survival was unchanged. The SIR of a second primary tumour in this young cohort increased 31-fold. Further research is needed to study this increasing incidence and optimise treatment outcome in these young patients.

INTRODUCTION

Malignancies in the young age group 15 to 24 years are uncommon, with an estimated incidence of 291 per million per year in the year 2000 in the United States (1). Within the total burden of cancer incidence (4203 per million in Europe 2003 (2), and 4723 per million in the USA 1996-2000 (3)), the adolescent cancer patient population forms a minority, whereas malignancies in children (0-14 years) are even more uncommon (153 per million per year USA, 2000 (1) and 130.9 per million per year in Europe (4)).

Although the number of Adolescent and Young Adult (AYA) cancer patients remains low, reports have shown a marked increase in incidence. Over the past three decades, the cancer incidence among AYA's (15-24 years) in the United States increased with 0.85% per year, and European studies report an increase as well (1;5;6).

Survival has improved considerably during the past three decades for young children treated by paediatric oncology groups in Western countries, probably as a result of large scale enrolment into international cooperative multicentre clinical trials investigating new treatment strategies including multi-agent chemotherapy. In contrast, adolescents and young adults had a less favourable improvement in clinical outcome (5;7). Surveillance Epidemiology and End Results (SEER) data showed an annual increase in 5-year relative survival of only 0.75% in adolescents (15-24 years), compared to 1.53% in children (0-14 years) between 1975 and 1997 (1). Notably, due to the relatively low incidence of cancer in this age group, patients are getting dispersed between large numbers of patients at oncology departments.

Although improvement in survival in adolescents and young adults lags behind, the number of cancer survivors in this group has increased consistently over time. With the increasing age of survivors and more intensive treatment for cancer, awareness of an increased risk for developing second and treatment related secondary tumours in young cancer patients is important (8).

We assessed recent trends in incidence and outcome of cancer, with special attention on second primary tumours, among adolescent and young adult cancer patients in a population-based cohort of 12-24 year olds diagnosed between 1989 and 2003 in the North-Netherlands.

METHODS

Patients

Patients diagnosed between 1989 and 2003 in the Northern part of The Netherlands (covered by the cancer registry of the Comprehensive Cancer Centre North-Netherlands (CCCN)) aged from 12 to 24 years were included in this study. Patients were classified by cancer type, age group (12-15, 16-19, 20-24 years) and gender for the periods 1989-1993, 1994-1998 and 1999-2003. These age groups were constructed because 12-15 year-olds are generally treated at a paediatric oncology unit and most 20-24 year olds are treated at a medical oncology unit, whereas 16-19 year old patients form an intermediate group. Age-group therefore can be used as a proxy for treatment by a paediatric or adult medical oncology unit. Cancer type was defined using the ICD-0-3 classification (9) and classified by the Classification Scheme for Cancer in 15-24 years olds (version 5) according to Birch *et al* (10). Carcinoids of the appendix (mostly incidental findings) with malignant properties (ICD 8240-8245, ICD-0-3) were excluded from the analysis.

Data collection by the cancer registries

Data were collected by the regional cancer registry of the CCCN, covering the Northern Netherlands, a predominantly rural area with a population of about 2.2 million. The nationwide Dutch network and registry of histo- and cytopathology regularly submits reports of all diagnosed malignancies to the cancer registries. The national hospital discharge databank, which receives discharge diagnoses of admitted patients from all Dutch hospitals, completes case ascertainment. After notification, trained registry personnel collect data on diagnosis, staging, and treatment from the medical records, including pathology and surgery reports, using the registration and coding manual of the Dutch Association of Comprehensive Cancer Centres. Vital status was established either directly from the patient's medical record or through linkage of cancer registry data with the municipal population registries, which record information on their inhabitant's vital status. There were no death certificates only in the cohort. The closing date of the study was December 31st 2004, resulting in follow-up less than 5 years in 17.4% of the patients. A second primary tumour was defined as a new primary tumour registered in the regional cancer registry, as reported by the clinician or pathologist. Furthermore, when a second primary tumour was suspect to be a relapse (same histology) we checked medical files of the patients. A melanoma was considered a second primary only when its location was obviously different from the first tumour. Furthermore, a contralateral testicular tumour was considered to be a second primary tumour (11).

Statistical analysis

The population at risk for each year was retrieved from Statistics Netherlands (*http://statline.cbs.nl/StatWeb*). The annual age-specific incidence rates for each tumour type were calculated per 100,000 according to gender and period of diagnosis. In addition, three-year moving means of the incidence were calculated for patients aged 12-15, 16-19 and 20-24 years. Trends in incidence rates were estimated based on a linear regression of the log-transformed incidence rates with year of diagnosis as the regressor variable. The regression coefficient for year of diagnosis was used to estimate the Annual Percentage Change (EAPC). The association of age, year and sex

with incidence was performed using a multivariate Poisson model. Survival rates at 3, 5 and 10 years were calculated for all ages together by the life table method. Differences in survival rates were analysed using the log-rank test. Multivariate Cox proportional hazard analysis was performed to determine factors associated with survival for this age group. To asses the risk of second primary tumours a standardized incidence ratio (SIR) was calculated, which compares the observed number of secondary tumours with the expected number of tumours. The expected number of second tumours was calculated using age-, year- and gender specific incidence rates in the general population derived from the cancer registry. Cumulative incidence of second primary tumours over the study period and confidence intervals were estimated, with death as competing risks (12;13).

RESULTS

Patients

A total of 1118 patients aged 12-24 years were diagnosed with cancer in the period 1989-2003 in the Northern Netherlands. In this cohort, 203 patients (18.2%) were 12-15 years, 385 (34.4%) were 16-19 years and 530 (47.4%) were 20-24 years at diagnosis, with a male:female (M:F) ratio of 1.32. Of these patients, 1055 were diagnosed and treated in the Northern Netherlands and the remaining 63 were treated in regions covered by other cancer registries.

Incidence

Figure 1a shows the distribution of the tumour types by age-group and gender. Table 1a shows the age-specific incidence rates for the main tumour types, by age group, gender and time period. The annual cancer incidence (rate per 100,000) was lowest among 12-15 year-olds: 13.4 (M) and 13.9 (F). This incidence increased to 27.3 (M) and 20.9 (F) in 16-19 year-olds and 27.9 (M) and 20.8 (F) in 20-24 year olds.

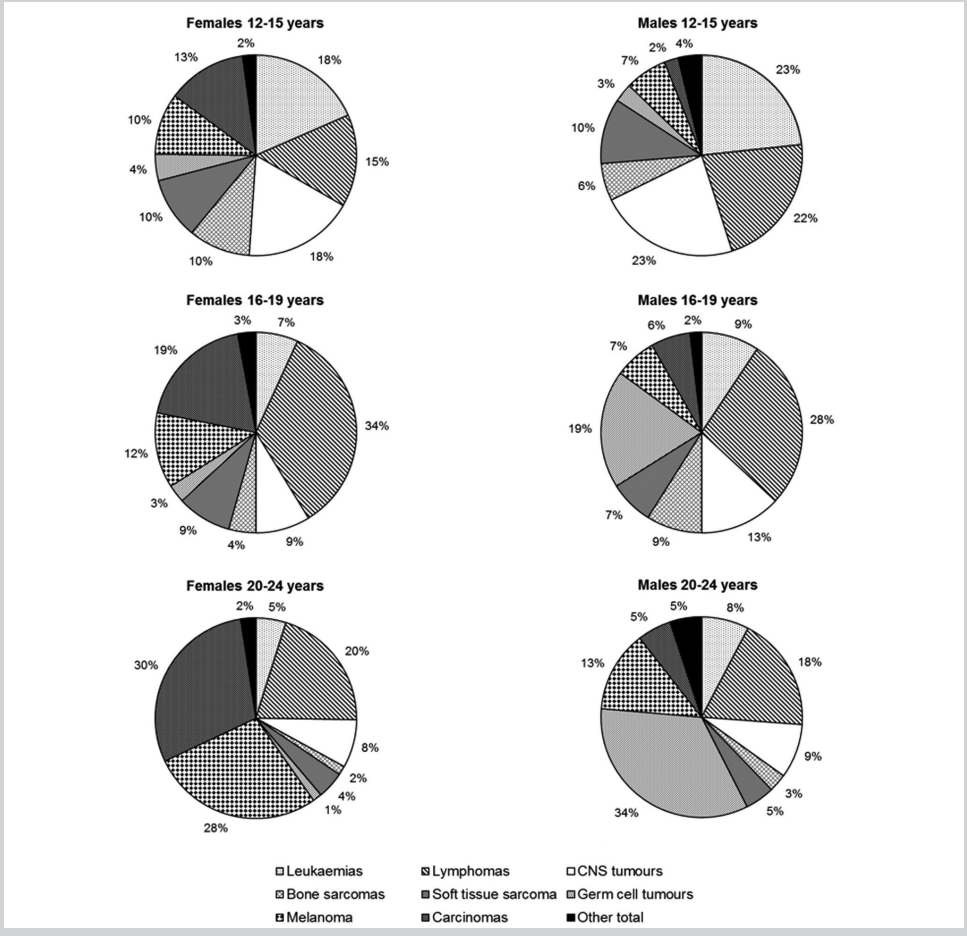
The highest incidence in males was seen for germ cell tumours with the highest incidence in 20-24 year-olds (9.3). Non-seminomatous testicular cancer (ICD-9065, ICD-0-3) was the predominant morphological subtype (79.6%). Lymphomas were the second most frequent tumour type in males with the highest incidence in 16-19 year olds (7.4). In females, lymphomas represented the most frequent cancer type with a highest incidence of 7.2 for 16-19 year-olds.

Cancer incidence increased markedly over time with an estimated annual percentage change (EAPC) of 2.15% ($p= 0.001$, unadjusted for age group and sex). The age-specific incidence (3-year moving means) of all cancer types in both sexes between 1989 and 2003 is shown in Figure 1b. We observed an increase in incidence in the age groups of 16-19 and 20-24 years for males. In females, the incidence increased for all age-groups, especially after 2000 for age-group 16-19 years.

Tables 1b and 1c explore the association of age, gender and year of diagnosis with incidence in a multivariate regression analysis. Increasing age was associated with increasing incidence in lymphoma ($p<0.001$), germ cell tumours ($p<0.001$), melanoma ($p= 0.001$), and carcinomas ($p<0.001$). While the incidence of leukaemia did not differ significantly by age group, it should be

mentioned that with increasing age the contribution of acute lymphatic leukaemia (ALL) to the total of cases of leukaemia declined, whereas the contribution of acute myeloid leukaemia (AML) increased. For CNS tumours, incidence decreased significantly with increasing age (p=0.03). Later year of diagnosis was associated with an increase in incidence for lymphomas (p=0.008), germ cell tumours (p<0.001), and carcinomas (p= 0.002). A remarkably high lymphoma incidence was found during the period 1999-2003 (11.7 and 9.9 among males and females, respectively), due to a high incidence of Hodgkin's lymphoma in 16-19 year olds in the period 1999-2003. Germ cell tumour incidence was significantly lower for females (p<0.001), and incidence of melanoma and carcinoma was higher in females than among males (p<0.001 for both). This is largely due to the high incidence of thyroid cancer in females.

Figure 1a
The distribution of all malignancies, displayed by age group and sex.



*CNS tumours = central nervous system tumours; **Lymphomas= Hodgkin's lymphoma and non-Hodgkin lymphoma added up; Numbers are incidence rates, displayed per 100,000 within the figures.

Table 1a: Age-specific incidence rates per 100,000 for different tumour types in Adolescents and Young Adults (AYA), males and females in the different periods in Northern Netherlands over the period 1989-2003.

Tumor	Age	Male				Female			
		1989-1993	1994-1998	1999-2003	Total	1989-1993	1994-1998	1999-2003	Total
Leukemias	12-15	3.2	3.2	3.1	3.1	2.5	1.7	3.6	2.6
	16-19	2.3	3.5	1.9	2.5	1.0	1.6	1.6	1.4
	20-24	2.4	1.1	3.0	2.1	1.3	1.1	0.6	1.0
	Total	**2.6**	**2.4**	**2.7**	**2.5**	**1.5**	**1.4**	**1.8**	**1.6**
Lymphomas	12-15	5.1	2.0	1.5	2.9	2.1	2.6	1.6	2.1
	16-19	5.9	4.7	11.7	7.4	4.8	7.0	9.9	7.1
	20-24	3.6	5.9	6.0	5.1	4.3	4.8	3.5	4.2
	Total	**4.7**	**4.5**	**6.4**	**5.2**	**3.9**	**4.8**	**4.9**	**4.5**
Central Nervous	12-15	3.9	2.8	2.3	3.0	2.9	2.6	2.0	2.5
System tumours	16-19	2.6	3.5	4.5	3.5	1.4	1.6	2.4	1.8
	20-24	1.9	3.8	1.5	2.4	1.5	1.7	1.6	1.6
	Total	**2.7**	**3.4**	**2.7**	**2.9**	**1.8**	**1.9**	**2.0**	**1.9**
Bone sarcomas	12-15	0.8	0.8	0.8	0.8	0.4	1.3	2.4	1.4
	16-19	2.3	3.1	1.9	2.4	0.7	1.2	0.8	0.9
	20-24	0.9	1.1	0.3	0.8	0.5	0.3	-	0.3
	Total	**1.3**	**1.6**	**0.9**	**1.3**	**0.5**	**0.8**	**1.0**	**0.8**
Soft tissue	12-15	-	2.4	1.9	1.4	1.2	2.1	0.8	1.4
sarcomas	16-19	1.6	1.2	3.0	1.9	2.1	2.1	1.2	1.8
	20-24	1.9	0.8	1.2	1.3	1.3	0.6	1.0	0.9
	Total	**1.3**	**1.4**	**2.0**	**1.5**	**1.5**	**1.4**	**1.0**	**1.3**
Germ cell tumours	12-15	0.4	-	0.8	0.4	0.8	0.4	0.4	0.6
	16-19	3.6	5.5	6.4	5.1	0.7	0.4	0.8	0.6
	20-24	6.2	8.6	14.1	9.3	0.5	-	0.3	0.3
	Total	**3.9**	**5.3**	**7.7**	**5.5**	**0.6**	**0.2**	**0.5**	**0.5**
Melanoma	12-15	0.8	0.4	1.5	0.9	0.4	0.4	3.2	1.4
	16-19	1.3	2.4	1.9	1.8	2.1	2.5	3.2	2.5
	20-24	3.3	3.8	3.6	3.6	5.3	5.1	7.0	5.7
	Total	**2.0**	**2.4**	**2.4**	**2.3**	**3.0**	**3.0**	**4.7**	**3.5**
Carcinomas	12-15	-	-	0.8	0.3	2.5	1.3	1.6	1.8
	16-19	0.7	3.1	1.5	1.7	3.1	3.7	5.2	3.9
	20-24	0.9	2.4	1.2	1.5	4.3	6.5	7.9	6.1
	Total	**0.6**	**1.9**	**1.2**	**1.2**	**3.5**	**4.2**	**5.2**	**4.2**
All malignancies	**12-15**	**14.6**	**12.1**	**13.4**	**13.4**	**13.7**	**12.3**	**15.7**	**13.9**
	16-19	**20.4**	**28.3**	**33.1**	**26.9**	**16.8**	**20.1**	**25.9**	**20.7**
	20-24	**23.3**	**28.5**	**31.9**	**27.5**	**20.1**	**20.1**	**22.2**	**20.7**

Figure 1b
Incidence rates per 100,000 for males (a) and females (b) 1989-2003. Three-year moving means.

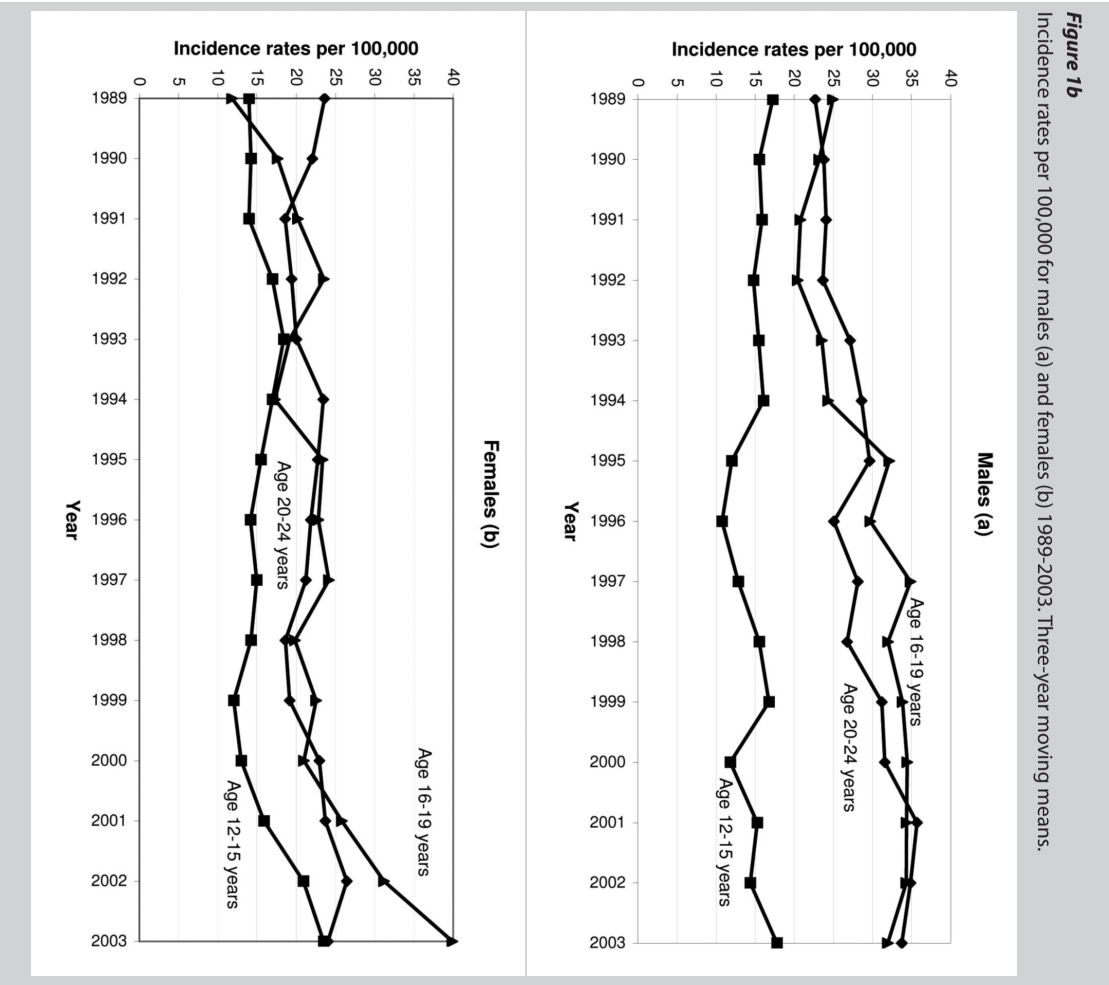


Table 1b: Multivariate analyses of incidence (model with age, year and sex) of malignancies in AYA over the period 1989-2003.

	Leukemias			**Lymphomas**			**CNS tumours**			**Bone tumours**			**Soft Tissue Sarcoma**		
	IRR	95%CI	p-value	IRR	95%CI	p-value	IRR	95%CI	p-value	IRR	95%CI	p-value	IRR	95%CI	p-value
Age group			0.168			**<0.001**			**0.029**			0.191			0.327
12-15	Ref			Ref			Ref			Ref			Ref		
16-19	0.86	0.62-1.19		2.22	1.71-2.90		0.86	0.63-1.17		0.99	0.63-1.57		1.09	0.73-1.62	
20-24	0.71	0.50-1.01		1.35	1.02-1.78		0.64	0.45-0.89		0.59	0.31-1.13		0.78	0.49-1.24	
Year			0.980			**0.008**			0.170			0.379			0.815
	1.00	0.97-1.01		1.03	1.01-1.05		1.02	0.99-1.06		1.02	0.97-1.07		1.00	0.97-1.04	
Sex			0.285			0.966			0.092			0.386			0.974
Male	Ref			Ref			0.79	0.61-1.04		0.83	0.55-1.26		1.01	0.70-1.44	
Female	0.85	0.64-1.14		1.00	0.83-1.21										

Table 1c: multivariate analyses of incidence (continued) of malignancies in AYA over the period 1989-2003.

	Germ cell tumours			**Melanoma**			**Carcinoma**			**All tumours**		
	IRR	95%CI	p-value	IRR	95%CI	p-value	IRR	95%CI	p-value	IRR	95%CI	p-value
Age group			**<0.001**			**0.001**			**0.0002**			**<0.001**
12-15	Ref			Ref			Ref			Ref		
16-19	1.82	1.03-3.23		0.94	0.62-1.43		1.83	1.21-2.78		1.29	1.14-1.45	
20-24	3.15	1.80-5.51		1.56	1.07-2.29		2.30	1.54-3.43		1.32	1.17-1.49	
Year			**<0.001**			0.132			**0.002**			**<0.001**
	1.06	1.03-1.09		1.02	0.99-1.05		1.05	1.02-1.08		1.03	1.02-1.04	
Sex			**<0.001**			**0.001**			**<0.0005**			0.512
Male	Ref			Ref			2.07	1.52-2.82		0.97	0.89-1.06	
Female	0.33	0.21-0.51		1.55	1.20-2.02							

Multivariate analyses of incidence (model with age, year and sex). The association of age, year and sex with incidence was performed using a multivariate Poisson model. IRR= incidence rates ratio. 95%CI= 95% confidence interval, P<0.05 is considered significant

Survival

Survival (3-, 5-, and 10-year) of the most prevalent tumour types are shown by age group in Table 2a. The median follow-up time in the cohort was 5.5 years (range 0.0-16.0). The 5-years survival was 80.8% (CI 78.1-83.1).

High survival rates were found for germ cell tumours, and melanoma, with 5-year survival rates exceeding 90%. Intermediate survival was found for carcinomas (87.1%), and lymphomas (87.0%). The 5-year survival rates were rather low for central nervous system (CNS) tumours, leukaemia, bone tumours, and soft tissue sarcomas (STS), not exceeding 70%. However, the numbers at risk for bone tumours and STS were small.

Multivariate survival analysis adjusting for age group and gender did not show any increase in survival over time for the whole group (HR 0.96, CI 0.93-1.0, p=0.033), nor for the different tumour types (Table 2b and 2c).

Table 2a
Survival for the different AYA tumours diagnosed in both sexes.

Tumour	N at risk	3-years		5-years		10-year	
		Survival	95%CI	Survival	95%CI	Survival	95%CI
Leukemias	110	63.3	52.9-71.9	60.4	49.8-69.5	60.4	49.8-69.5
Lymphomas	256	88.6	83.8-92.0	87.0	81.9-90.7	86.4	81.2-90.2
CNS tumours	128	76.6	68.2-83.1	65.5	55.9-73.5	58.6	48.2-67.6
Bone tumours	55	66.4	51.6-77.7	61.3	45.9-73.5	58.5	43.0-71.2
Soft tissue sarcoma	76	78.0	66.1-86.1	68.4	55.1-78.5	66.3	52.7-76.8
Germ cell tumours	162	92.3	86.8-95.5	92.3	86.8-95.5	91.2	85.1-94.8
Melanoma	153	95.0	89.9-97.6	95.0	89.9-97.6	91.6	83.5-95.8
Carcinomas	142	88.1	81.3-92.5	87.1	0.81-0.92	87.1	0.81-0.92
Total	1118*	83.8	81.4-85.9	80.8	78.1-83.1	78.8	76.0-81.4

This table displays 3-year, 5-year and 10-year survival for the different AYA tumours diagnosed in both sexes over 1989-2003 with their 95%-confidence intervals. Survival was calculated using the life table method. * N=1118 includes also patients with "other tumours" (N=36)

Table 2b: Multivariate regression analysis of survival (model with age, year and sex) in AYA for the period 1989-2003.

	Leukaemias			Lymphomas			CNS tumours			Bone tumours			Soft Tissue Sarcoma		
	HR	95%CI	p-value	HR	95%CI	p-value	HR	95%CI	p-value	HR	95%CI	p-value	HR	95%CI	p-value
Age group			0.0008			0.604			0.377			0.523			0.338
12-15	Ref			Ref			Ref			Ref			Ref		
16-19	1.22	0.49-2.99		1.54	0.50-4.70		0.85	0.37-1.96		1.39	0.45-4.29		2.38	0.73-7.83	
20-24	3.68	1.72-7.89		1.10	0.35-3.49		1.41	0.70-2.81		0.69	0.15-3.20		2.14	0.63-7.24	
Year			0.054			0.522			0.122			0.270			0.758
(continue)	0.94	0.87-1.00		0.97	0.89-1.06		0.93	0.86-1.02		1.07	0.95-1.21		1.02	0.92-1.12	
Sex			0.948			0.503			0.436			0.197			0.712
Male	Ref			Ref			Ref			Ref			Ref		
Female	0.98	0.50-1.91		0.78	0.38-1.61		1.27	0.70-2.32		0.48	0.16-1.46		1.18	0.49-2.81	

Table 2c: multivariate regression analysis of survival (continued) in AYA for the period 1989-2003.

	Germ cell tumours			Melanoma			Carcinoma			All tumours		
	HR	95%CI	p-value	HR	95%CI	p-value	HR	95%CI	p-value	HR	95%CI	p-value
Age group			0.780			0.282			0.958			0.755
12-15	} Ref			Ref			} Ref			Ref		
16-19				0.15	0.01-1.67					1.03	0.70-1.53	
20-24	1.19	0.35-3.98		0.39	0.08-1.95		1.03	0.39-2.71		0.92	0.64-1.34	
Year			0.590			0.201			0.450			0.033
(continue)	0.97	0.85-1.10		0.90	0.76-1.06		1.05	0.93-1.18		0.96	0.93-1.00	
Sex			0.921			0.118			0.470			0.123
Male	Ref			Ref			Ref			Ref		
Female	1.11	0.14-9.13		0.40	0.13-1.26		0.68	0.24-1.94		0.80	0.61-1.06	

Multivariate regression analysis of survival. Multivariate Cox proportional hazard analysis was performed to determine factors associated with survival for this agegroup. HR= Hazards ratio. 95% CI= 95% confidence interval, P<0.05 is considered significant

Second primary tumours

With a median follow-up time of 5.5 years (range 0.0-16.0), 26 patients developed a second tumour (table 3). This corresponds with a standardized incidence ratio (SIR) of 30.55 (CI 19.96-44-76, p<0.05). The cumulative incidence of second primary tumours at 10 years was 2.83% (CI 1.7-4.3, cumulated over 1989-2003; figure 2). The median interval between first and second primary malignancy was 6.19 years (range 0.02-12.9 years). Of the patients with a second malignancy, 21 patients are alive with a median follow up after the second tumour of 8.69 years (range 1.5-15.7 years).

Two patients developed a secondary tumour in the radiation field after treatment for Hodgkin's Lymphoma. Five patients with melanoma developed a localized second melanoma at a distinct place. Only one patient with multiple melanomas was known with familial atypical multiple mole melanoma (FAMMM) syndrome. Four patients with a primary testicular tumour in adolescence developed a contralateral testicular tumour during follow-up.

Figure 2
Cumulative incidence of second primary tumours in AYA over the period 1989-2003 including 95% Confidence Intervals (CI).

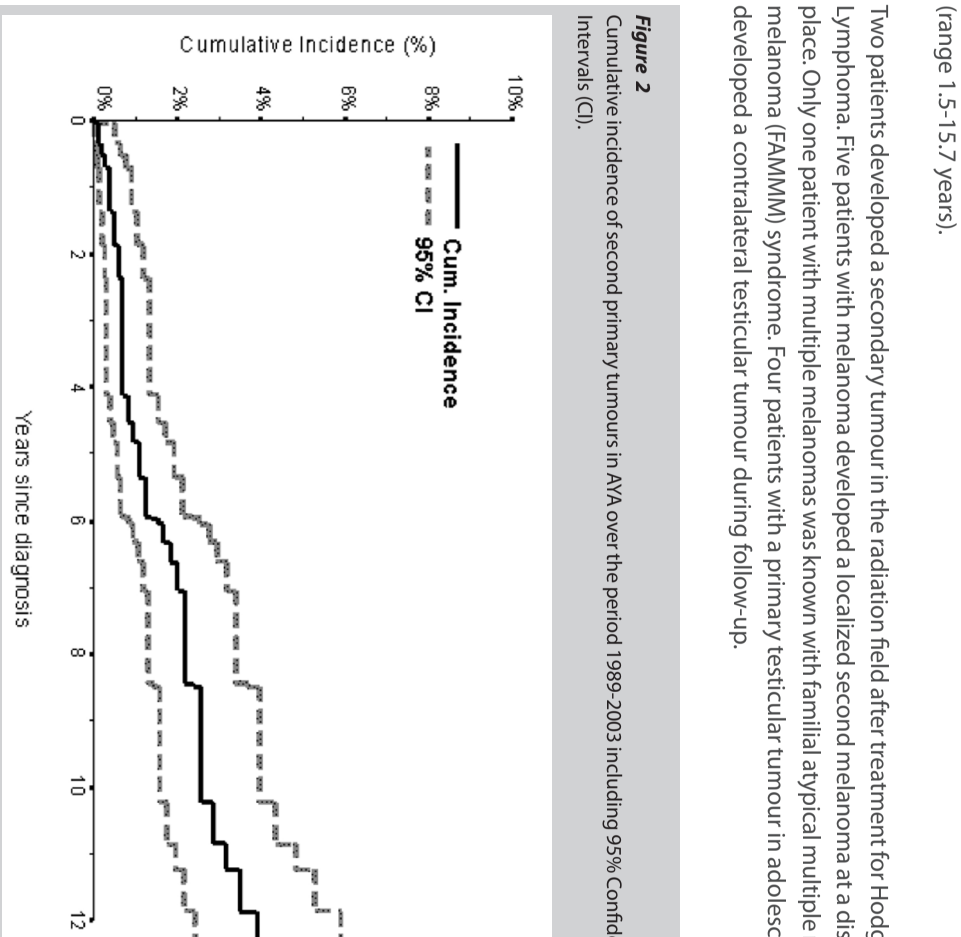


Table 3. Second primary tumours in AYA (12-24 years) during 1989-2003.

First primary	Age at diagnosis	Year of diagnosis	Therapy	Second primary tumour	Interval (yr)
Acute Lymphatic Leukaemia (ALL)	14	1998	Chemotherapy	Melanoma	6.3
Epidermoid carcinoma cervix	23	2003	Surgery	Epidermoid carcinoma vulva	0.7
Medulloblastoma	14	1995	Surgery & radiotherapy	Osteosarcoma	5.9
Astrocytoma	22	1995	Surgery	Breast cancer	5.3
Adenocarcinoma small intestine	22	1996	Surgery	Epidermoid carcinoma bladder	0.3
Germcell tumour endocrine gland	22	1996	Surgery & radiotherapy	Melanoma	4.1
Hodgkin lymphoma	17	1989	Surgery & radiotherapy	Gastric adenocarcinoma*	12.4
Hodgkin lymphoma	20	1989	Radiotherapy	Breast cancer*	12.9
Hodgkin lymphoma	18	1996	Chemotherapy	Astrocytoma	8.4
Hodgkin lymphoma	17	1991	Chemotherapy	Acute Myeloid Leukaemia (AML)	2.4
Melanoma	23	1995	Surgery	Melanoma	6.1
Melanoma**	21	1992	Surgery	Melanoma	7.1
Melanoma	18	1991	Surgery	Melanoma	12.8
Melanoma	14	2002	Surgery	Melanoma	0.5
Melanoma	20	1997	Surgery	Melanoma	1.9
Melanoma	24	1989	Surgery	Astrocytoma	10.9
Renal cell cancer	21	1996	Surgery	Astrocytoma	4.8
Non-Hodgkin lymphoma	18	2002	Chemotherapy	Kaposi sarcoma[1]	0.0
Non-seminomatous testicular cancer	20	1992	Surgery & chemotherapy	Thyroid cancer	6.6
Non-seminomatous testicular cancer	23	1993	Surgery	Non-seminomatous testicular cancer	12.7
Non-seminomatous testicular cancer	20	1999	Surgery & chemotherapy	Non-seminomatous testicular cancer	7.0
Non-seminomatous testicular cancer	21	1999	Surgery & radiotherapy	Non-seminomatous testicular cancer	1.4
Seminomatous testicular cancer	17	1990	Surgery	Non-seminomatous testicular cancer	11.2
Non-seminomatous testicular cancer	21	2000	Surgery	Renal cell cancer	4.8
Liposarcoma	20	1996	Surgery & radiotherapy	Melanoma	8.5
Soft tissue sarcoma (not otherwise specified)	16	1994	Surgery	Hodgkin lymphoma	11.5

[1] HIV-positive patient developed non-Hodgkin lymphoma and Kaposi sarcoma synchronously. The standard incidence ratio (SIR) of a second tumour was 30.55 (CI 19.96-44.76; p<0.05). The cumulative incidence at 10 years was 2.83% (1.7%-4.3%). The median interval between primary tumour and second malignancy was 6.19 years (range 0.02-12.9 years). * The second tumour occurred in the radiation field. ** Patient familial atypical multiple mole melanoma (FAMMM) syndrome

DISCUSSION

Interest in oncology in the AYA group is emerging (14-16). In order to better define the current magnitude of the cancer problem for this age group we started an inventory study on the incidence and survival of 12-24 year-olds, with emphasis not only on the primary tumour, but also on the second primary tumours.

The incidence of cancer in AYA (20.5 per 100,000 per year 1989-2003) was particularly low when compared to the overall cancer incidence, with a reported annual incidence of 415.8/100.000 (European Standardized Rate (ESR) (17) in 2002 in the same district.

Within the AYA group the incidence increases with increasing age from 13.7 in 12-15 year-olds to 24.1 and 24.4 in 16-19 and 20-24 year-olds, respectively. These age groups were constructed to be used as a proxy for treating unit. All 12-15 year-olds are generally treated at a paediatric oncology unit, 20-24 year olds at a medical oncology unit, whereas 16-19 year-olds will be seen at both departments. Most studies however routinely utilize five-year increments for their analyses, considering patients up to 14 years as children, and 15 years and older into the adolescent and younger adult group (1;5;18).

When comparing our data with those from other countries the total incidence of cancer in 12-15 year-olds greatly resembles SEER (USA)(1) (10-14 year-olds) and British data (18) (10-14 year-olds), but the incidence in our 16-19 year-olds population is slightly higher than that reported in the USA as well as in Europe (24.1 compared to 20.3 in the USA 1975-2000 (1), and 18.6 in Europe 1988-1997 in 15-19 year-olds (19)). This might be due to the difference in age group or study period. The incidence we showed in 20-24 year-olds (24.4) is lower than in the USA (35.2 per 100,000 per year, 1975-2000 (1)) and comparable to British data (22.6 per 100,000 per year, 1979-1997 (18)).

We found a significant increase in incidence of all malignancies over time in 12-24 year-olds. When corrected for age-group and gender, increase in germ cell malignancies, lymphomas and carcinomas was found, confirming the findings from other recent reports in the Western countries (1;5-7;10;18;20). Increasing incidence for germ cell malignancies in males (e.g. testicular cancer) seems to be a world-wide phenomenon (1;21), and is not fully understood thus far. An increase in incidence for lymphomas was observed in both sexes. This increase in incidence was caused mainly by an increase in Hodgkin's lymphoma in 16-19 year-olds, which was previously reported by Reedijk *et al* and Clavel *et al* (5;22). Interestingly, in England Birch *et al* observed a decrease for both Hodgkin's lymphoma and Non-Hodgkin's lymphoma in 15-24 year-olds in the same period (10). Moreover, SEER data show an Estimated Annual Percentage Change (EAPC) of 3.6% for Non-Hodgkin's lymphoma, compared to only 0.2% for Hodgkin's lymphoma in 20-24 year-olds and a decrease in 10-19 year olds during 1975-2000 (1). The variation in reported trends for lymphomas over time might be due to improvement of the quality of data collection, uniformity in classification, and homogeneity in international coding and classification practices, as suggested by Clavel *et al*. (22).

We found no improvement in survival between 1989 and 2003. When compared to children, AYA benefited less from the general survival improvement during the past decades as shown by several large studies in Western countries. As stated before, from 1975 to 2000, SEER data (USA) reported a twice higher annual increase of 5-year relative survival in children in comparison to adolescents (1). In contrast, Reedijk *et al*. reported equal 5-year survival increases in adolescents as well as in children (adolescents: 64 to 82%, children: 56% to 75% 1973-1999)(5). We performed our study on data from 1989 up to date, which might suggest that following the large improvements made in the seventies and eighties with the introduction of chemotherapy, the increase in survival of AYA has come to a steady state during the last 10 years.

The cooperation of experts in developing large comprehensive children's cancer studies in the USA as well as in Europe has led to world-wide improvement in children's cancer care. Whereas 90% of children under the age of 15 are entered into a clinical trial, only 10% of adolescents (15-19 years) and 2% of young adults (20-24 years) in the USA are entered on to clinical trials of the paediatric or adult cooperative groups (23). Survival rates for specific tumour types in AYA are still less than 70%. These malignancies include Non-Hodgkin lymphomas, soft-tissue and bone sarcomas, CNS tumours and leukaemias. Treatment optimization, especially for these malignancies, is of great importance. Because of the rarity of these tumours, international cooperation and inclusion in clinical trials for these patients is warranted.

Importantly, adolescents may access oncologic care from paediatric or adult medical centres. In adolescent patients with leukaemia, especially acute lymphoblastic leukaemia, childhood protocols improve survival (24-27). Furthermore, a recent study from Utah (USA) investigated the site of oncologic specialty care for older adolescents (15-19 years), concluding that 66% of these patients were never seen by a paediatric oncologist, and there was a trend to worse survival for adolescents not treated by a paediatric oncologist for all malignancies except non-Hodgkin's lymphoma, germ cell tumours, and carcinomas (28). Unfortunately we were not able to assess the treatment unit for the patients in our cohort, as data concerning inclusion in clinical trials was incomplete in the cancer registry.

Besides these treatment variables, other factors as patient and/or doctors delay in referral and/or diagnosis may play a role in the final outcome of the patients, presuming that AYA patients are diagnosed at a relatively higher stage of disease than children do. A very recent study from the Canadian Childhood Cancer Surveillance and Control Program also confirms this idea and shows a longer delay in both referral and diagnosis in 10-19 year-olds when compared to children <10 years, due to both patients and physicians (29). Furthermore, more aggressive tumour biology is also suggested to play a role in a disadvantage in outcome for adolescents (30).

Remarkably, we observed an unexpectedly high risk of second malignancies in our cohort (SIR 30.55) which has been not reported previously. Because the median time from first to second malignancy was longer (6.19 years) than the median follow-up (5.5 years), we might have missed the bulk of second malignancies in our study. This means that the observed SIR in our study might be even an underestimation of the actual risk. Since more young people survive

from cancer nowadays, the potential risk of late effects after primary cancer treatment have increasingly become a major topic of interest over time (31-33).

A reasonable origin of these second tumours could be both intrinsic (genetic susceptibility) and/or extrinsic (environmental exposure, treatment induced). For example, melanoma of skin shows association with sun exposure but also with total and dysplastic nevi and genetic predisposition and this may lead to an increased risk to develop second or even third melanomas (34). In general, the younger sequential tumours become overt, the higher the chance that intrinsic –genetic- factors play an important etiological role. The occurrence of second tumours of the same histology in eight patients (melanoma and non-seminomatous testicular cancer), with only one patient known with a familiar predisposition for melanoma (FAMM syndrome), confirms the hypothesis that extrinsic factors as well as unknown genetic predisposition play an important role in carcinogenesis in these young patients and should be further investigated.

Despite the growing interest in AYA oncology in general, population-based studies with an overview of all second primary tumours in this group are scarce. The only reported data found a SIR of 12.4 in 15-24 year-olds and a peak incidence in 15-19 year-olds (35). In contrast with the scarcity of data on AYA, data on second primary malignancies in childhood cancer survivors (up to 18-20 years of age) are widely available, reporting a 5- to 6-fold increased risk for second primary malignancies (8;36-38).

In conclusion, we found an increase in incidence of malignancies in AYA, with no survival improvement during 1989-2003. Large-scale enrolment and treatment of adolescents and young adults in international cooperative multi-centric clinical trials could be the solution in optimising care in this neglected group of young patients world-wide. The prevalence of survivors of adolescent cancer is increasing. In our cohort we showed that these survivors are at high risk for developing second primary malignancies. Further research is needed to study the increasing incidence of cancer in AYA and the incidence of second malignancies to optimize care for these young patients.

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CHAPTER 3

Cancer in adolescents and young adults (15-29 years): a population-based study in the Netherlands 1989-2009

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ABSTRACT

Background: Cancer among adolescents and young adults (AYAs; 15-29 years old) is relatively rare but its incidence is increasing worldwide. To define the extent and nature of the AYA patients, this population-based study was performed to explore trends in cancer incidence, survival and risk of second primary cancers in AYAs.

Material and Methods: Data from all AYAs diagnosed with cancer between 1989-2009 were obtained from the Netherlands Cancer Registry. Age-standardized incidence rates with estimated annual percentage of change (EAPC) and 5-year relative survival rates were calculated. Relative survival was used as a good approximation of cause-specific survival. All analyses were stratified by gender, 5-year age-group and calendar period. In addition, Standardized Incidence Ratios were determined to evaluate the risk of second primary cancers.

Results: 23,161 AYAs were diagnosed with cancer between 1989-2009. Since 1989 the cancer incidence has increased significantly from 28 to 43 per 100,000 person years in males (EAPC: 1.9) and from 30 to 40 per 100,000 person years in females (EAPC: 1.4). The most frequently diagnosed cancers in male AYAs included testicular cancer, melanoma and Hodgkin's disease, whereas in females melanoma, breast cancer and Hodgkin's disease were the most frequently occurring cancers. Five-year relative survival rates were 80% and 82% for males and females, respectively. Over time, the 5-year relative survival increased from 74% to 86% and from 79% to 86% in males and females, respectively. The risk of developing a second primary cancer was increased 3-6 times in males and 2-5 times in females, depending on rules for counting second primary cancers.

Conclusions: Although the overall survival has improved over time, the progress made in AYAs for specific cancers is still less compared to improvements made in children and adults. This and the increasing incidence and high risk of second primary cancers warrants further research.

BACKGROUND

Cancer in adolescents and young adults (AYAs) with an age at diagnosis between 15 and 29 years is relatively rare but its incidence is increasing (1-4). The age-adjusted incidence rate of cancer (European Standardized Rate; ESR) in AYAs in the Netherlands was 41 per 100,000 person years in 2009. Compared to the total incidence of cancer in the Netherlands, AYAs only represent a small part, i.e. slightly over 1%. However, compared to cancer incidence in children (ESR, <15 years) which was 12 per 100,000 person years in 2009, the incidence among AYAs is more than 3 times higher *(http://www.cancerregistry.nl)*.

Former studies showed that the progress made in survival of AYAs with cancer is limited as compared to the steadily increase in survival in adults and the large improvements that have been achieved in many childhood cancers (5;6). This lack of survival improvement might be due to differences in biological characteristics or etiology. Insufficient tailor-made professional attention for AYAs with cancer and lack of specialized guidelines may also be responsible for suboptimal outcome in this particular subset of young cancer patients.

Furthermore, although the improvement in survival of AYAs with cancer lags behind, the majority of the patients survive their cancer and attention should be paid to their life time risk of developing second primary cancers. In a small Dutch study including patients diagnosed between 1989-2003 at the age of 12-24 in the Northern part of the Netherlands a strongly increased risk of developing a second primary cancer was found (standardized incidence ratio: 31, 95% Confidence Interval: 20.0-44.8) (7).

In order to improve the current care of AYAs with cancer and to develop services tailored to their needs, it is important to define the extent and nature of this patient population by use of analyses of population-based, accurate and recent data. As recent, population-based information on the cancer occurrence in AYAs is scarce, we performed the current study including all cancer patients diagnosed at the age of 15 till 29 years between 1989-2009 in the Netherlands in order to give an extensive overview. Cancer-specific trends in incidence, survival and risk of second primary cancers in the AYA population were evaluated by gender, age group and calendar period.

MATERIAL AND METHODS

Data were obtained from the Netherlands Cancer Registry (NCR), a nationwide population-based registry that includes all new diagnoses of cancer since 1989 except for basal cell carcinomas of the skin. The completeness of the Netherlands Cancer Registry since 1989 is estimated to be over 95%. All patients diagnosed with a primary malignant cancer at the age of 15 to 29 in the period 1989 to 2009 were included. Benign tumors, tumors of uncertain malignancy (including borderline ovarian tumors, myelodysplastic syndrome, carcinoid of the appendix) were excluded. Patient- and tumor characteristics such as age at diagnosis, date of diagnosis, topography, morphology, invasiveness, WHO grade of differentiation, lateralization and stage as well as follow-up information concerning vital status and second primary cancers were retrieved. Vital status of all patients recorded in the NCR is updated annually by record linkage to the Dutch Municipal Personal Records Database which keeps information about vital status of all inhabitants in the Netherlands. All cancers in the NCR are classified using the International Classification of Diseases for Oncology (ICD-O). Cancers diagnosed between 1989-1992 were coded using the first edition of the ICD-O. Between 1993-2000 the second edition of the ICD-O was used and since 2001 the ICD-O third edition. Although, this classification on topography can be used for all cancers, it is largely satisfactory for late age of onset cancers, which mainly exist of carcinomas. As in young people the carcinomas only represent a small part of all diagnosed cancers a classification based on the histology is more appropriate to use. In the current study all primary tumors were therefore re-classified using the histology-based classification scheme as proposed by Birch *et al*. (8) which gives an accurate and balanced overview of cancers in the younger age groups.

Cancer incidence

Cancer incidence rates were calculated by gender, 5-year age groups, and calendar period (1989-1995, 1996-2002 and 2003-2009). Rates were age-adjusted by standardization to the European standard population (European Standardized Rates, ESR). Gender, age and calendar year specific population data were annually retrieved from Statistics Netherlands (*http://statline.cbs.nl*).

Relative survival

Because the cause of death of the patients is not available in the cancer registry, disease specific survival could not be calculated. Instead, relative survival analyses were performed according to Dickman as a good approximation of disease-specific survival (9). This method adjusts crude survival rates amongst cancer patients for the expected mortality according to annual life tables of the general population matched on age, gender and calendar period (as annually retrieved from Statistics Netherlands). In the survival analyses end of follow-up was defined as date of death, date of emigration or 1-1-2010 whichever came first. Five-year tumor specific relative survival rates by gender and age group were calculated. To evaluate trends over time in survival, 5-year survival rates were calculated for the top ten most frequently diagnosed tumors in males and females by calendar period (1989-1993, 1994-1998, 1999-2003, 2004-2009) as well.

Risk of second primary cancers

All analyses on second primary cancers only included primary cancers, i.e. recurrences or progressive disease were not included in the analyses. In order to evaluate the risk of second primary cancers, Standardized Incidence Ratios (SIR) with 95% Confidence Intervals (CI) assuming a Poisson distribution were calculated. Person-years at risk were accumulated for each person in the cohort from the date of diagnosis of the first primary cancer up to the date of diagnosis of second primary cancer, date of death, date of emigration or 1-1-2010, whichever came first. The expected number of second primary cancers was calculated by applying the 5-year age, calendar year and gender-specific incidence rates for each cancer in the Netherlands to the person-years at risk among the AYA cohort. SIRs were calculated as the ratio of the observed number of second primary cancers and the expected incident number of given tumors in a specified gender- and age group.

All analyses on second primary cancers were performed three times. Once including all second primary cancers, regardless of the time between first and second primary cancer and type of cancer. In this way we are able to evaluate the risk of developing a second primary cancer, including the contra-lateral or same site tumors (for example for testicular cancer, breast cancer and melanoma). Secondly, in order to exclude the effect of detection bias, cancers presented concurrently at the time of first diagnosis (rather than subsequent to the first diagnosis) and second primary cancers diagnosed within 3 months of the first cancer were excluded. Thirdly, in order to be able to compare the calculated SIRs with results reported by other studies, the International rules concerning multiple cancers as proposed by the International Agency for Research on Cancer / International Association of Cancer Registries (IARC/IACR) were applied (10). Using this latter definition the recognition of a second primary cancer is independent of time and only one primary tumor arising in an organ as defined by the three character topography code can be considered as primary cancer. Analyses were performed using the statistical software package SAS 9.2.

RESULTS

Overall 23,161 AYAs were diagnosed with cancer between 1989 and 2009 in the Netherlands. Fifty-one percent were male, 18% were diagnosed between 15-19 years, 30% between 20-24 years and 52% between 25-29 years.

Cancer incidence over time

Table 1 displays the cancer incidence rates by tumor type, gender and by period (table 1a) and age (table 1b). Incidence rates were based on tumor instead of patients (n=23,360 tumors diagnosed between 1989-2010, at age 15-29). Overall, the cancer incidence among males AYAs has increased sharply since 1989. The most prominent trend over time concerned testicular cancer (gonadal germ cell tumor) in males; testicular cancer incidence has doubled from 7 per 100,000 person years in 1989-1995 to 14 per 100,000 in 2003-2009 (EAPC=4.90, $p<0.01$). When excluding testicular cancer a moderate rise in incidence is still evident. Significant rising trends ($p<0.01$) were seen for chronic myeloid leukemia (CML), Hodgkin's disease, head and neck carcinomas and

carcinomas of the respiratory tract. A decreasing trend was observed for astrocytoma. In females, a clear increase in cancer incidence is seen as well; significant increases were observed for CML, Hodgkin's disease, melanoma, thyroid cancer and breast cancer. Astrocytoma and gonadal germ cell tumors were significantly decreased. In figure 1 the trends in cancer incidence over time by age and gender are presented. Over time the incidence of cancer among AYAs has increased significantly (EAPC=1.88, p<0.01 for males and EAPC=1.37, p<0.01 for females).

Table 1: Incidence rates by tumor and gender
a) By period

	Male					Female				
Birch category cancer	**1989-1995**	**1996-2002**	**2003-2009**			**1989-1995**	**1996-2002**	**2003-2009**		
	ESR[a]	**ESR[a]**	**ESR[a]**	**EAPC[b]**	**p-value**	**ESR[a]**	**ESR[a]**	**ESR[a]**	**EAPC[b]**	**p-value**
All cancer sites	30.1	34.0	39.1	1.88	<0.01	30.7	34.0	37.1	1.38	<0.01
All cancer sites (excluding gonadal germ cell tumors)*	22.8	23.2	24.9	0.58	<0.01					
Acute lymphatic leukemia	0.99	1.21	1.04	0.45	0.61	0.49	0.73	0.76	2.58	0.01
Acute myeloid leukemia	0.76	0.87	0.80	0.20	0.87	0.67	0.77	0.90	1.83	0.08
Chronic myeloid leukemia	0.28	0.38	0.55	5.50	<0.01	0.24	0.30	0.44	4.48	<0.01
Other leukemia	0.14	0.15	0.11	1.38	0.54	0.09	0.05	0.09	-1.68	0.30
Non-Hodgkin lymphoma	2.60	2.30	2.86	0.68	0.27	1.40	1.69	1.83	2.15	0.01
Hodgkin's disease	3.44	4.07	4.32	1.52	<0.01	3.31	3.97	4.16	1.71	<0.01
Astrocytoma	1.32	1.02	0.71	-4.17	<0.01	1.00	0.83	0.57	-4.33	<0.01
Other glioma	0.35	0.36	0.43	0.77	0.69	0.36	0.36	0.31	-1.48	0.38
Ependymoma	0.21	0.16	0.17	-3.11	0.10	0.20	0.17	0.16	-0.38	0.87
Medulloblastoma	0.16	0.28	0.25	1.75	0.47	0.17	0.11	0.19	0.18	0.93
Other central nervous system tumor	0.57	0.45	0.74	1.80	0.25	0.28	0.35	0.59	5.81	0.01
Osteosarcoma	0.69	0.42	0.65	-0.45	0.80	0.36	0.42	0.37	0.42	0.81
Chondrosarcoma	0.22	0.20	0.44	4.79	0.03	0.15	0.23	0.32	3.96	0.08
Ewing's tumor	0.33	0.35	0.41	2.18	0.27	0.23	0.21	0.14	-0.10	0.96
Other bone tumor	0.13	0.07	0.15	0.60	0.75	0.17	0.17	0.16	-2.97	0.03
Fibrosarcoma	0.02	0.10	0.03	-0.89	0.72	0.04	0.11	0.04	0.71	0.72
Rhabdomyosarcoma	0.24	0.28	0.24	-0.49	0.83	0.16	0.10	0.14	-1.72	0.38
Other soft tissue sarcoma	1.94	1.70	1.48	-1.80	0.04	1.40	1.46	1.37	0.46	0.58
Gonadal germ cell tumor	7.31	10.87	14.37	4.92	<0.01	1.38	1.23	0.99	-2.05	<0.01
Non-gonadal germ cell tumor	0.53	0.50	0.49	-1.77	0.28	0.24	0.18	0.13	-1.95	0.35
Melanoma	3.70	3.99	4.42	1.31	<0.01	7.41	9.13	10.23	2.35	<0.01
Non-melanoma skin cancer	0.23	0.23	0.23	0.32	0.88	0.27	0.19	0.32	1.40	0.55
Carcinoma of thyroid	0.63	0.52	0.73	1.64	0.44	1.71	1.89	2.37	2.41	<0.01
Carcinoma of head and neck	0.32	0.65	0.69	5.27	<0.01	0.47	0.62	0.63	2.29	0.11
Carcinoma of trachea, bronchus, lung and pleura	0.17	0.21	0.30	5.11	<0.01	0.26	0.21	0.42	3.39	0.15
Carcinoma of breast	0.01	-	0.05	0.71	0.88	3.08	3.76	4.38	2.58	<0.01
Carcinoma of genito-urinary tract	0.75	0.78	0.92	1.05	0.22	2.92	2.67	3.37	0.85	0.23
Carcinoma of gastrointestinal tract	1.48	1.42	1.30	-1.23	0.15	1.71	1.57	1.41	-1.40	0.17
Carcinoma of other ill-defined sites	0.15	0.11	0.08	-1.55	0.42	0.12	0.10	0.11	1.29	0.52
Embryonal tumors	0.04	0.02	0.03	-0.79	0.70	0.07	0.04	0.02	0.47	0.69
Other rare miscellaneous specified neoplasms	0.03	0.02	0.04	2.90	0.29	0.02	0.03	-	2.31	0.74
Unspecified malignant neoplasms not elsewhere specified	0.34	0.36	0.30	-2.96	0.17	0.26	0.29	0.16	-1.17	0.62

Table 1 *(continued)*
b) By age

	Males				Females			
Birch category cancer	**15-19 yrs**	**20-24 yrs**	**25-29 yrs**	**15-29 yrs**	**15-19 yrs**	**20-24 yrs**	**25-29 yrs**	**15-29 yrs**
	ESR[a]	**ESR[a]**	**ESR[a]**	**ESR[a]**	**ESR[a]**	**ESR[a]**	**ESR[a]**	**ESR[a]**
All cancer types	22.1	33.5	46.8	34.2	18.1	29.5	53.4	33.7
Acute lymphatic leukemia	1.73	1.02	0.48	1.08	0.85	0.70	0.42	0.66
Acute myeloid leukemia	0.75	0.69	0.97	0.80	0.62	0.73	0.98	0.78
Chronic myeloid leukemia	0.21	0.42	0.55	0.39	0.22	0.32	0.42	0.32
Other leukemia	0.07	0.18	0.15	0.13	0.04	0.08	0.11	0.08
Non-Hodgkin lymphoma	1.97	2.32	3.45	2.58	1.23	1.56	2.07	1.62
Hodgkin's disease	3.31	4.32	4.11	3.91	3.80	4.23	3.32	3.78
Astrocytoma	0.54	0.99	1.59	1.04	0.48	0.77	1.18	0.81
Other glioma	0.20	0.34	0.58	0.38	0.17	0.30	0.57	0.35
Ependymoma	0.14	0.20	0.19	0.18	0.23	0.17	0.12	0.18
Medulloblastoma	0.25	0.27	0.17	0.23	0.16	0.22	0.09	0.16
Other central nervous system tumor	0.53	0.56	0.66	0.58	0.32	0.37	0.51	0.40
Osteosarcoma	1.12	0.42	0.23	0.59	0.77	0.19	0.18	0.38
Chondrosarcoma	0.30	0.22	0.31	0.28	0.21	0.19	0.28	0.23
Ewing's tumor	0.61	0.33	0.15	0.36	0.31	0.19	0.08	0.19
Other bone tumor	0.14	0.11	0.10	0.12	0.19	0.15	0.16	0.16
Fibrosarcoma	-	0.06	0.08	0.05	0.06	0.06	0.07	0.06
Rhabdomyosarcoma	0.42	0.20	0.13	0.25	0.22	0.13	0.05	0.13
Other soft tissue sarcoma	1.21	1.44	2.53	1.73	1.04	1.16	2.03	1.41
Gonadal germ cell tumor	4.18	11.28	16.21	10.56	0.81	1.23	1.61	1.21
Non-gonadal germ cell tumor	0.56	0.56	0.40	0.51	0.11	0.08	0.36	0.18
Melanoma	1.56	3.86	6.61	4.01	3.00	8.67	14.81	8.83
Non-melanoma skin cancer	0.11	0.22	0.37	0.23	0.10	0.15	0.53	0.26
Carcinoma of thyroid	0.38	0.55	0.94	0.62	1.14	1.85	2.93	1.97
Carcinoma of head and neck	0.41	0.43	0.77	0.54	0.33	0.52	0.85	0.57
Carcinoma of trachea, bronchus, lung and pleura	0.11	0.13	0.43	0.22	0.11	0.22	0.54	0.29
Carcinoma of breast	-	0.03	0.03	0.02	0.11	1.65	9.32	3.69
Carcinoma of genito-urinary tract	0.24	0.73	1.47	0.81	0.32	1.85	6.74	2.97
Carcinoma of gastrointestinal tract	0.81	1.05	2.32	1.40	0.92	1.37	2.42	1.57
Carcinoma of other ill-defined sites	0.05	0.14	0.15	0.11	0.08	0.11	0.14	0.11
Embryonal tumors	-	0.05	0.03	0.03	0.06	0.05	0.02	0.04
Other rare miscellaneous specified neoplasms	0.01	0.02	0.06	0.03	0.01	0.02	0.02	0.01
Unspecified malignant neoplasms not elsewhere specified	0.14	0.33	0.54	0.34	0.09	0.21	0.42	0.24

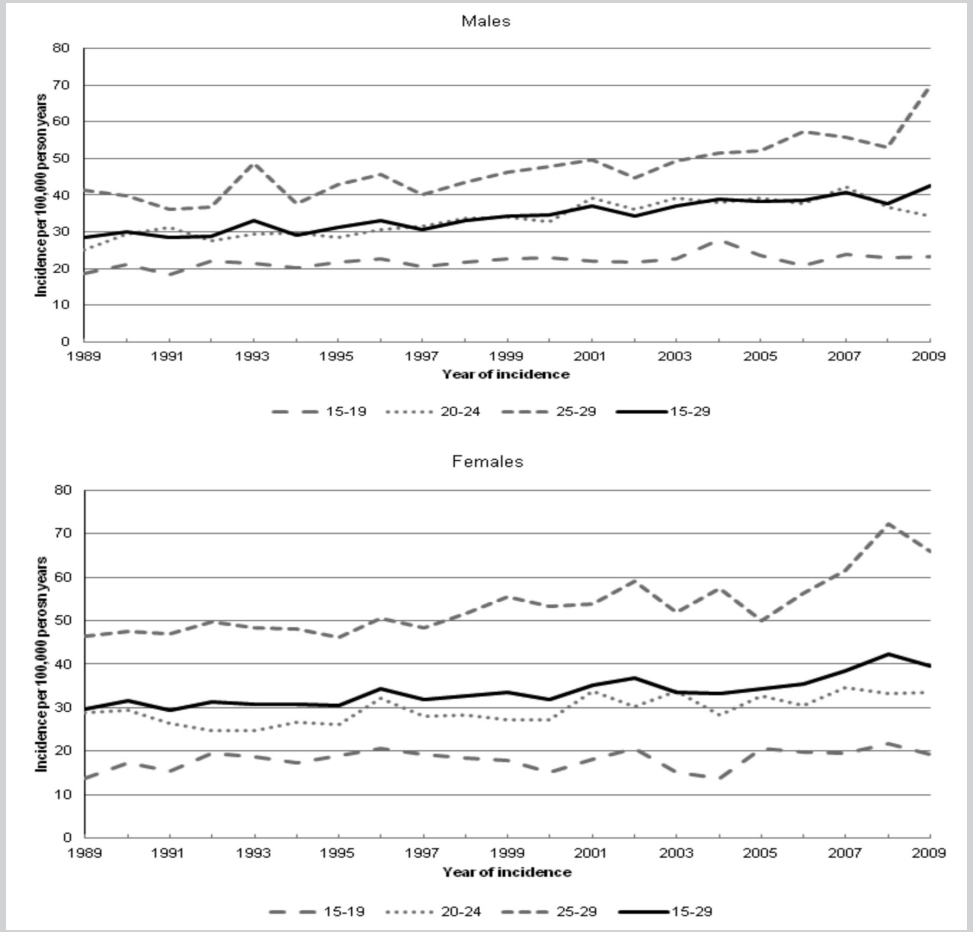
* Only presented for males, a ESR: European Standardized Rate (per 100,000), b EAPC: Estimated Annual Percentage of Change

Cancer incidence by age

Within the AYA population, the incidence increased sharply with age. Each 5-year age category showed a different pattern of observed cancer types. In male AYAs with age 15 to 19 testicular cancer was the most frequently observed cancer (19%), followed by Hodgkin's disease (15%) and non-Hodgkin lymphoma (9%). In 20-24 year olds testicular cancer comprised one-third of all cancers, followed by Hodgkin's disease (13%) and melanoma (12%). In the oldest group (age 25-29), testicular cancer was still diagnosed in 35% of all patients. Melanoma (14%) and Hodgkin's disease (9%) were second and third most frequent cancers.

In female AYAs with age 15 to 19 Hodgkin's disease (21%), melanoma (17%) and non-Hodgkin's lymphoma (7%) were most frequently diagnosed. In females aged 20 to 24 melanoma accounted for 29% of all cancers, followed by Hodgkin's disease (14%) and thyroid cancer (6%). In the oldest female AYAs (25 to 29) melanoma still represented the most frequent tumor (28%) with breast cancer (17%) and cancer of the genito-urinary tract (mainly cervical cancer) (13%) as number two and three.

Figure 1
Trends in incidence of cancer in AYAs over time (1989-2009) by age (5-year age groups and total) and gender



Relative survival

In table 2, the 5-year tumor specific relative survival rates by gender and age are presented. Overall, the five year relative survival in males was slightly worse compared to females (80% versus 82%, p<0.001). A fairly good survival (at least 80%) was observed in male patients with Hodgkin's disease, ependymoma, chondrosarcoma, other bone tumors, fibrosarcoma, gonadal germ cell tumors, melanoma, non-melanoma skin cancer, thyroid cancer, head and neck cancer and cancer of the genito-urinary tract (>80% was kidney or bladder cancer). Patients with acute myeloid leukemia (AML), acute lymphatic leukemia (ALL), other leukemia, other central nervous system tumors, Ewing's tumor, osteosarcoma, rhabdomyosarcoma, and unspecified malignant neoplasms fare worse with a 5-year survival of approximately 50% or less. Largely similar to males, a 5-year survival of at least 80% was seen in female patients with Hodgkin's disease,

chondrosarcoma, other bone tumors, fibrosarcoma, gonadal germ cell tumors, non-gonadal germ cell tumors, melanoma, non-melanoma skin cancer, thyroid cancer, head and neck cancer and cancer of the genito-urinary tract. Female patients with AML, central nervous system tumors, Ewing's tumor, rhabdomyosarcoma, and unspecified malignant neoplasms have a fairly poor survival of less than 50%. In addition, the 5-year relative survival over time was evaluated (data not shown). Since 1989, a distinct improvement in the overall relative survival of approximately 74% to 86% in males and 79% to 86% in females was observed. A clear survival improvement in patients with non-Hodgkin lymphoma in both males and females was seen. Among males, the survival of gonadal germ cell tumors and melanoma showed a slight but steady increase over time. The 5-year survival rates of the majority of other cancers (with the exception of Hodgkin's disease and AML) seemed to be improved as well although the 95% Confidence Intervals are wide. In contrast to males, females showed a minor survival improvement for Hodgkin's disease. The survival of female breast cancer patients improved significantly as well, though the survival of all other cancers among females remained similar or decreased.

Overall, with a median follow-up time of 6.8 years, 884 patients (3.8% of all AYAs) were diagnosed with at least one second primary cancer. In 105 of these 884 patients (11.9%), the second tumor occurred within 3 months after diagnosis of the first tumor. A large part of the remaining 779 patients with non-simultaneous second primary cancers consist of melanoma, contra-lateral testicular cancer, and contra-lateral breast cancer. With the application of the IARC/IACR rules 412 AYAs (1.8% of all AYAs) are defined to have a second primary tumor. In table 3 the SIRs are presented for all primary sites combined. Male AYAs diagnosed with cancer have a more than 6 times increased risk of developing a second primary cancer. After application of the IACR/IARC rules, this risk is still more than 3 times increased. For females, the risk of developing a second primary cancer is almost 5 and 2 times increased, respectively. Overall, melanoma is the most frequently diagnosed second primary cancer among AYAs. The risk of developing a melanoma after a first primary cancer was 6 times increased for males and 8 times increased for females. This risk was even higher in patients with a melanoma as first primary cancer (females SIR: 22.8 and males SIR: 27.2, not shown in table 3). After application of the IARC/IACR rules for multiple cancers, the most frequently diagnosed second primary cancers in males were hematological malignancies (n=40, SIR: 3.6, 95%CI: 2.6-4.9), gastrointestinal tumors (n=32, SIR: 5.2, 95%CI: 3.6-7.4) and gonadal germ cell tumors (n=28, SIR: 1.9, 95%CI: 1.2-2.7). For females these second primary cancers included breast cancer (n=72, SIR: 1.8, 95%CI: 1.4-2.2), melanoma (n=25, SIR: 1.2, 95%CI: 0.8-4.5) and hematological malignancies (n=23, SIR: 2.6, 95%CI: 1.8-4.0). We also explored the risks of second primary cancers after tumor-specific first primary cancers (data not shown). The most striking result was the more than 7 times increased risk of breast cancer after Hodgkin's lymphoma (n=26 SIR: 7.4, 95%CI: 4.8-10.8).

Table 2: Five year relative survival rates by age and gender
Males

Birch category cancer #		**15-29 yrs**		**15-19 yrs**		**20-24 yrs**		**25-29 yrs**
	N	**5-year relative survival (95% CI*)**	**N**	**5-year relative survival (95% CI*)**	**N**	**5-year relative survival (95% CI*)**	**N**	**5-year relative survival (95% CI*)**
All cancer sites	11790	79.9 (79.2-80.7)	2299	76.2 (74.3-77.9)	3757	80.8 (79.5-82.1)	5734	80.9 (79.8-82.0)
Acute lymphatic leukemia	355	50 (44.4-55.4)	182	58.2 (50.1-65.5)	114	42.6 (33-51.9)	59	39.7 (26.9-52.3)
Acute myeloid leukemia	271	43.8 (37.5-49.8)	78	42.6 (31.1-53.6)	78	38.5 (27.4-49.5)	115	48.4 (38.5-57.6)
Chronic myeloid leukemia	136	68.3 (58.7-76.1)	22	63.2 (37.6-80.8)	47	69.3 (51.8-81.6)	67	69.5 (55.8-79.8)
Other leukemia	42	48.5 (32.0-63.3)	7		18		17	39.1 (19.3-58.6)
Non-Hodgkin lymphoma	886	71.8 (68.6-74.8)	203	74.1 (67.1-79.9)	260	71.6 (65.4-76.8)	423	70.9 (66.1-75.2)
Hodgkin's disease	1341	90.9 (89.1-92.4)	347	91.7 (87.9-94.3)	485	92.4 (89.4-94.6)	509	89 (85.6-91.6)
Astrocytoma	364	54.3 (48.8-59.5)	56	51.1 (37.3-63.4)	111	53.7 (43.4-62.9)	197	55.6 (48-62.5)
Other glioma	130	64.8 (55.5-72.7)	22	57.2 (33.6-75.1)	38	58.1 (40.1-72.4)	70	70.8 (57.8-80.4)
Ependymoma	60	86 (73.8-92.9)	15		21	90.8 (67.2-97.8)	24	91.6 (69.7-98.1)
Medulloblastoma	75	65 (52.1-75.2)	26	59.1 (36.8-75.9)	29	80.3 (58.2-91.6)	20	50.8 (26.2-71)
Other central nervous system tumor	203	41 (33.7-48.2)	54	45.4 (31.3-58.5)	67	41.9 (29-54.2)	82	37.3 (26-48.5)
Osteosarcoma	193	45.4 (37.6-52.9)	116	45.6 (35.6-55.1)	48	44.8 (29.2-59.3)	29	45.9 (25.1-64.5)
Chondrosarcoma	95	91.9 (83.2-96.2)	31	87.2 (69.1-95.2)	25	95.2 (69-99.6)	39	94.3 (78.2-98.8)
Ewing's tumor	119	45.8 (35.9-55.1)	64	48.2 (34.5-60.7)	37	44.2 (27-60.2)	18	
Other bone tumor	40	84.5 (68.4-92.9)	15	92.6 (56.8-99.2)	12		13	92.7 (56.8-99.3)
Fibrosarcoma	17	94.2 (64.4-99.4)	0		7		10	
Rhabdomyosarcoma	83	36.1 (25.4-47)	44	49.2 (32.6-63.8)	23		16	
Other soft tissue sarcoma	595	66.7 (62.6-70.5)	129	75 (66.2-81.8)	158	65.4 (57-72.6)	308	63.9 (58-69.2)
Gonadal germ cell tumor	3700	96.3 (95.6-96.9)	435	93.8 (90.8-95.8)	1266	96.8 (95.6-97.7)	1999	96.6 (95.6-97.4)
Non-gonadal germ cell tumor	172	76.3 (69.1-82.1)	61	71.2 (57.6-81.2)	62	82.5 (70.5-90)	49	74.6 (59.3-84.8)
Melanoma	1403	87.8 (85.8-89.5)	162	90.9 (84.9-94.6)	433	86.4 (82.5-89.5)	808	87.9 (85.2-90.1)
Non-melanoma skin cancer	82	90.8 (81.3-95.7)	12		25	91.7 (69.9-98.1)	45	90.7 (76.5-96.6)
Carcinoma of thyroid	215	96.2 (92.3-98.3)	39		62	95.3 (85.5-98.7)	114	95.4 (88.9-98.3)
Carcinoma of head and neck	185	81.7 (74.7-87)	42	92.5 (78-97.7)	49	75.6 (58.7-86.4)	94	80.1 (69.7-87.3)
Carcinoma of trachea, bronchus, lung and pleura	77	62.6 (50.2-72.8)	10		14		53	57.8 (42.8-70.3)
Carcinoma of breast	6				3		3	
Carcinoma of genito-urinary tract	280	85.7 (80.6-89.5)	24	81.2 (56.6-92.7)	81	87.4 (77.7-93.2)	175	85.3 (78.5-90.1)
Carcinoma of gastrointestinal tract	483	60.6 (55.9-65)	84	80.7 (70.3-87.8)	119	60.2 (50.4-68.7)	280	54.4 (48.1-60.3)
Carcinoma of other ill-defined sites	38	50.9 (33.7-65.8)	4		16	67.9 (39-85.4)	18	
Embryonal tumors	10		0		6		4	
Other rare miscellaneous specified neoplasms	10		1		2		7	
Unspecified malignant neoplasms not elsewhere specified	124	31 (22.9-39.5)	14		40	34.8 (20.5-49.4)	70	24.5 (14.7-35.6)

Table 2 *(continued)*
Females

Birch category cancer #		15-29 yrs		15-19 yrs		20-24 yrs		25-29 yrs
	N	5-year relative survival (95% CI*)	N	5-year relative survival (95% CI*)	N	5-year relative survival (95% CI*)	N	5-year relative survival (95% CI*)
All cancer sites	11371	82.3 (81.6-83.1)	1806	81.8 (79.8-83.5)	3220	83.4 (82.0-84.7)	6345	82.0 (80.9-83.)
Acute lymphatic leukemia	210	52.3 (44.8-59.2)	85	59.5 (47.7-69.5)	75	45.2 (32.7-56.9)	50	50.4 (35.1-63.9)
Acute myeloid leukemia	256	47.9 (41.4-54.1)	60	60.9 (47.1-72.1)	79	38.4 (27.4-49.3)	117	47.7 (37.8-56.9)
Chronic myeloid leukemia	108	76.5 (66.2-84)	22		35	68 (47.3-82.1)	51	80.9 (66.2-89.7)
Other leukemia	26	44.0 (23.9-62.0)	4		9		13	
Non-Hodgkin lymphoma	536	74.8 (70.8-78.4)	121	76 (66.9-83)	170	72.8 (65.2-79.1)	245	75.7 (69.5-80.8)
Hodgkin's disease	1243	94.2 (92.6-95.4)	380	92.9 (89.5-95.2)	465	94.3 (91.5-96.2)	398	95.2 (92.4-97)
Astrocytoma	277	57.4 (51-63.2)	48	64.8 (49-76.8)	86	56.8 (45.5-66.7)	143	55.2 (46.2-63.4)
Other glioma	115	65.7 (55.8-73.9)	17		31	70.8 (51.3-83.7)	67	66.5 (52.6-77.2)
Ependymoma	57	69.4 (54.7-80.1)	23	71.9 (47.6-86.4)	19	59 (32.3-78.2)	15	77.9 (45.6-92.5)
Medulloblastoma	51	59.2 (43-72.3)	16		24	65.2 (42.1-80.9)	11	
Central nervous system tumor	131	44.6 (35.1-53.5)	32	39.9 (22.3-56.9)	41	50.9 (32.8-66.3)	58	44.2 (30.6-57)
Osteosarcoma	119	70 (60.4-77.8)	77	71.3 (59.4-80.4)	20	76.6 (48.7-90.6)	22	59.2 (34.3-77.3)
Chondrosarcoma	75	92.8 (83.3-97)	21	94.4 (65.9-99.3)	21	90.2 (66-97.6)	33	93.6 (76.2-98.5)
Ewing's tumor	62	39.4 (26.7-51.8)	31		21		10	
Other bone tumor	54	88.1 (75.1-94.5)	19	89 (62.4-97.2)	16	87 (57-96.7)	19	88 (59.3-97)
Fibrosarcoma	21	94.9 (68.2-99.4)	6		7		8	
Rhabdomyosarcoma	42	38.5 (23.7-53)	22		14		6	
Other soft tissue sarcoma	471	77.8 (73.6-81.4)	103	78.8 (68.9-85.9)	129	73.5 (64.5-80.5)	239	79.7 (73.8-84.5)
Gonadal germ cell tumor	408	81.4 (77.1-84.9)	81	83.2 (72.8-90)	134	83.5 (75.7-88.9)	193	79.1 (72.5-84.4)
Non-gonadal germ cell tumor	63	90 (78.8-95.5)	11		9		43	85.6 (70.5-93.4)
Melanoma	3004	95.7 (94.9-96.4)	300	95.9 (92.7-97.8)	946	96.6 (95-97.6)	1758	95.2 (94-96.2)
Non-melanoma skin cancer	90	97.7 (90.7-99.6)	10		16		64	100,0
Carcinoma of thyroid	666	99.5 (98.4-99.9)	114	100,0	203	100,0	349	98.9 (96.7-99.7)
Carcinoma of head and neck	192	84.9 (78.5-89.5)	33	81.2 (60.3-91.9)	57	92.1 (80-97.1)	102	81.9 (72.4-88.5)
Carcinoma of trachea, bronchus, lung and pleura	99	63.3 (52.2-72.4)	11		24	90.1 (65.1-97.6)	64	48.9 (35.4-61.1)
Carcinoma of breast	1299	74.6 (71.9-77.1)	11		181	73.9 (65.8-80.4)	1107	74.6 (71.6-77.3)
Carcinoma of genito-urinary tract	1035	82.1 (79.5-84.4)	32	67.4 (47.8-81)	199	84.9 (78.9-89.3)	804	81.9 (78.9-84.6)
Carcinoma of gastrointestinal tract	527	69.7 (65.3-73.6)	92	85.3 (75.9-91.2)	147	72.2 (63.7-79)	288	63.3 (57.2-68.8)
Carcinoma of other ill-defined sites	35	62.2 (43.8-76.1)	8		11		16	
Embryonal tumors	14		6		5		3	
Other rare miscellaneous specified neoplasms	5		1		2		2	
Unspecified malignant neoplasms not elsewhere specified	80	29.9 (20.3-40.2)	9		23		48	31.3 (18.9-44.5)

Ref (13)

* 95% CI: 95% Confidence Interval

Table 3: Second primary cancers after a first primary cancer in AYAs
All primary cancers

Males	**All second primary cancers**		**Excluding simultaneous tumors**		**IARC/IACR and excluding simultaneous tumors**	
Second primary cancer	**Observed**	**SIR***	**Observed**	**SIR***	**Observed**	**SIR***
All cancer types	372	6.2 (5.6-6.9)	321	5.3 (4.8-6.0)	187	3.1 (2.7-3.6)
Blood, bone marrow & lymph nodes	50	4.6 (3.4-6.0)	45	4.1 (3.0-5.5)	40	3.6 (2.6-4.9)
Bone & soft tissue cancer	20	6.5 (3.9-10.0)	18	5.8 (3.4-9.2)	16	5.2 (2.9-8.4)
Head & neck cancer	9	4.7 (2.2-9.0)	8	4.2 (1.8-8.3)	8	4.0 (1.7-7.9)
Non-melanoma skin cancer	11	5.8 (2.9-10.4)	10	5.3 (2.5-9.7)	9	4.6 (2.1-8.8)
Melanoma	57	6.1 (4.6-7.9)	51	5.4 (4.0-7.1)	21	2.2 (1.4-3.4)
Cancer male genital organs	2	6.1 (0.7-21.9)	2	6.1 (0.7-21.9)	2	6.1 (0.7-21.9)
Cancer respiratory tract	9	4.6 (2.1-8.8)	9	4.6 (2.1-8.8)	8	4.1 (1.7-8.0)
Cancer gastrointestinal tract	39	6.5 (4.6-8.8)	33	5.4 (3.7-7.6)	32	5.2 (3.6-7.4)
Kidney cancer	12	10.2 (5.2-17.8)	10	8.3 (4-15.3)	4	3.3 (0.9-8.5)
Bladder, renal pelvis, ureter	6	3.0 (1.1-6.5)	6	3.0 (1.1-6.5)	4	2.0 (0.5-5.0)
Other localizations	15	3.1 (1.7-5.1)	15	3.1 (1.7-5.1)	11	2.2 (1.1-4.0)
Breast cancer	2	20 (2.2-72.2)	2	16.7 (1.9-60.2)	2	16.7 (1.9-60.2)
Unknown primary cancer	2	2.9 (0.3-10.3)	2	2.9 (0.3-10.3)	2	2.8 (0.3-10.2)
Gonadal germ cell tumor	137	9.3 (7.8-11.0)	110	7.4 (6.1-9.0)	28	1.9 (1.2-2.7)
Non-gonadal germ cell tumor	1	2.9 (0.03-15.9)	0		0	

Females	**All second primary cancers**		**Excluding simultaneous tumors**		**IARC/IACR and excluding simultaneous tumors**	
All cancer types	512	4.8 (4.4-5.2)	458	4.2 (3.9-4.7)	225	2.0 (1.8-2.3)
Blood, bone marrow & lymph nodes	27	3.2 (2.1-4.6)	26	3.1 (2.0-4.5)	23	2.6 (1.8-4.0)
Bone & soft tissue cancer	19	8.3 (5.0-12.9)	17	7.4 (4.3-11.8)	15	6.3 (3.5-10.3)
Head & neck cancer	7	4.7 (1.9-9.6)	6	4.0 (1.5-8.7)	4	2.7 (0.7-6.8)
Non-melanoma skin cancer	17	6.5 (3.8-10.5)	15	5.8 (3.2-9.5)	13	4.8 (2.6-8.3)
Melanoma	162	8.1 (6.9-9.4)	145	7.2 (6.1-8.5)	25	1.2 (0.8-4.5)
Cancer respiratory tract	7	2.3 (0.9-4.7)	6	1.9 (0.7-4.2)	7	2.2 (0.9-4.5)
Cancer gastrointestinal tract	21	3.4 (2.1-5.1)	16	2.6 (1.5-4.2)	15	2.3 (1.3-3.9)
Kidney cancer	7	9.5 (3.8-19.5)	6	8.1 (3.0-17.6)	3	3.9 (0.8-11.5)
Bladder, renal pelvis, ureter	4	4.2 (1.1-10.7)	4	4.2 (1.1-10.7)	3	3.1 (0.6-8.9)
Female genital organs	30	2.7 (1.8-3.8)	24	2.1 (1.4-3.2)	13	1.1 (0.6-1.9)
Other localizations	20	3.0 (1.8-4.6)	18	2.7 (1.6-4.3)	19	2.8 (1.7-4.4)
Breast cancer	174	4.4 (3.7-5.1)	160	4.0 (3.4-4.7)	72	1.8 (1.4-2.2)
Unknown primary cancer	3	3.3 (0.7-9.6)	3	3.3 (0.7-9.6)	3	3.2 (0.6-9.4)
Gonadal germ cell tumor	14	5.1 (2.8-8.6)	12	4.4 (2.3-7.7)	10	3.6 (1.7-6.6)

* SIR: Standardized Incidence Ratio

DISCUSSION

Recent, population-based data about cancer among AYAs is scarce. This study was performed to provide insight in cancer incidence, survival and risk of second primary cancers among AYAs in the Netherlands. This information is necessary to define the extent and nature of the AYA patient population and provides input for further research to improve quality of care in this specific group of patients. Similar to earlier findings, the incidence of cancer in AYAs has increased significantly between 1989 and 2009 in the Netherlands (1-4). A comparison with incidence data from the SEER registry shows a slightly higher cancer incidence among AYAs in the US *(http://seer.cancer.gov/)* (1). The most prominent observed trend over time in male AYAs concerned testicular cancer which incidence has more than doubled in the period 1989-2009. This increase in testicular cancer has been reported worldwide (11). An accumulating body of evidence suggests that testicular cancer may originate already during fetal life, possibly associated with impaired spermatogenesis, cryptorchidism and hypospadias. As a common causal factor for this combination (also called Testicular Dysgenesis Syndrome) exposure to endocrine disrupting chemicals has been postulated (12). The other most striking result is the observed steep increase in melanoma incidence among older adult females (and males) as well, which is probably largely due to a combination of more UV exposure (sun bathing and sunbed use) and increased awareness (13;14). The same explanation might be underlying the observed rise in melanoma in female AYAs. A recent study performed in Australia, including patients with melanoma between 18-39 years old, found that sunbed use during adolescence and early adulthood was associated with increased risk of early onset melanoma (15). No obvious explanation is available for the observed increase in breast cancer over time in AYAs. The known life style factors associated with breast cancer risk such as age at birth of first child, number of pregnancies, hormonal therapy, breast feeding are in these AYAs not very likely causes, as in our analyses AYAs only include women younger than 30 years. A decreasing trend in astrocytomas is observed. This was also reported by Bleyer and colleagues (1) but no clear explanation is available. Next to variation in incidence over time, considerable variation exists in the incidence of specific cancer types across the AYA continuum. Within the male AYA population, the gonadal germ cell tumors are the most frequently diagnosed cancer at each age, but the proportion of these tumors increased strongly with increasing age (from 19% to 35%). In the youngest males, Hodgkin's disease, non-Hodgkin lymphoma and ALL jointly represent over 30% but this proportion decreased towards the older age groups. Melanoma, on the other hand, showed a clear increase with increasing age. Similar to males, in young female AYAs Hodgkin's disease and non-Hodgkin's lymphoma represented the majority of diagnosed cancers but with increasing age, the predominant cancers become melanoma and breast cancer. Similar patterns are reported in the US by Bleyer and colleagues (1).

This study showed a typical distribution of cancer types among AYAs, very different from the pattern reported for children and older adults. Frequently occurring cancers during childhood such as neuroblastoma, nephroblastoma (Wilm's tumor), other embryonal tumors and retinoblastoma are uncommon among AYAs. ALL is the most common hematological malignancy among children (5). The different types of leukemia in AYAs reflect a clear transition of a childhood pattern, with ALL as most frequently diagnosed in the AYAs aged 15-19, to an adult pattern dominated by

AML and rising incidence of CML. Concerning soft tissue and bone cancers a shift is seen as well; younger AYAs are more frequently diagnosed with 'childhood and adolescent types' such as osteosarcoma, Ewing's tumor and rhabdomyosarcoma whereas older AYAs are diagnosed with other types of soft tissue and bone cancers. Non-Hodgkin lymphomas are diagnosed in children, AYAs and adults, but a different distribution of histological subtypes has been reported (16). Frequently diagnosed carcinomas in adults such as colorectal, lung and prostate cancer are very rare among children and AYAs (8), with the exception of breast cancer and melanoma in females. However, the underlying etiology might be different in AYAs compared to older women.

The overall 5-year relative survival of AYAs diagnosed with cancer in the Netherlands is 80-82%. This is better compared to survival rates for AYAs in the US (US female AYAs have a similar survival but US male AYAs have a 5-year survival of approximately 70%) (1), but these figures relate to a more distant time period (1975-1999). The EUROCARE study reported a 5-year survival rate of 87% for patients diagnosed at age 15-24 (6). Time trends were studied as well in EUROCARE and a small improvement in the 5-year survival rate in the period 1995-1999 versus 1990-1994 was reported. In our study, we observed an improvement in the 5-year survival in males as well as females since 1989, from 74% to 86% in males and from 79% to 86% in females. Part of the survival improvement in males might be caused by the increased proportion of patients with testicular cancer which have a very good prognosis. Therefore, survival over time was assessed after exclusion of these tumors as well. A similar trend and improvement in survival was observed (5-year survival of 67% in the period 1989-1993 and 79% in the period 2004-2009). It can be concluded that the progress made in survival is not due to the strongly increased number of patients with testicular cancer. The most prominent survival improvement is seen in patients with non-Hodgkin lymphoma. This is in line with findings from EUROCARE in which a significant increased survival was reported in these patients as well (6). In general the 5-year survival rates of the most frequently diagnosed tumors in male AYAs seem to improve over time. As the number of patients by tumor site are relatively small, observed trends should be interpreted with care. During the study period there might have been influence of changing clinical practice, with more centralization and more patients discussed in multidisciplinary teams and a better adherence to tumor directed guidelines. However whether these factors have played a role in our study population is unclear as literature on this topic in AYA cancers is very scarce. Also, stage migration over time might have had a positive effect on survival. An elaborate discussion on stage migration was beyond the scope of this overview paper. In female AYAs time trends by tumor site are less clear; next to non-Hodgkin lymphoma, only survival of breast cancer and Hodgkin's disease patients has clearly improved over time. In the most recent period the survival of AYAs with breast cancer seems similar compared with the survival of adult breast cancer patients (5-year survival of 84% versus 86%) *(http://www.cancerregistry.nl)*. The survival improvement seen in young breast cancer patients was also observed in other European countries (6). However, a US study based on SEER data reported 5-year survival rates of young breast cancer patients which were less than 80% and stated that improvement over time was less compared to the adult breast cancer population, probably because of age-dependent biological differences (17).

A relatively poor survival is noted among AYAs diagnosed recently with ALL (5-year survival of 50-52%), AML (5-year survival of 44-48%), astrocytoma (5-year survival of 54-57%), rhabdomyosarcoma (5-year survival of 36-39%), Ewing's tumor (5-year survival of 40-46%) and osteosarcoma among males (5-year survival of 45%). The survival of these cancers in children (0-14 years) in the Netherlands is much better: Children with ALL have a 5-year survival of approximately 80%, with AML 53%, with astrocytoma 76%, with rhabdomyosarcoma 65%, with Ewing's tumor 65% and with osteosarcoma (63%) (unpublished data from the Netherlands Cancer Registry, notably from a later part of the whole time period than this AYA analysis has been performed, 2003-2007). Similar survival rates in children were reported by Linabery and Ross (2008) (18) and by the EUROCARE study (6). The poor survival among AYAs with these cancers which are also prevalent among children may be partly explained by the low participation rate of AYAs in clinical trials (19;20) but tumor biology may be different as well (21). Another explanation might be that AYAs are diagnosed at a more advanced disease stage due to patient's and doctor's delay (22). There is an ongoing debate about the question whether to treat AYAs with cancer as children or as adults (23). For ALL adolescents have better survival in case they are treated with pediatric rather than adult protocols (24).

In this study we had to use relative survival as an approximation for disease-specific survival because disease-specific survival cannot be estimated as no information on the cause of death was available to the Netherlands Cancer Registry due to specific legislation concerning Statistics Netherlands, holder of information on causes of death in the Netherlands. This relative survival adjusts for the general survival of the Dutch population taking gender, age, and calendar year into account. This might be interpreted as a limitation of this study, however, it can be argued that relative survival is the most appropriate method to use in population-based cancer survival studies, as misclassification of cancer specific deaths, result in biased estimates for cancer-specific survival (25). Furthermore, it should be noted that Death Certificate Only (DCO) registrations are not available in the Netherlands Cancer Registry due to the same reason as mentioned before. However, these patients would not affected the survival results as they would be excluded from the analyses.

Next to incidence and survival, we assessed the risk of second primary cancers in this study. A striking result was that AYAs with cancer have a more than 6 times (in case of male AYAs) or almost 5 (in case of females) increased risk to develop a second primary cancer. A large part of these second primary cancers are bilateral or of the same type (breast cancer, testicular cancer and melanoma). But also after excluding these bilateral/same-type- tumors, the risk of developing a second primary was approximately 3 times increased in males and 2 times in females. Although in the current study the mean follow-up time was almost 10 years and the median follow-up time was almost 7 years, part of the second primary cancers may occur later in life. The reported risk of second primary cancers may therefore be an underestimation. Several explanations can be given for the increased risk of second primary cancers: patients could be genetically susceptible for (different types of) cancer; environmental factors early in life may account for both the first and the second tumor; the second primary cancers may be treatment-induced; and/or general survival improvement of the first primary cancer may result more second primary cancers.

Compared to the earlier performed Dutch study from van Gaal *et al* (7), the reported risk on second primary cancers is much lower (i.e. 5-6 fold increased risk versus 31 fold). Our data are more recent and much more precise due to a 20 year national coverage of all AYA tumors in The Netherlands. However, it should be mentioned that due to fact that the NCR is complete since 1989, first primary tumors prior to 1989 may not be recorded and therefore, secondary primary tumors may not be identified as such. Although, all available information before 1989 was incorporated to identify first primary cancers, this could have led to an underestimation of the risk of secondary primary cancers.

As far as we know no studies have been performed in this age group evaluating the risk of second primary cancers. Studies including children with cancer reported on average a 6-fold increased risk of second primary cancers (26;27).

In conclusion, this study demonstrates that the incidence of cancer at AYA age is rising. In general, the survival has improved over time, but for specific tumor sites survival still lacks behind compared to children. The risk of second primary cancers is high and might even increase in the future as a result of survival progress. Cancer specific studies are needed to explain these unique features and to translate the figures into useful prognostic information for patients. Specialized treatment and follow-up of all AYAs after treatment of the primary tumor is needed to diagnose second primary cancers in a stage where cure can still be reached. Finally, tailor made treatment regimes should be developed for this age group as AYAs with cancer live in a completely different psycho-social context compared to older cancer patients, with specific dynamics in education, careers, social networks, sexual relations and family life.

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CHAPTER 4

The impact of age on outcome of embryonal and alveolar rhabdomyosarcoma patients. A multicenter study

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ABSTRACT

Background: The prognosis of rhabdomyosarcoma (RMS) in children and adolescents has improved since the introduction of multiagent chemotherapy. However, outcome data of adults with RMS are scarce. This multicenter retrospective study investigated the effect of age on outcome of RMS.

Patients and Methods: Data were collected from three Dutch University Medical Centers between 1977-2009. The effect of age and clinical prognostic factors on relapse-free and disease-specific survival (DSS) were analyzed.

Results: Age as a continuous variable predicted poor survival in multivariate analysis. Five-year DSS was highest for non-metastatic embryonal RMS, followed by non-metastatic alveolar RMS and was poor in metastatic disease. Higher age correlated with unfavorable histological subtype (alveolar RMS) and with metastatic disease at presentation in embryonal RMS. In non-metastatic embryonal RMS and all alveolar RMS, higher age was an adverse prognostic factor of outcome.

Conclusion: This study indicates that age is a negative predictor of survival in patients with embryonal and alveolar RMS. Whether this is due to nature (biological make-up) or nurture (treatment) deserves further research.

INTRODUCTION

Rhabdomyosarcoma (RMS) is a soft tissue sarcoma (STS) that occurs predominantly in children; 70% of cases are diagnosed within the first decade of life (1, 2). There is a second peak in incidence during adolescence (age 15-19 years), with RMS accounting for 1.7% of all malignancies in this age group (3). In adulthood, RMS is extremely rare, given that STS accounts for fewer than 1% of all malignancies, and as RMS comprises only 3.3 % of all STS (4).

The two main histological subtypes of RMS that occur in both adults and children are embryonal and alveolar RMS. Alveolar RMS, which occurs mostly in older children and adolescents, has a worse outcome compared to embryonal RMS, which is more common in young children.

The prognosis in children with RMS has improved dramatically during the past decades because of the introduction of multiagent chemotherapy in consecutive multidisciplinary clinical trials and treatment in a centralized setting (5-9). The Intergroup Rhabdomyosarcoma Study Group (IRSG) publications demonstrated successive increases in five-year survival rates between 1972 and 1997; from 55% on IRS-I (5) to 74% on IRS-IV protocols (8). In contrast, data on adults with RMS are scarce and in general show a worse outcome compared to the disease in children (10, 11). Additionally, a recent Surveillance, Epidemiology and End Results (SEER) report of 2,600 patients with RMS, confirms that no improvements in adults have been made over the past decades (12).

The reasons underlying the worse outcome in adult patients with RMS compared to children remain to be revealed. From larger pediatric studies, several prognostic factors have been established, such as site of the primary tumor (8), size below or above 5 cm diameter (12), histological subtype (14), stage of disease (15) and age at diagnosis (16, 17). In the scarce data on adult RMS, nearly identical factors have been described (10-12, 18-22).

To further substantiate the differences in outcomes between children and adults with RMS, the present Dutch multicenter study was conducted to investigate the role of age and other putative prognostic factors on outcome in a large cohort of both children and adult patients with RMS.

Patients
We selected all patients with RMS in PALGA, the Dutch National Histopathological Database System, treated at either the Pediatric Oncology Unit of the Department of Pediatrics or the Department of Medical Oncology at the University Medical Center Groningen, the Erasmus Medical Center Rotterdam, or the Radboud University Nijmegen Medical Centre diagnosed between 1977 and 2009 (N= 226). Patients were excluded if medical files were not available (N=10), or histological diagnoses were other than embryonal or alveolar RMS (N=47) as pathology reports were reviewed.

Data collection
The following data were collected: age at diagnosis, gender, site and size of the primary tumor, presence of metastases at diagnosis, metastatic site, lymph node involvement, treatment modalities (intention-to-treat principle), and follow-up status. Histological subtype was defined as embryonal or alveolar RMS. Botryoid RMS (a subtype of embryonal RMS occurring almost exclusively in infants and toddlers with a superior outcome) was analyzed separately.

Staging
Staging at diagnosis started with physical diagnosis. Evaluation of the location, size and local extent of the tumor was additionally evaluated with computerized tomography (CT) and/or magnetic resonance imaging (MRI). Regional lymph nodes were considered positive when suspicious at physical examination, and when confirmed by CT scanning and/or histological or cytological evaluation. Further staging included chest X-ray and/or CT scan, bone scintigraphy and bone marrow aspiration. Pre-treatment staging in children was based on the IRSG classification (23). Adult patients were staged according to the TNM classification, based on local tumor extension and/or fixation to surrounding tissues (T), tumor size <5 cm of ≥5 cm, lymph node involvement (N) and presence of metastatic disease (M), and were translated into IRSG classification to facilitate comparison. Favorable locations of the primary tumor included the orbit, head and neck but not parameningeal, and paratesticular sites, whereas unfavorable locations included all other sites, as stated by the IRSG studies. Postsurgical clinical grouping (CG) staging was based on the extent of residual tumor after the initial resection, according to the IRSG clinical grouping system.

Treatment
Most children were treated according to the Children's Oncology Group (COG) or International Society of Paediatric Oncology (SIOP) guidelines (24, 25). Local control consisted of primary surgery or surgery after neoadjuvant chemotherapy and/or radiotherapy. Adjuvant systemic treatment consisted of combinations of vincristine, D-actinomycin, and either iphosphamide or cyclophosphamide. Anthracyclines were added in the case of advanced disease. Treatment of adults consisted of surgery and/or radiotherapy for local control following the STS protocols. Additionally, chemotherapy, mainly consisting of anthracyclines in combination with one or more of vindesine/vincristine, iphosphamide, and etoposide, was administered to the majority of adult patients.

Statistical analyses
Follow up data was collected in a database and statistical analyses were performed by Statistics 16.0 (SPSS, Chicago, IL, USA). Frequency distribution of patient characteristics and prognostic factors for the different histological subtypes were assessed with Chi-square test. The relation between age and prognostic factors was assessed using non-parametric testing with Kruskal Wallis, or Mann Whitney U-test when appropriate.

Overall survival (OS) probabilities were assessed using the Kaplan Meier method and compared with log-rank test. OS was defined as the time from onset of disease until death from any cause. A multivariate Cox proportional hazards analysis including age and other prognostic factors was used to analyze whether age was an independent prognostic factor in disease-specific survival (DSS) and relapse-free survival (RFS), using a backward conditional model. Furthermore, we tested interaction between the effect of age and histological subtype on DSS and RFS. Treatment modalities were selectively tested based on clinical relevance. DSS was defined as the time from disease onset until death due to disease. RFS was defined as the time from the end of treatment with complete remission (CR) until local and/or distant recurrence. CR was defined as no evidence of residual disease at the end of treatment, confirmed by radiography and/or histopathology. Patients who never experienced an event were censored at the last contact date with the hospital and/or medical correspondence.

RESULTS

Patients
A total of 169 patients were eligible for analysis. The median age at diagnosis was 8 years (range 0-73 years). Because all patients younger than 16 years were treated by a pediatric oncologist in all centers, we separated our patients into age groups <16 and ≥16 years. Patients' and tumor characteristics are summarized in Table 1.

A significantly higher rate of alveolar subtype and tumors arising at unfavorable sites (mainly involving parameningeal and extremity sites), more lymph node involvement, a higher rate of distant metastasis, a lower probability of CR, a higher rate of relapse, and a trend towards higher IRS stage was demonstrated in patients ≥16 years in comparison to patients <16 years.

Pre-treatment prognostic factors
Age was significantly related to histological subtype ($p<0.001$, Table 1, Figure 1A and B). Embryonal RMS occurred mainly in young children (median age=7 years). Botryoid RMS mostly occurred in infants and toddlers (median age=4 years). Alveolar RMS (median age=15 years) showed two peaks in incidence: one in young children and one in adolescence and young adulthood.

Because of this strong correlation, we compared prognostic factors for the different histological subtypes (Table 2). Embryonal RMS was characterized by a significantly higher rate of favorable primary sites, absence of regional lymph node involvement and metastatic disease, with a consequently lower IRS stage at diagnosis when compared to alveolar RMS.

Table 1
Patient characteristics.

Patient characteristics	Total		Age <16 years		Age ≥16 years		
	N	%	N	%	N	%	*p*-Value[a]
No.	**169**	100	118		51		
Median age (range), years	**8 (0-73)**		**4 (0-15)**		**21 (16-73)**		
Sex (male/female)	97/72	57.1/42.9	70/48	58.8/41.2	27/24	52.9/47.1	0.441
Type N (%)							***0.001***
Embryonal	103	60.9	78	66.1	25	49.0	
Botryoid	15	8.9	14	11.9	1	2.0	
Alveolar	51	30.2	26	22.0	25	49.0	
Site							***0.041***
Favorable	59	34.9	47	39.8	12	23.5	
Orbit	7	4.1	7	5.9	0	-	
Head/neck (non-PM)	13	7.7	9	7.6	4	7.8	
Genito-urinary tract[b]	39	23.1	31	26.3	8	15.7	
Unfavorable	110	65.1	71	60.2	39	76.5	
Parameningeal	39	23.1	21	17.8	18	35.3	
Extremities	22	13.0	13	11.0	9	17.6	
Bladder/prostate	15	8.9	13	11.0	2	3.9	
Trunk	7	4.1	4	3.4	3	5.9	
Retroperitoneal	7	4.1	7	5.9	0	-	
Perineal/peri-anal	5	3.0	2	1.7	3	5.9	
Other	15	8.9	11	9.3	4	7.8	
Size							0.332
<5 cm	45	26.6	32	27.1	13	25.5	
>5 cm	67	39.6	53	44.9	14	27.5	
Unknown	57	33.7	33	28.0	24	47.1	
Local tumor extension							0.497
T1	27	16.0	19	16.1	8	15.7	
T2	62	36.7	39	33.1	23	45.1	
Unknown	80	47.3	70	59.3	20	39.2	
Lymph node involvement[c]							***0.011***
N1	53	31.4	30	25.4	23	45.1	
N0	116	68.6	88	74.6	28	54.9	
Metastatic disease							***0.02***
M1	37	21.9	20	16.9	17	33.3	
M0	131	77.5	97	82.2	34	66.7	
Unknown	1	0.6	1	0.8	0	-	
IRS stage[d]							0.052
I	56	33.1	45	38.1	11	21.6	
II	10	5.9	6	5.1	4	7.8	
III	54	32.0	38	32.2	16	31.4	
IV	37	21.9	20	16.9	17	33.3	
Unknown	12	7.1	9	7.6	3	5.9	
Complete remission (%)[e]	135	79.9	99	83.9	36	70.6	***0.048***
Relapse	58	34.3	30	25.4	28	54.9	***<0.001***
Local relapse	21	12.4	14	11.9	7	13.7	
Distant relapse	30	17.8	10	8.5	20	39.2	
Local and distant relapse	7	4.1	6	5.1	1	2	
Time to relapse (months)[f]	5.2 (0.1-73.6)		5.4 (0.1-73.6)		4.5 (0.1-54.5)		
Survival after relapse	4.3 (0.3-177.5)		4.4 (0.3-52.0)		3.8 (0.7-177.5)		
Follow-up time[g]	29.0 (0.1-328.4)		41.7 (0.1-328.4)		16.0 (0.2-197.0)		
Alive at last follow-up (%)	85	50.3	74	62.7	11	21.6	***<0.001***

[a]Chi-square test; [b]non-prostate, non-bladder; [c]Evaluated by either histological evaluation, by CT scanning or by clinical examination; [d]IRS: Intergroup Rhabdomyosarcoma Study Group (IRSG) pre-operative staging; [e]Complete remission (CR) was defined as no evidence of residual disease at the end of treatment, confirmed by radiography and/or histopathology; [f]Time to relapse is defined as the time from the end of treatment with CR until disease relapse; [g]Follow-up time is the time between time of diagnosis until death or the last contact moment to the hospital

Table 2
Pre-treatment prognostic factors by histological subtype.

	Histological subtype						
	Embryonal RMS		Alveolar RMS		Botryoid RMS		
	N	%	N	%	N	%	*p*-Value [a]
All patients	103		51		15		
Age, years	7 (0-73)		15 (0-54)		4 (1-19)		
Site							***<0.001***
Favorable	48	46.6	7	13.7	4	26.7	
Unfavorable	55	53.4	44	86.3	11	73.3	
Size							*0.685*
≤5 cm	31	30.1	11	21.6	3	20.0	
>5 cm	47	45.6	13	25.5	7	46.7	
Lymph node involvement							***<0.001***
N0	81	78.6	27	52.9	11	73.3	
N1	22	21.4	24	47.1	4	26.7	
Metastastatic disease							***<0.001***
M0	91	88.3	27	52.9	13	86.7	
M1	11	10.7	24	47.1	2	13.3	
Local tumor extension							***0.029***
T1	23	22.3	4	7.8	0	-	
T2	26	25.2	18	35.3	8	53.3	
IRS stage[b]							***<0.001***
IRS 1	47	45.6	5	9.8	4	26.7	
IRS 2	4	3.9	5	9.8	1	6.7	
IRS 3	34	33.0	14	27.5	6	40.0	
IRS 4	11	10.7	24	47.1	2	13.3	

[a]Chi-square test; [b]IRS stage: Intergroup Rhabdomyosarcoma Study Group (IRSG) pre-operative staging.
This table displays the frequency distribution of the prognostic factors by histological subtype.

In the largest group, namely embryonal RMS, higher age was related to a higher rate of metastatic disease at diagnosis (p=0.001) and IRS stage (p=0.002). Within the group of alveolar RMS, higher age was related to IRS stage (p=0.026), and there was a trend for a relation between higher age and more lymph node involvement (p=0.079). Tumors arising at unfavorable primary sites (p=0.068), such as parameningeal versus other primary locations (p<0.001), were also related to higher age.

Treatment

Of all 169 patients, six patients did not receive any treatment due to varying reasons (supplementary Table 1). Of the remaining patients (N=163), 22 did not complete treatment.

Surgery was performed slightly more frequently in patients <16 years (p=0.051), whereas radiotherapy was more frequently administered to patients ≥16 years (p=0.003, Table 3). A significantly lower number of patients ≥16 years underwent chemotherapy when compared to

Figure 1A
Frequency distribution by age and histological subtype.

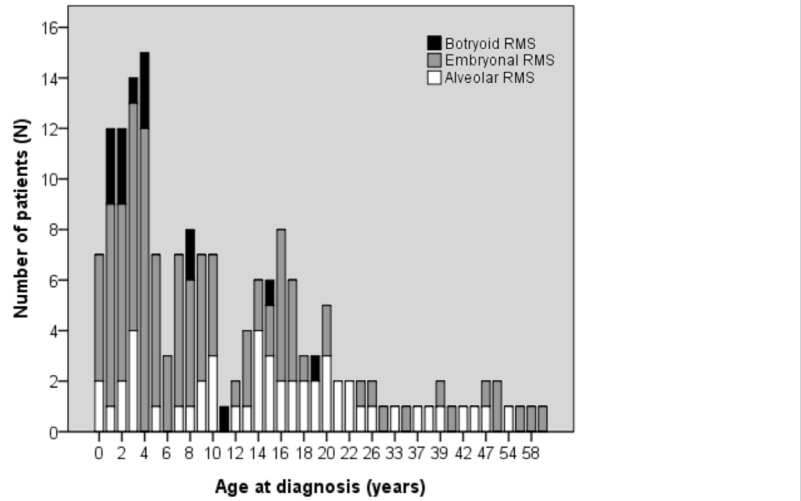
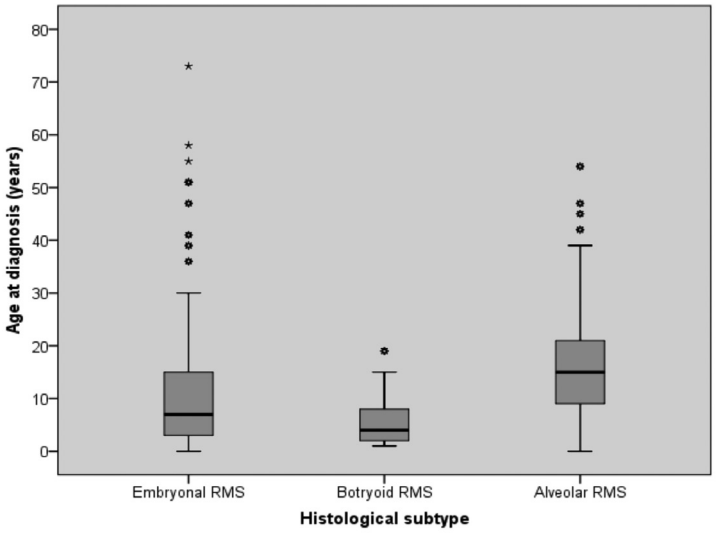


Figure 1B
Age distribution by histological subtype.



This figure shows the box plots of the age distribution by histological subtype. Stepwise non-parametric testing was performed. Age differed significantly between patients with alveolar (median=15 years) and those with embryonal RMS (median=8 years, p<0.001) and botryoid RMS (median=4 years, p<0.001). There was a trend for a difference in age between embryonal and botryoid RMS (p=0.084).

patients <16 years (p=0.003). The reason why not all patients received chemotherapy could not be retrieved in all cases. Some patients were in a poor clinical condition, impairing administration of chemotherapy; others had had a resection of a small tumor and were apparently deemed to have been optimally treated in earlier years of this retrospective study.

Supplementary table 1
Treatment intention and follow-up.

Treatment intention	N		N		N		N
No curative treatment	6	Died before start of treatment	3				
		Palliative treatment intention	2				
		Unknown	1				
Curative treatment	163	Did not complete treatment	22	Died to toxic side effects	9		
				Ended treatment due to severe side-effects	2		
				Progression during treatment	10	Distant failure	9
						Local progression	1
				Unknown	1		
		Completed treatment	141	Refractory disease	5		
				Complete remission	135	Local failure	21
						Distant failure	30
						Local+ distant failure	7
						NED[a]	77

[a]NED=no evidence of disease. This table displays the number of patients included in the study and the treatment intention, as well as data on completement of treatment and follow-up.

Most children received multiagent chemotherapy but no anthracyclines (63.5%), with the addition of anthracyclines only to patients with advanced disease (29.6%). Adults mainly received anthracycline-based multiagent chemotherapy, resulting in a more frequent use of anthracyclines in patients ≥16 years when compared to patients <16 years (p<0.001).

Survival

Five-year OS for the whole cohort was 52.0±4.0%. Patients ≥16 years had a disadvantage in outcome when compared to patients <16 years (5-year OS 21.4±6.4% versus 64.8±4.5%, p<0.001, Figure 2A).

A significant difference in survival was seen for the different histological subtypes (Figure 2B). Botryoid RMS had the best 5-year OS (78.3±11.1%), whereas alveolar RMS demonstrated poor outcome (5-year OS=21.9±6.1%). Patients with embryonal RMS had an intermediate 5-year OS of 63.7±4.9%.

For embryonal RMS, a superior survival was seen in patients <16 years (5-year OS=71.3±5.2%) versus patients ≥16 years (5-year OS=37.4±11.0%, p=0.001, Figure 2C). For alveolar RMS, an advantage in survival for patients <16 years when compared to patients ≥16 years was also seen (5-year OS=35.8±9.7% versus 8.5±5.8%, p=0.020, Figure 2D). In Figure 2E, we show OS in non-metastatic embryonal RMS (5-year OS=69.1±5.0%), non-metastatic alveolar RMS (5-year OS=34.1±9.5%), and metastatic embryonal and alveolar RMS (5-year OS=11.7±5.9%). Furthermore, within the subgroup of non-metastatic embryonal RMS, there was a significant disadvantage in survival for patients ≥16 years (5-year OS=40.0±13.0%) when compared to

Table 3
Treatment characteristics by age group.

	Total		Age <16 years		Age ≥16 years		
Treatment characteristics	N	%	N	%	N	%	*p*-Value[a]
All patients	169		118		51		
Curative treatment intent	163	100	115	100	48	100	
Chemotherapy							***0.003***
Yes	154	94.5	113	98.3	41	85.4	
No	8	4.9	2	1.7	6	12.5	
Unknown	1	0.6	0	-	1	2.1	
Drug regimen[b]							
VA containing	125	76.7	108	93.9	17	35.4	***<0.001***
VA	18	11.0	17	14.8	1	2.1	
VAC/VAI	64	39.3	56	48.7	8	16.7	
VAIA/VACA	42	25.8	34	29.6	8	16.7	
Other combinations with VA	1	0.6	1	0.9	0	-	
DIME	5	3.1	0	-	5	10.4	
EVI	8	4.9	0	-	8	16.7	
Other/unknown	16	9.8	5	4.3	11	22.9	
Anthracyclines							***<0.001***
Yes	64	39.3	36	31.3	28	58.3	
No/unknown	90	55.2	77	67.0	13	27.1	
Radiotherapy							***0.003***
Yes	81	49.7	48	41.7	32	66.7	
No	81	49.7	66	57.4	15	31.3	
Unknown	2	1.2	1	0.9	1	2.1	
Surgery							0.051
Yes	104	63.8	79	68.7	25	52.1	
Primary tumor	90	55.2	71	61.7	19	39.6	
Primary tumor + metastases	12	7.4	8	7.0	4	8.3	
Metastases	2	1.2	0	-	2	4.2	
No	55	33.7	33	28.7	21	43.8	
Unknown	5	3.1	3	2.6	2	4.2	
Result of surgery							0.082
Radical	48	29.4	32	27.8	16	33.3	
Not radical	43	26.4	37	32.2	6	12.5	
Unknown	13	8.0	7	6.1	3	6.3	
Clinical group[c]							0.051
I	38	23.3	25	21.7	13	27.1	
II	25	15.3	22	19.1	3	6.3	
III	53	32.5	38	33.0	14	29.2	
IV	31	19.0	17	14.8	14	29.2	
Unknown	17	10.4	13	11.3	4	8.3	

[a]Chi-square test. [b]Treatment regimens were divided into vincristine and actinomycin (VA)-containing and non-VA-containing. VAC/VAI(A), vincristine, D-actinomycin, cyclophosphamide/ iphosphamide, (doxorubicin); DIME, doxorubicine, iphosphamide, mesna, etoposide; EVI, etoposide, vindesine/vincristine and iphosphamide. [c]Clinical group was based on the extent of residual tumor after the initial surgical resection, according to the clinical grouping system of the Intergroup Rhabdomyosarcoma Study Group. Percentages of treatment modalities (chemotherapy, radiotherapy, surgery and clinical group) were calculated as the percentage of all patients treated with curative intention of treatment (N=163).

children <16 years (75.9±5.1%, p=0.002, Figure 2F). Exclusion of patients who did not receive chemotherapy resulted in a 5-year OS 49.2±15.4% in patients ≥16 years versus 78.1±5.0% in patients <16 of age (p= 0.021).

Prognostic factors
In all RMS patients (Table 4), increasing age (p<0.001, HR=1.028), unfavorable primary site (p=0.012, HR=2.51), lymph node involvement (p=0.023, HR=1.94) and the presence of metastatic disease (p=0.009, HR=2.16) were multivariate predictors of poor DSS. Increasing age (p<0.001, HR=1.031) was the only significant multivariate prognostic factor of poor RFS, along with a trend towards a negative prognostic effect of alveolar subtype (p=0.057, HR=1.96) and metastatic disease (p=0.094, HR=1.89). To further exclude the interaction between the effect of age and histological subtype on outcome, we analyzed embryonal and alveolar RMS separately.

Embryonal RMS
In embryonal RMS (Table 5), increasing age (p=0.001, HR=1.038), unfavorable primary site (p=0.006, HR=4.21), and lymph node involvement (p<0.001, HR=5.47) were significant multivariate predictors of poor DSS, whereas a trend was seen for metastatic disease (p=0.063, HR=2.45). Higher age (p<0.001, HR=1.043), unfavorable primary site (p=0.033, HR=2.50), and lymph node involvement (p=0.015, HR=3.47) were multivariate predictors of poor RFS.

To eliminate a possible effect of metastatic disease in older patients as mentioned before, non-metastatic embryonal RMS was analyzed separately (Table 6). Increasing age (p=0.001, HR=1.045), unfavorable primary site (p=0.011, HR=4.00), and lymph node involvement (p<0.001, HR=6.44) were significant multivariate predictors of poor DSS, whereas higher age (p<0.001, HR=1.043), and unfavorable primary site (p=0.045, HR=2.52) were predictors of poor RFS.

Furthermore, we investigated the effect of treatment modalities (chemotherapy, radiotherapy, surgery, and Clinical Group I-IV) on DSS in non-metastatic embryonal RMS. In univariate analysis, no administration of chemotherapy (p=0.011, HR=5.19, 95% CI=1.47-18.63), no surgery for primary tumor and/or metastases (p=0.004, HR=3.66, 95% CI=1.515-8.83), and Clinical Group III versus I (p=0.013, HR=5.06, 95% CI=1.41-18.20) predicted poor DSS. In a multivariate model including age, lymph node involvement, primary tumor location, Clinical Group, radiotherapy, chemotherapy and surgery (N=79), higher age (p=0.005, HR=1.07, 95% CI=1.019-1.115), lymph node involvement (p=0.003, HR=6.51, 95% CI=1.90-22.25), Clinical group III versus I (p=0.021, HR=23.45, 95% CI=1.61-342.34), and no radiotherapy (p=0.048, HR=3.68, 95% CI=1.01-13.37) were predictors of poor DSS.

Alveolar RMS
In alveolar RMS (Table 7), there was a trend for poorer DSS with increasing age (p=0.078, HR=1.02), and metastatic disease (p=0.095 HR=1.82). A nearly identical result was seen for RFS.

Figure 2
Rhabdomyosarcoma survival.

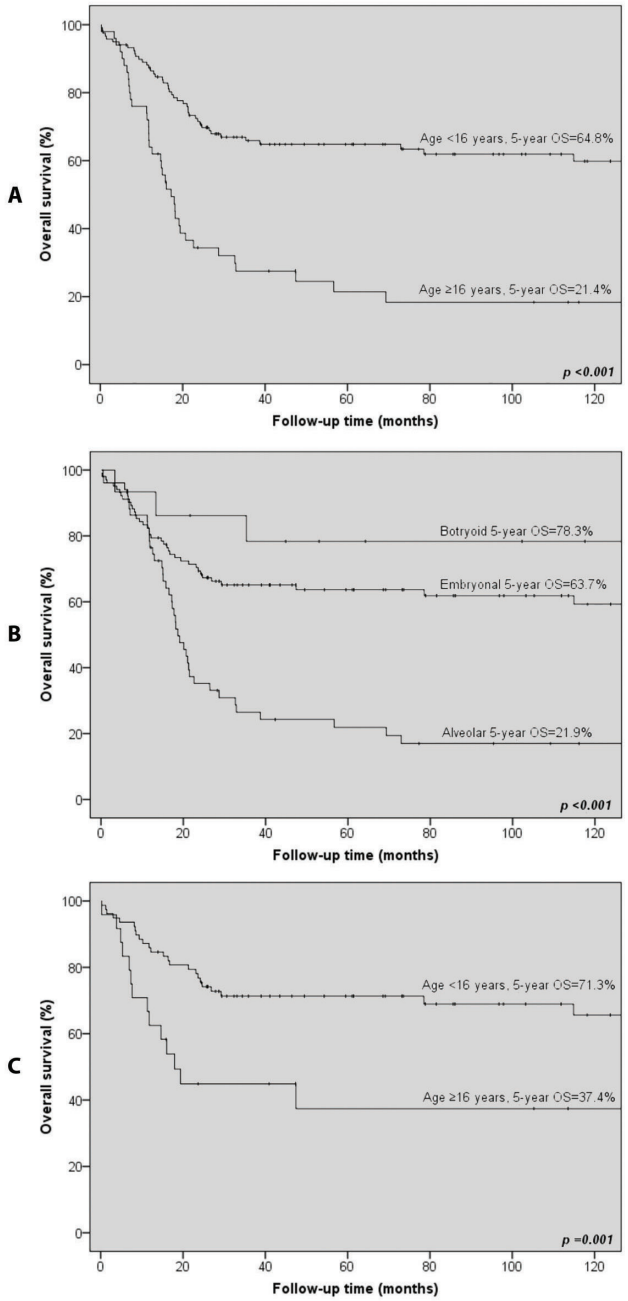
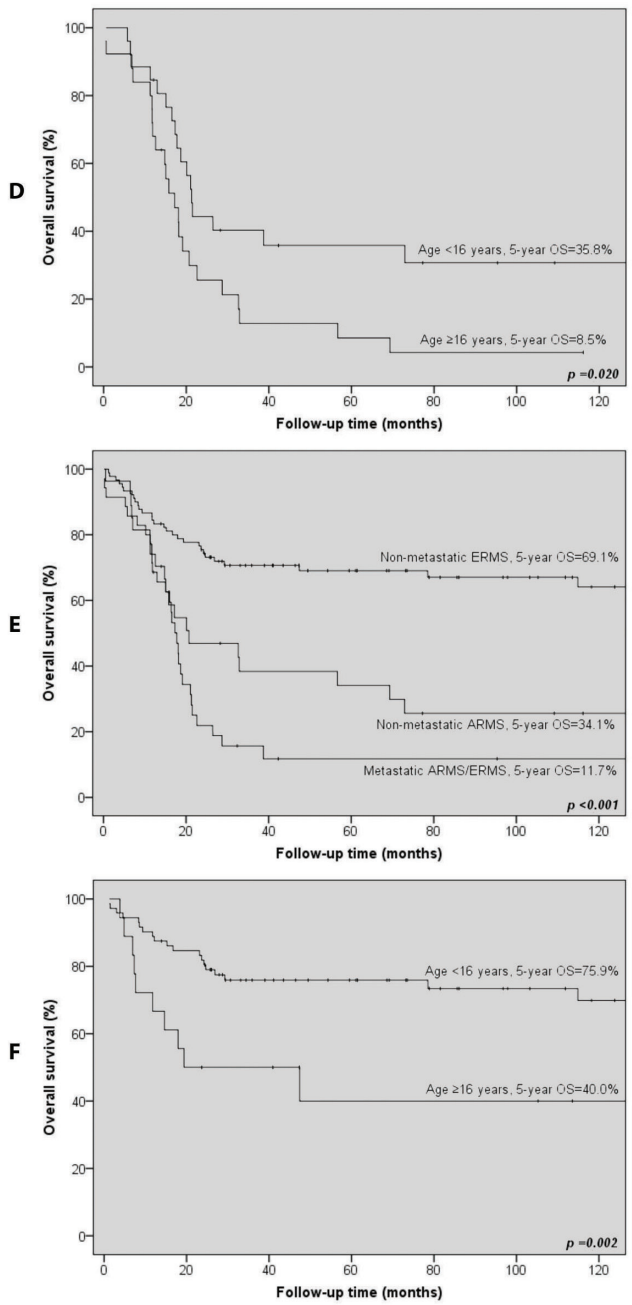


Figure 2 *(continued)*
Rhabdomyosarcoma survival.



Kaplan-Meier overall survival (OS) curves for patients with rhabdomyosarcoma (RMS) by age group (A), patients <16 years: N=118 patients, N at 5 years=51, patients ≥16 years: N=50 patients, N at 5 years=7; by histological subtype (B), embryonal RMS: N=102 patients, N at 5 years=41, botryoid RMS: N=15 patients, N at 5 years=8, and alveolar RMS: N=51 patients, N at 5 years=9; by age group for embryonal RMS (C), patients <16 years: N=78 patients, N at 5 years=36, patients ≥16 years: N=24 patients, N at 5 years=5; by age group for alveolar RMS (D), patients <16 years: N=26 patients, N at 5 years=7, patients ≥16 years: N=25 patients, N at 5 years=2; for non-metastatic embryonal RMS, non-metastatic alveolar RMS and metastatic embryonal and alveolar RMS (E), non-metastatic embryonal RMS: N=90 patients, N at 5 years 40, non-metastatic alveolar RMS: N=27 patients, N at 5 years=8, metastatic embryonal and alveolar RMS: N=35 patients, N at 5 years=2; and by age group for non-metastatic embryonal (F), patients <16 years at diagnosis: N=72 patients, N at 5 years=36, patients ≥16 years at diagnosis: N=18 patients, N at 5 years=4.

Table 4
Prognostic factors in Cox proportional hazards analysis for all patients.

Patient group	Prognostic factor	Disease-specific survival						Relapse-free survival					
		Univariate			Multivariate			Univariate			Multivariate		
		p-Value	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value	HR	95% CI
All RMS	Age	**<0.001**	1.042	1.028-1.056	**<0.001**[a]	1.028	1.012-1.044	**<0.001**	1.039	1.023-1.054	**<0.001**[b]	1.031	1.013-1.048
	Type	**<0.001**			0.643			**<0.001**			0.162		
	Embryonal	Reference			Reference			Reference			Reference		
	Botryoid	0.778	0.860	0.302-2.453	0.715	0.818	0.280-2.394	0.955	1.031	0.358-2.963	0.753	1.19	0.402-3.527
	Alveolar	**<0.001**	3.626	2.206-5.960	0.578	1.283	0.675-2.441	**<0.001**	3.501	2.009-6.104	0.057	1.964	0.979-3.938
	Local extension	**0.01**	3.993	1.382-11.192	NI			0.125	2.016	0.824-4.935	NI		
	Favorability	**<0.001**	4.226	2.159-8.272	**0.012**	2.512	1.221-5.167	**0.002**	2.697	1.446-5.032	0.154	1.642	0.830-3.245
	Lymph nodes	**<0.001**	3.307	2.055-5.332	**0.023**	1.935	1.095-3.420	**0.012**	2.027	1.169-3.517	0.615	1.179	0.620-2.242
	Metastases	**<0.001**	4.434	2.680-7.336	**0.009**	2.161	1.209-3.863	**<0.001**	3.826	2.046-7.153	0.094	1.893	0.900-3.757
	IRS stage	**<0.001**			NI			**<0.001**			NI		
	I	Reference						Reference					
	II	**0.023**	3.856	1.206-12.331				**0.019**	3.827	1.245-11.760			
	III	**0.008**	2.763	1.307-5.838				0.101	1.819	0.891-3.714			
	IV	**<0.001**	8.378	4.000-17.547				**<0.001**	5.821	2.683-12.628			
	Size	0.443	1.309	0.658-2.607	NI			0.313	1.440	0.709-2.928	NI		

NI, Not included; NS, not significant at p<0.05; HR, hazards ratio. CI, confidence interval. Embryonal rhabdomyosarcoma and IRS stage I was used as a reference for calculating significance and HR of Cox proportional hazards analysis when applicable. aMultivariate analysis on disease-specific survival (DSS) (N=167, 68 events), including age, histological type, site favorability (as defined by the IRSG), lymph node involvement, and the presence of metastatic disease. Invasiveness and size were not included because lack of availability on data on these items leading to very small patient numbers. A model with only age and type results in a significant effect of age (p<0.001, HR=1.034, 95% CI=1.018-1.050) and type (alveolar versus embryonal, p<0.001, HR=2.811, 95% CI=1.683-4.696) on outcome (N=168). Addition of IRS stage instead of lymph node involvement, site and metastatic disease, resulted in a significant effect of age (p<0.001, HR=1.036, 95%CI=1.020-1.054) and IRS stage III (p=0.033, HR=2.306, 95%CI=1.068-4.980) and IV (p<0.001, HR=4.801, 95% CI=2.073-11.120) versus 1 (N=156 patients). A trend towards an interaction between age and histological subtype on DSS was found (p=0.098). bMultivariate analysis on relapse-free survival (RFS), including all patients with complete remission at the end of treatment (N=130, 54 events). Prognostic factors age, histological type, site favorability (as defined by the IRSG), lymph node involvement, and the presence of metastatic disease were included. Invasiveness and tumor size were not included. A model with IRS stage instead of lymph node involvement, site and metastatic disease (N=120, 49 events), resulted in a significant effect of age (p<0.001, HR=1.044, 95% CI=1.025-1.064), IRS group IV versus I (p=0.025, HR=2.840, 95% CI=1.138-7.091). There was no interaction (p=0.313) between the effect of age and histological subtype on RFS.

Table 5
Prognostic factors in Cox proportional hazards analysis for patients with embryonal rhabdomyosarcoma (ERMS).

Patient group	Prognostic factor	Disease-specific survival						Relapse-free survival					
		Univariate			Multivariate			Univariate			Multivariate		
		p-Value	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value	HR	95% CI
Embryonal RMS	Age	**<0.001**	1.043	1.022-1.064	**0.001**[a]	1.038	0.015-1.062	**0.001**	1.037	0.016-1.059	**<0.001**[b]	1.043	1.021-1.067
	Local extension	**0.038**	3.739	1.074-13.019	NI			0.187	2.003	0.714-5.621	NI		
	Location	**0.002**	4.682	1.779-12.321	**0.006**	4.214	1.513-11.736	**0.033**	2.500	1.078-5.798	**0.033**	2.656	1.080-6.537
	Lymph nodes	**<0.001**	4.411	2.091-9.302	**<0.001**	5.466	2.407-12.411	0.171	1.898	0.758-4.756	**0.015**	3.468	1.270-9.469
	Metastases	**<0.001**	5.721	2.364-13.846	0.063	2.450	0.951-6.312	0.178	2.656	0.623-11.329	0.411	1.872	0.420-8.339
	IRS stage	**<0.001**			NI			**0.011**			NI		
	I	Reference						Reference					
	II	**0.027**	6.379	1.233-32.986				**0.002**	8.688	2.274-33.198			
	III	**0.022**	3.433	1.192-9.887				0.173	1.939	0.748-5.029			
	IV	**<0.001**	12.757	3.968-41.014				0.069	4.222	0.893-19.956			
	Gender	0.968	1.016	0.460-2.247	NI			0.872	1.071	0.462-2.483	NI		
	Size	0.239	1.848	0.665-5.135	NI			0.170	2.031	0.738-5.590	NI		

NI, Not included; NS, not significant at p<0.05; HR, hazards ratio. CI, confidence interval. IRS stage I was used as a reference for calculating significance and HR of Cox proportional hazards analysis when applicable. aMultivariate analysis on disease-specific survival (N=101, 27 events), including age, site favorability (as defined by the IRSG), lymph node involvement, and the presence of metastatic disease. Invasiveness and size were not included because lack of availability on data on these items leading to very small patient numbers. Addition of IRS stage instead of lymph node involvement, site and metastatic disease, resulted in a significant effect of age (p<0.001, HR=1.055, 95%, CI=1.025-1.085) and IRS stage III (p=0.014, HR=3.793, 95% CI=1.306-11.015) and IV (p<0.001, HR=9.257, 95% CI=2.886-29.893) versus 1 (N=95, 25 events). bMultivariate analysis on relapse-free survival, including all patients with complete remission at the end of treatment (N=82, 24 events). Prognostic factors age, site favorability (as defined by the IRSG), lymph node involvement, and the presence of metastatic disease were included. Invasiveness and tumor size were not included. A model with IRS stage instead of lymph node involvement, site and metastatic disease (N=77, 22 events), resulted in a significant effect of age (p<0.001, HR=1.059, 95% CI=1.033-1.085 and IRS II versus 1 (p=0,004, HR=7.391, 95% CI=1.911-28.583) and a trend towards a significant effect of IRS II (p=0.063, HR=2.544, 95% CI=0.949-6.815) and IRS IV (p=0.074, HR=4.172, 95%, CI=0.872-19.964) versus I respectively.

Table 6

Prognostic factors in Cox proportional hazards analysis for patient with non-metastatic embryonal rhabdomyosarcoma (ERMS, M0).

Patient group	Prognostic factor		Disease-specific survival						Relapse-free survival					
			Univariate			Multivariate			Univariate			Multivariate		
			p-Value	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value	HR	95% CI
Embryonal	Age		**<0.001**	1.045	1.021-1.070	**<0.001**[a]	1.045	1.019-1.070	**<0.001**	1.041	1.019-1.064	**<0.001**[b]	1.043	1.021-1.067
RMS, M0	Local extension		0.055	4.375	0.969-19.750	NI			0.112	2.482	0.809-7.617	NI		
	Favorability		**0.017**	3.416	1.241-9.404	**0.011**	3.966	1.379-11.409	0.067	2.252	0.944-5.371	**0.045**	2.515	1.022-6.191
	Lymph nodes		**0.001**	4.321	1.786-10.452	**<0.001**	6.439	2.490-16.647	0.285	1.723	0.635-4.671	0.058	2.802	0.964-8.147
	IRS stage		**0.029**			NI			**0.006**			NI		
		I	Reference						Reference					
		II	**0.025**	6.549	1.265-33.911				**0.001**	8.952	2.337-34.295			
		III	**0.022**	3.441	1.194-9.912				0.171	1.946	0.750-5.047			
	Size		0.184	2.156	0.695-6.688	NI			0.173	2.033	0.732-5.645	NI		

NI, Not included; NS, not significant at p<0.05; HR, hazards ratio. CI, confidence interval. IRS stage I was used as a reference for calculating significance and HR of Cox proportional hazards analysis when applicable. aMultivariate analysis on disease-specific survival (N=88, 20 events), including age, site favorability (as defined by the IRSG), and lymph node involvement. Addition of IRS stage instead of lymph node involvement, and site, resulted in a significant effect of age (p<0.001, HR=1.064, 95% CI=1.030-1.098) and IRS III versus I (p=0.013, HR=3.916, 95% CI=1.338-11.462 (N=82, 18 events). bMultivariate analysis on relapse-free survival, including all patients with complete remission at the end of treatment (N=78, 22 events). Prognostic factors age, site favorability (as defined by the IRSG), and lymph node involvement were included. A model with IRS stage instead of lymph node involvement, and site (N=73, 20 events), resulted in a significant effect of age (p<0.001, HR=1.065, 95% CI=1.037-1.093), IRS II (p=0.003, HR=7.692, 95% CI=1.982-29.855) and a trend for IRS III (p=0.057, HR=2.621, 95% CI=0.974-7.055) versus I, respectively.

Table 7

Prognostic factors in Cox proportional hazards analysis for patients with alveolar rhabdomyosarcoma (ARMS).

Patient group	Prognostic factor		Disease-specific survival						Relapse-free survival					
			Univariate			Multivariate			Univariate			Multivariate		
			p-Value	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value	HR	95% CI
Alveolar	Age		**0.043**	1.023	1.001-1.045	0.078[a]	1.02	0.998-1.043	0.077	1.024	0.997-1.052	0.091[b]	1.023	0.996-1.050
RMS	Local extension		0.217	3.613	0.470-27.772	NI			0.467	2.159	0.271-17.188	NI		
	Location		0.094	2.455	0.858-7.029	0.258	1.918	0.621-5.928	0.467	1.448	0.510-4.343	0.961	1.031	0.303-3.510
	Lymph nodes		0.154	1.625	0.834-3.165	0.840	1.078	0.520-2.235	0.286	1.526	0.702-3.317	0.821	1.117	0.449-2.780
	Metastases		0.098	1.767	0.901-3.407	0.095	1.824	0.901-3.694	0.073	2.107	0.932-4.761	0.091	2.091	0.890-4.913
	IRS stage		0.402			NI			0.318			NI		
		I	Reference						Reference					
		II	0.811	1.245	0.207-7.493				0.585	0.532	0.055-5.117			
		III	0.254	2.128	0.581-7.796				0.526	1.539	0.406-5.836			
		IV	0.131	2.607	0.752-9.031				0.179	2.455	0.663-9.082			
	Size		0.564	0.746	0.276-2.019	NI			0.580	0.734	0.245-2.197	NI		

NI, Not included; NS, not significant at p<0.05; HR, hazards ratio. CI= confidence interval. IRS stage I was used as a reference for calculating significance and HR of Cox proportional hazards analysis when applicable. aMultivariate analysis on disease-specific survival (N=50, 37 events), including age, histological type, site favorability (as defined by the IRSG), and lymph node involvement. Addition of IRS stage instead of lymph node involvement, and site, did not provide additional information (N=48). bMultivariate analysis on relapse-free survival, including all patients with complete remission at the end of treatment (N=36, 27 events). Prognostic factors age, site favorability (as defined by the IRSG), and lymph node involvement were included. A model with IRS stage instead of lymph node involvement, and site (N=73, 20 events), did not give any additional information.

DISCUSSION

In this multicenter retrospective clinical study, we demonstrated in multivariate analysis that increasing age as a continuous variable is a strong prognostic factor of a poor therapeutic outcome in patients with embryonal and alveolar RMS aged 0-73 years. Moreover, histological subtype and clinical presentation at diagnosis (e.g. tumor location, lymph node involvement and presence of metastatic disease) were prognosticators of outcome in the whole cohort.

Whereas prognostic factors in children with RMS have been investigated extensively over the past decades (5-8), until recently, data on prognostic factors in adult patients were scarce, most likely due to the rarity of these tumors and the dispersion of patients treated in adult oncology centers (11).

Our study confirms previous findings in cohorts aged 0-75 years, with additional correction for treatment modalities. La Quaglia *et al*. were the first who described both adults and children with RMS and found age, TNM stage, and histological subtype as prognostic factors of survival (22). Furthermore, a population-based study including 2,600 patients of all ages with RMS was published recently (12), indicating that age, histological subtype, primary site location, stage, and local control with surgery and/or radiation were significant predictors of survival. In contrast to this large study, we attempted to add in this present study an analysis of chemotherapy schedules that were administered and excluded pleiomorphic RMS, which occurs exclusively in adults.

Histological subtype is an established prognostic factor of survival in RMS in children. This holds true with the present findings for the whole cohort in univariate analysis. Moreover, results of our multivariate model for the whole cohort indicate that this disadvantage in outcome for alveolar RMS might have a stronger relation to an unfavorable clinical presentation (e.g. unfavorable primary site and the presence of lymph node and distant metastases) than to alveolar histology itself, as reported by Sultan *et al* (12). In addition, a trend for unfavorable primary location (i.e. parameningeal) and lymph node involvement at a higher age in alveolar RMS was found, which might explain the worse outcome in older patients. However, it should be mentioned that the documentation and assessment of lymph node involvement was suboptimal during the early time period. Nevertheless, the reported effect of lymph node involvement on survival in the current study is in line with a recent report, and supports the view that lymph node involvement should be considered as an important prognosticator, especially in alveolar RMS (26). Regarding histological subtype, it should be taken into account that refinement of the histological diagnosis of RMS with molecular and genetic diagnostic techniques has taken place over time during our study (7, 27).

Metastatic disease is a strong adverse prognostic factor in all patients with RMS, leading to poor survival not exceeding 30% (11, 12, 28, 29). In our cohort, 5-year overall survival of patients with metastatic disease hardly exceeded 10%. Importantly, the presence of metastatic disease correlated with higher age at diagnosis, even in the restricted group of embryonal RMS. Unfavorable clinical presentation with increasing age was also reported by others (11, 20, 21).

Importantly, we found age was a prognosticator of DSS in non-metastatic embryonal RMS. Although age as a prognostic factor in this particular subset of patients has been previously reported (22), to our knowledge, the present study is the first that has evaluated age as a continuous factor in multivariate analysis including treatment modalities.

Apart from tumor-specific factors, treatment-related aspects play a role in the final outcome of RMS patients. Pediatric patients (up to 21 years) are generally treated on study protocols developed by, for example, the SIOP (Europe) and COG (formerly IRSG, United States), including systemic treatment for all patients. In adults, we show that systemic treatment is administered, although less frequently and comprising other agents, as was mentioned above. Of note, anthracyclines were applied more frequently as part of the primary treatment for adult patients. The underlying reason for this discrepancy in treatment approach is not fully clear. The lack of international protocols for the elder RMS population and the rarity of the disease within the adult oncologic population may play an important role (30). Although we found that age remained of prognostic significance after including treatment modalities in a multivariate model, we were not able to correct for the different regimens and dose intensities given. Whether these observed differences in chemotherapy schedules might play a role should be further investigated. Importantly, Ferrari *et al*. previously hypothesized that survival in adults would be comparable to that of children, if they received the appropriate treatment as prescribed in the current childhood regimens (11).

Other suggested explanations for poor survival of adults with RMS are differences in oncogenesis and biological behavior. Advanced clinical presentation with increasing age, as well as age per se being a prognostic factor in uniformly treated children, support this idea (17, 26, 31, 32). Despite investigations focusing on understanding oncogenesis and biological behavior, limited data are available with regard to age-related biological differences of RMS. Younger onset of RMS is associated with several rare syndromes that harbor specific genetic alterations, including of the germline *p53* tumor-suppressor gene, *HRAS* oncogene, and neurofibromin *(NF1)* gene, suggesting a different genetic background for early RMS genesis within these patients (33). Furthermore, the *PAX7/FKHR* translocation *t(1;13)(p36;q14)* in alveolar RMS, is associated with younger onset of disease, primaries of the extremity, localized disease, and better outcome, whereas the more common *PAX3/FKHR* translocation *t(2;13)(q35;q14)* is associated with higher age at diagnosis and represents a highly malignant phenotype with a predilection for bone marrow involvement and worse outcome (34, 35). However, although a recent study confirmed the relation of *PAX3/FKHR* with a higher age and metastatic potential, it did not confirm a higher rate of bone marrow involvement and worse outcome for patients with these tumor types (36). A higher expression of drug-efflux pumps in adult RMS, thereby potentially contributing to worse response to chemotherapy, was also proposed to explain differences in terms of biological behavior (37).

CONCLUSION

In conclusion, age is a strong adverse prognostic factor of survival in patients with embryonal and alveolar RMS. Whether the dismal outcome is caused by a biological or treatment effect remains to be elucidated. With the introduction of more homogeneous treatment protocols for both children and adults, the contribution of age-related biological factors can be further explored. Given the presumably lower tolerance of adults to the high doses of chemotherapy administered in childhood RMS (20), dose schedules should be adapted. Based on the results of our study and the recently published SEER data, collaboration between pediatric and medical oncologists regarding patients with RMS is urgently needed, and should ideally take place at a global level, given the rarity of these tumors (30).

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CHAPTER 5

Anaplastic lymphoma kinase aberrations in rhabdomyosarcoma: clinical and prognostic implications

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ABSTRACT

Purpose: The aim of this study is to investigate anaplastic lymphoma kinase (ALK) protein expression and underlying genetic aberrations in rhabdomyosarcoma, with special attention to clinical and prognostic implications.

Patients and methods: A total of 189 paraffin-embedded rhabdomyosarcoma tumor specimens of 145 patients were collected on tissue microarray. ALK protein expression was evaluated by immunohistochemistry. *ALK* gene (2p23) copy number and translocations were determined by *in situ* hybridization. cDNA sequencing of the receptor tyrosine kinase domain of the *ALK* gene was assessed in 43 samples.

Results: Strong cytoplasmic ALK protein expression was more frequently observed in alveolar rhabdomyosarcoma (ARMS) than in embryonal rhabdomyosarcoma (ERMS) (81% versus 32%, respectively, p<0.001). *ALK* gene copy number gain was detected in the vast majority of ARMS (88%), compared to 52% of ERMS (p<0.001). *ALK* copy number correlated with protein expression in primary tumors (N=107). We identified one point mutation (2%) and seven tumors harboring whole exon deletions (16%). In ERMS, specific *ALK* gain in the primary tumor correlated with metastatic disease (100% in metastatic versus 29% in non-metastatic disease, p=0.004), and poor overall survival (OS) (5-years OS 62% versus 82%, p=0.046).

Conclusion: As ALK aberrations on genomic and protein level are frequently found in rhabdomyosarcomas, in particular ARMS, and are associated with disease progression and outcome in ERMS, ALK may play a role in tumor biology and may provide a potential therapeutic target for these tumors. Future research should aim at the oncogenic role of ALK and the potential effect of ALK-inhibitors in rhabdomyosarcoma.

INTRODUCTION

Rhabdomyosarcomas (RMS) are the most common soft tissue sarcomas of childhood (1, 2). The two main histological subtypes are embryonal RMS (ERMS, 60-70%) and alveolar RMS (ARMS, 20-30%) (1). ARMS is typically characterized by the specific t(2;13) translocation (*PAX3-FOXO1*, 55%), or a variant t(1;13) translocation (*PAX7-FOXO1*, 22%) and is known for its aggressive clinical behavior (3). Although dramatic survival improvements have been reached with the introduction of intensive chemotherapy schedules, survival rates for patients at high risk to develop metastatic disease or already having metastases at initial diagnosis, remain disappointing (<50%) (4-9).

In an attempt to identify genome-wide key players in oncogenesis as well as potential molecular targets for therapy, gene expression profiling studies recently identified the presence of distinct molecular signatures based on the presence or absence of the specific translocations in RMS (10-14). In these studies, translocation-positive ARMS was repeatedly associated with *anaplastic lymphoma kinase (ALK)* overexpression (10-12). Moreover, whole genome analysis for PAX3-FOXO1 protein binding sites recently showed a very high affinity for binding to *ALK*'s 3rd intron yielding increased *ALK* transcription. In the same study, introduction of lentiviral shRNA against *PAX3-FOXO1* into a *PAX3-FOXO1* positive cell line consequently downregulated *ALK* mRNA levels *in vitro* (15).

In line with these gene expression data, ALK protein expression was reported in RMS, predominantly in ARMS but also in ERMS (16, 17). In addition, FISH analysis of a small subset of ALK overexpressing ARMS points towards the presence of *ALK* copy number gain/amplification in RMS (17).

The ALK receptor is a member of the insulin receptor family of receptor tyrosine kinases (RTKs) which is capable of activating the STAT3 (18, 19), AKT/PI3K (20), and RAS/ERK (21) pathways. These pathways are involved in biological processes including cell proliferation, migration and survival. In normal tissues, ALK expression is essentially limited to the central and peripheral nervous system, where it plays a role in neuronal differentiation during embryogenesis (22). However, multiple malignancies are known to harbor ALK alterations, for example anaplastic large cell lymphomas (ALCL), non small cell lung cancer (NSCLC) (23, 24), inflammatory myofibroblastic tumors (IMT) (25, 26), and neuroblastoma (27-31). These alterations encompass translocations (ALCL/NSCLC) (32-34), germline or somatic mutations (neuroblastoma) (27) and genomic gains or amplifications (neuroblastoma, NSCLC) (24, 27). Impressive anti-tumor activity of an agent targeting ALK in NSCLC (35) underlines the clinical relevance that ALK can play in certain tumor entities.

Although the previous investigations point towards ALK as an important factor in RMS, there are limited data concerning expression and genetic alterations of ALK in this disease. The aim of the present multi-center cohort study is to investigate ALK protein expression and underlying genetic aberrations in RMS, with special attention to clinical and prognostic implications in a representative cohort of RMS patients with complete clinical follow-up data.

MATERIAL AND METHODS

Patients and tissue samples

A total of 189 RMS tissue samples of 145 patients were included. One-hundred-thirty-seven samples of patients diagnosed between 1979-2009 were retrieved from the files of the Pathology Departments from the author's affiliations. Fifty-two additional samples were selected from PALGA, the nationwide network and registry of histo- and cytopathology in the Netherlands (36). The study was performed in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands *(http://www.federa.org/?s=1&m=99)*. Slides were reviewed by an expert pathologist (U.E. Flucke) and the diagnosis was based on histological and immunohistochemical (IHC) criteria according to the WHO classification (37). Translocation status could be evaluated by reverse transcriptase polymerase chain reaction (RT-PCR) in 84.4% of ARMS, showing a *PAX3-FOXO1* translocation in 65.8%, *PAX7-FOXO1* translocation in 5.3%, *PAX3/7-FOXO1* translocation in 18.4%, and 10.5% were translocation negative. Paraffin-embedded tumor specimens were collected on tissue micro-arrays (TMAs) containing 1-3 cores of 1-3 mm diameter for each sample.

Immunohistochemistry

ALK IHC was performed on 4 µm thick formalin-fixed, paraffin-embedded TMAs using the mouse monoclonal antibody CD246 (ALK protein, clone ALK1, Dako, Denmark). A selected positive RMS case was used as positive control and substitution of the primary antibody with 1% BSA-PBS was used as a negative control. The IHC protocol is discussed in more detail in the supplementary methods. ALK IHC score was categorized as - (negative), +/- (weak), + (moderately strong), and ++ (strong), with a minimum cut-off at 10% of tumor cells. IHC was scored by three independent investigators. Additionally, a binary scoring system was used for statistical analysis, in which negative or weak staining was considered negative, whereas (moderately) strong staining was considered positive.

Chromogenic / Fluorescence *in situ* hybridization (CISH/FISH)

FISH procedures were performed on identical TMAs using an *ALK* (2p23) split-signal FISH DNA probe (Dako) to detect alternative rearrangements involving the ALK gene and to determine copy numbers. The TMAs were subsequently DuoCISH-stained (Dako DuoCISH kit, Dako, Denmark) and analyzed using bright field microscopy. *ALK* copy number was counted in 30 cells per core and categorized as normal (0-4 copies), low level gain (>4-6 copies), high level gain (>6-10 copies) and amplification (>10 copies) with a minimum cut-off at 10% of the cells. A *MYCN* (2p24) and *LAF* (2q11) Repeat-Free control probe (Kreatech Diagnostics) was used as reference for chromosome 2(p) alterations in cases with abnormal *ALK* copy number (>4 copies of *ALK* in >10% of cells). Cut-off points used were *ALK/LAF* or *MYCN/LAF* ratio <1.5, 1.5-2 and >2. A ratio >1.5 was considered a specific *ALK* or *MYCN* gain.

ALK RTK domain sequence analysis

Fresh-frozen tissue samples with an amount of tumor cells ≥60% (n=43;24 ERMS, 19 ARMS) and four cell lines (RD, Rh18, Rh30 and Rh41, kindly provided by Dr. Peter Houghton, pediatric preclinical testing program, Nationwide Children's Hospital, Columbus, OH) were analyzed by cDNA sequencing (primers and sequencing method are explained in supplementary methods). Mutations were confirmed by repeat amplification/sequencing and by consensus among two investigators.

Statistical analysis

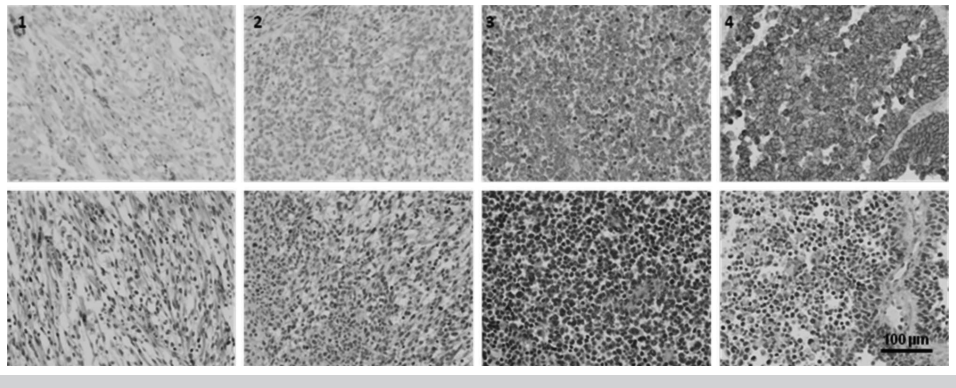
A possible relation between categorical data was calculated using Chi-2 testing or Fisher's exact test when appropriate. Differences in median age and median ALK copy numbers for different IHC groups were assessed using Kruskal Wallis with close-testing. Linear correlation between two continuous parameters was evaluated using Spearman correlation. Kaplan-Meier survival analyses with Log-rank test were performed to evaluate the effect of ALK protein expression and ALK copy number on disease-specific survival (DSS). DSS was defined as time from diagnosis until tumor-specific death. Patients who never experienced an event were censored at the last contact date to the hospital. A $p<0.05$ was considered significant. All statistical analyses were performed using SPSS version 16.0.

RESULTS

ALK protein expression

A total of 183 (97%) samples were evaluable for ALK protein expression. ALK protein staining showed a dot-like or diffuse cytoplasmic staining pattern (figure 1A). Nuclear staining was occasionally observed (7% overall). (Moderately) strong ALK protein expression was identified in tumor samples of different origins (primary 51%, distant/lymph node metastases 56%, postchemotherapy resections 29%, and local recurrences 42%). Strong ALK protein expression was predominantly seen in ARMS (81% overall, 93% primary tumors) and to a lesser extent in ERMS (32% overall, 38% primary tumors, $p<0.001$).

Figure 1A *(color figure page 196)*
ALK protein expression by IHC with the ALK1 antibody (Dako) in RMS.

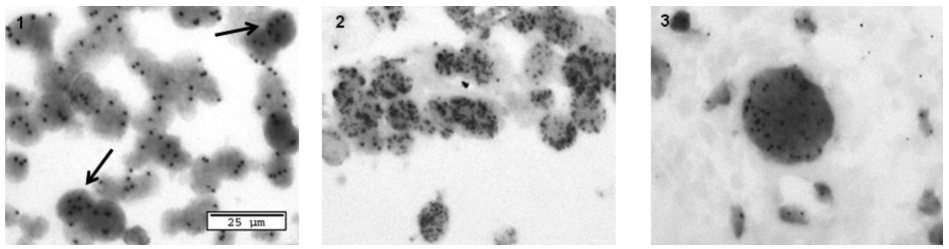


Upper row: 1. ALK negative staining in ERMS, 2. ALK weak staining inERMS, 3. ALK moderately strong staining in ARMS, 4. ALK strong staining in ARMS. Lower row: corresponding heamatoxylin and eosin (H&E) staining. Magnification: 20x

ALK copy number alterations

ALK gene copy numbers could be evaluated in 167 (88%) samples (figure 1B). There was no evidence for the presence of *ALK* translocations. Copy number gain was present in tumor specimens of different origin (primary tumors 59%, distant/lymph node metastases 72%, postchemotherapy resections 65%, and local recurrences 78%). *ALK* copy number gain was observed more frequently in ARMS (88% overall, 92% primary tumors) compared to ERMS tumors (52% overall, 49% primary tumors, p<0.001) (table 1).

Figure 1B *(color figure page 196)*
Chromogenic *in situ* hybridization (CISH) of the ALK gene in RMS.



1. low level ALK gain up to 6 copies per nucleus (marked by black arrows) 2. Primary ARMS showing ALK amplification in all cells 3. Amplification of the ALK gene with >30 ALK copies in an ERMS lymph node metastasis during treatment. Magnification: 40x.

A close-up on copy numbers in primary tumors of ARMS histology, revealed two cases (8%) showing normal *ALK* copy number, 14 cases (58%) showed low level gain, six cases (25%) showed high level gain and two cases (8%) showed true amplification. In ERMS primary tumors, 42 (51%) showed normal *ALK* copy numbers, 32 (39%) showed low level gain, 9 (11%) showed high level gain, and no amplifications were observed. Characteristics of all 17 primary tumors with high level gain or amplification are summarized in table 2.

True amplification of the *ALK* gene was limited to six specimens of unique patients (4%, table 2), including two primary ARMS, one ARMS lymph node metastasis, and three ERMS postchemotherapy resections.

Specific ALK gain and MYCN alterations

In primary tumors with evidence of *ALK* gain/amplification, we evaluated *LAF* (near-centromere; 2q11) and *MYCN* (neighboring gene; 2p24) signals as a reference for the specificity of *ALK* aberrations. No difference in the mean *ALK/LAF* ratio between ARMS and ERMS was seen, ranging from 1.10-2.72 in ERMS (median 1.58 N=36) and from 0.95-12.59 in ARMS (median 1.47 N=20, p=0.584).

In ERMS, 61% (22/36) of the evaluated samples showed specific *ALK* gain (*ALK/LAF* ratio >1.5), which corresponds to an overall rate of 28%. Also, 14% (5/36) showed an indication for whole chromosome 2 gain (defined as >10 % of cells harboring >4 LAF copies), involving a single case with an additional specific *ALK* gain. *MYCN* aberrations were absent in ERMS.

Table 1
Patient characteristics and ALK immunohistochemistry (IHC) score and ALK gene copy number by Chromogenic *In Situ* Hybridization (CISH)

	All patients		ALK IHC negative		ALK IHC positive		p	normal ALK copy number		ALK gain/ amplification		p
	No	%	No	%	No	%		No	%	No	%	
ALL TUMORS	189	100	87	47,5	96	52,5		62	37,1	105	62,9	
Tissue origin							0,144					0,466
primary tumor	119	63	57	49,1	59	50,9		44	41,1	63	58,9	
treatment effect	31	16,4	20	71,4	8	28,6		9	34,6	17	65,4	
metastases (N+M)	27	14,3	12	44,4	15	55,6		7	28	18	72	
local failure	12	6,3	7	58,3	5	41,7		2	22,2	7	77,8	
Histology							**<0.001**					**<0.001**
ERMS	128	67,7	85	68	40	32		56	48,3	60	51,7	
ARMS	61	32,3	11	19	47	81		6	11,8	45	88,2	
PRIMARY TUMORS	119											
Histology							**<0,001**					**<0,001**
ERMS	91	76,5	55	61,8	34	38,2		42	50,6	41	49,4	
ARMS	28	23,5	2	7,4	25	92,6		2	9,1	22	91,7	
Gender							0,826					0,222
Male	73	62,4	34	47,9	37	52,1		30	46,2	35	53,8	
Female	44	37,6	22	50	22	50		14	34,1	27	65,9	
Location							0,456					0,438
Favorable	60	50,8	31	52,5	28	47,5		25	44,6	31	55,4	
Unfavorable	58	49,2	26	45,6	31	54,4		19	37,3	32	62,7	
Size							0,087					0,078
≤5 cm	21	38,9	8	40	12	60		5	26,3	14	73,7	
>5 cm	33	61,1	22	67,7	11	33,3		17	54,8	14	45,2	
Invasiveness							1,00					0,739
T1	14	31,1	7	53,8	6	46,2		5	38,5	8	61,5	
T2	31	68,9	16	51,6	15	48,4		14	48,3	15	51,7	
Lymph node involvement							0,104					0,249
N0	56	75,7	31	57,4	23	42,6		22	43,1	29	56,9	
N1	18	24,3	6	33,3	12	66,7		4	23,5	13	76,5	
Metastatic disease							**0,033**					**0,022**
M0	61	82,4	34	57,6	25	42,4		25	44,6	31	55,4	
M1	13	17,6	3	23,1	10	76,9		1	8,3	11	91,7	
IRS stage							0,184					**0,032**
1	31	46,3	17	56,7	13	43,3		10	34,5	19	65,5	
2	3	4,5	2	67,7	1	33,3		1	33,3	2	67,7	
3	20	29,9	11	55	9	45		11	61,1	7	38,9	
4	13	19,4	3	23,1	10	76,9		1	8,3	11	91,7	
Clinical Group							0,063					**0,006**
I	20	31,3	10	50	10	50		10	52,6	9	47,4	
II	12	18,8	6	50	6	50		1	8,3	11	91,7	
III	19	29,7	13	72,2	5	27,8		9	52,9	8	47,1	
IV	13	20,3	3	23,1	10	76,9		1	8,3	11	91,7	
Age median (range years)	8 (0-72)		6 (0-64)		13 (0-72)		**0,041**	6,5 (1-64)		11 (0-72)		0,424

NOTE missing data were excluded from the analysis. T= Tumor invasiveness; T1= non-invasive, T2= invasive to surrounding tissues, Tx= unknown. N= lymph node involvement; N0= no clinical/CT/histological evidence for lymph node involvement, N1= evidence for lymph node involvement. M= metastatic disease; M0= no distant metastases, M1= distant metastases present. IRS= pre-treatment staging system. Clinical group= post-surgical clinical group.

Table 2
ALK characteristics of the primary tumors with high level gain or amplification and non-primary tumor samples with amplification

ID	Type	Translocation	ALK copy number	RANGE ALK CN	MEAN ALK CN	MEAN LAF CN	MEAN MYCN CN	Chrom 2 gain*	ALK/LAF ratio	MYCN/LAF ratio	ALK IHC
Primary tumor samples with high level gain/amplification											
RMS175	ERMS		hlg	2-14	4,62	2,08	2,12	no	2,22	1,02	+/-
RMS24	ERMS		hlg	2-25	5,28	2,37	2,07	no	2,23	0,87	+/-
RMS5	ERMS		hlg	1-15	3,83	2,50	2,56	no	1,53	1,02	-
L-347	ERMS		hlg	1-14	4,67	2,52	2,44	no	1,85	0,97	-
RMS61	ERMS		hlg	3-9	5,07	2,67	2,57	no	1,90	0,96	-
RMS212	ERMS		hlg	2-12	4,99	2,92	3,17	yes	1,71	1,08	+
RMS45	ERMS		hlg	2-15	4,92	2,98	3,04	no	1,65	1,02	+
RMS150	ERMS		hlg	2-8	4,60	3,39	3,19	yes	1,36	0,94	+/-
L-818	ERMS		hlg	2-14	4,99	3,94	4,11	yes	1,26	1,04	+
L-878	ARMS	PAX3	hlg	1-13	4,70	2,43	3,50	no	1,93	1,44	++
L-250	ARMS	PAX3	hlg	1-9	4,35	2,54	3,09	no	1,71	1,21	+
L-315	ARMS	PAX3	hlg	1-18	4,75	2,75	2,55	no	1,73	0,93	++
RMS191	ARMS	NEGATIVE	hlg	2-12	6,28	3,15	3,12	no	1,99	0,99	+
L-193	ARMS	PAX3	hlg	1-12	4,00	4,20	4,18	yes	0,95	1,00	+
RMS132	ARMS	PAX3	hlg	2-10	5,37	n.a.	n.a.	n.a.	n.a.	n.a.	+
RMS103	ARMS	n.a.	ampl	30-30	30,00	2,38	2.32	no	12,59	0,97	++
RMS205	ARMS	PAX3	ampl	2-15	6,07	2,98	2.81	yes	2,04	0,94	+
Non-primary tumor samples with ALK amplification											
RMS89	ARMS	PAX3/7	ampl	2-20	10.10	2.24	2.32	no	4.50	1.03	++
RMS15	ERMS	-	ampl	2-31	6.43	12.6	4.67	yes	0.51	0.37	+
RMS154	ERMS	-	ampl	2-15	6.17	2.32	1.97	no	2.67	0.85	-
RMS5	ERMS	-	ampl	1-12	5.43	2.02	1.97	no	2.69	0.98	+/-

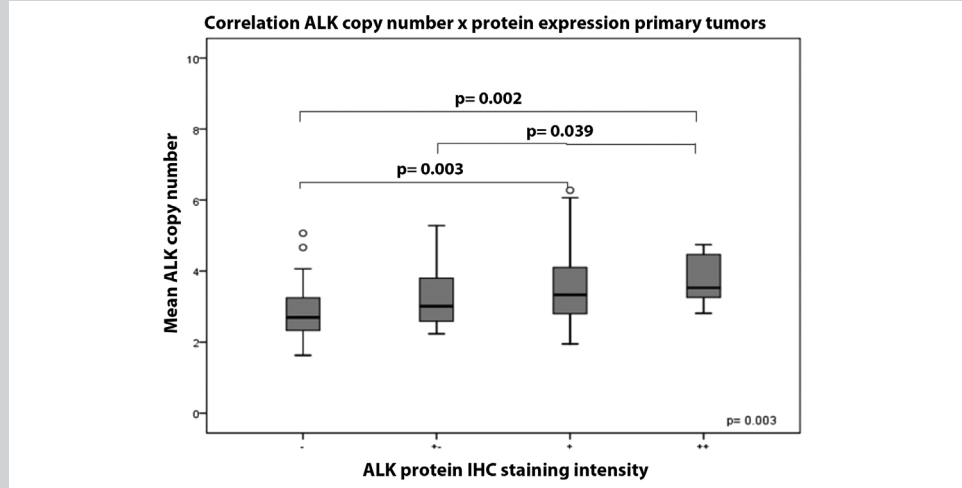
(RMS89 represents a lymph node metastasis, RMS 15, RMS154 and RMS5 postchemotherapy specimens). *whole chromosome 2 gain was defined as a minimum of ≥10% of nuclei containing more than 4 LAF signals. Hlg= high level gain ampl= amplification n.a.= could not be assessed

In ARMS, *ALK* specific gain was observed in 45% (9/20), which corresponds to an overall rate of 41% in all primary ARMS (9/22). Whole chromosome 2 gain was present in 25% (5/20) of ARMS, including one case with an additional specific *ALK* gain. *MYCN* amplification was observed in three cases, all without specific *ALK* gain.

Relation between ALK copy number and protein expression

The mean *ALK* copy number distribution by protein expression was evaluated in primary tumors (figure 1C). There was a significant positive correlation of mean *ALK* gene copy number and protein expression (Spearman's rho p<0.001, correlation coefficient (cc) 0.352, N=107). No significant correlation between mean copy number and protein expression was found in ARMS (N=24, p=0.109, cc 0.335), and ERMS (N=83, p=0.085, cc 0.190) separately. Specific *ALK* gain was not related to protein expression.

Figure 1c
ALK IHC by mean ALK copy number.



This figure displays the distribution of the mean ALK copy number in RMS tumors showing negative (-), weakly (+/-), moderately strong (+), or strong (++) ALK staining in primary tumors. P-value's calculated by Kruskal-Wallis test and close-testing of the separate groups by Mann-Whitney-U test.

Sequencing of the ALK RTK domain

Eight out of 43 (19%) investigated tumors harbored genetic alterations in mRNA encoding for the *ALK* RTK domain. One patient showed a missense mutation c.3673G>A; p.Asp1225Asn. Matched germline DNA was not available to determine whether this was a germline or a somatic mutation. We furthermore identified a solitary exon 23 deletion (n=2 patients, and Rh18), a solitary exon 27 deletion (n=3 patients and Rh41), a combined exon 23 and 25 deletion (n=1 patient) and a combined deletion of 2 nucleotides in exon 25 and a whole exon 27 deletion (n=1 patient). Assuming there are no alterations of *ALK* outside the sequenced domain, these deletions will result in the formation of an unglycosylated ALK protein of ~130 (exon 23), ~140 (GA deletion exon 25 and exon 27), and ~170 (exon 25) kDa. *ALK* copy number and protein expression data of these patients and their clinical characteristics are summarized in table 3 and 4. No obvious relation between these genetic aberrations and protein expression was observed.

Longitudinal ALK expression

Paired tumor samples of single patients (primary tumor/chemotherapy effect/lymph node or distant metastasis/local recurrence) were analyzed for longitudinal changes in ALK expression. ALK protein expression (n=47 pairs, (moderately) strong versus weak/negative) was similar in 68.1%, whereas *ALK* copy number (n=40 pairs, normal versus gain/amplification) was identical in 80.0%. ALK protein expression was lower in postchemotherapy resections than in the related primary tumor (n=21, p=0.02, z=-2.333) and a tendency for decreased *ALK* copy number in post-chemotherapy resections compared to the related primary tumor was observed (n=17, p=0.08, z=-1.732).

Table 3
ALK characteristics in cases with genetic alterations of the *ALK* RTK domain.

NO	ID	GENETIC ALTERATION			TYPE	PAX/FOXO1	ALK CN (MEAN)	ALK RANGE	CN group	MYCN CN (MEAN)	ALK/LAF ratio	ALK IHC	whole chrom 2 gain
1	61	c.3516_3645del = p.Ser1172_Pro1215del	exon 23 deletion	frameshift	ERMS	na	5,07	3-9	HLG	2,57	1,90	_	NO
2	205	c.3516_3645del = p.Ser1172_Pro1215del	exon 23 deletion	frameshift	ARMS	PAX3-FOXO1	6,07	2-15	AMPL	2,81	2,04	+	yes
3	116	c.3516_3645del = p.Ser1172_Pro1215del	exon 23 deletion	frameshift	ARMS	PAX3/7-FOXO1	3,31	2-6	LLG	3,51	0,95	++	NO
		c.3744_3836del = p.Arg1248_Arg1279del	exon 25 deletion	in frame									
4	203	c.3938_4073del = p.Trp1313_Val1357del	exon 27 deletion	frameshift	ERMS	NEGATIVE	3,03	2-5	NORMAL	1,78	1,53	_	NO
5	114	c.3938_4073del = p.Trp1313_Val1357del	exon 27 deletion	frameshift	ERMS	na	3,97	2-11	LLG	2,35	1,59	+	NO
6	213	c.3938_4073del = p.Trp1313_Val1357del	exon 27 deletion	frameshift	ERMS	NEGATIVE	1,87	1-3	NORMAL	2,41	0.75	+	NO
7	209	c.3938_4073del = p.Trp1313_Val1357del	exon 27 deletion	frameshift	ERMS	na	3,03	2-8	LLG	3,27	1,03	_	NO
		c.3745_3746delGA	2 nucleotide deletion (exon 25)	frameshift									
8	85	c.3673G>A = p.Asp1225Asn	Mutation		ERMS	na	2.53	2-4	NORMAL	2,37	1,43	+/-	NO

LLG= low level gain, HLG = high level gain, AMPL=amplification. Whole chromosome 2 gain was defined as a minimum of 10% of nuclei displaying >4 LAF copies. na= not assessable. CN = copy number No. 3 (ID116) represents a lymph node metastasis at diagnosis, whereas the other samples were all primary tumors.

Table 4
Clinical characteristics of the cases with genetic alterations in the *ALK* RTK domain.

NO	ID	Age	Gender	Location	Favorability	Size	T	N	M	IRS	Clinical Group	CR	Event	EFS	Status FU	FU time (months)
1	61	3	MALE	EXTREMITY/BUTTOCK	UNFAV	>5	T2	N0	M0	3	1	YES	NED		DOC	115
2	205	37	MALE	PARAMENINGEAL	UNFAV		T2	N1	M0	3		YES	DF	15,3	DOD	17
3	116	10	MALE	EXTREMITY	UNFAV		Tx	N0	M1	4	4	NO	LF+DF	9,0	DOD	17
4	203	1	MALE	BLADDER/PROSTATE	UNFAV	>5	T2	N0	M0	3		YES	LF	26,0	DOD	27
5	114	3	FEMALE	PELVIS (BLADDER/OVARY)	UNFAV	>5	Tx	N0	M1	4	4	YES	NED		NED	22
6	213	2	FEMALE	PARAMENINGEAL	UNFAV	>5	T2	N1	M0	3		YES	LF	20,7	DOD	25
7	209	8	MALE	PARATESTTICULAR	FAV	>5	T1	N0	M0	1	1	YES	NED		NED	188
8	85	7	MALE	ORBIT	FAV	<5	T1	N0	M0	1	3	YES	NED		NED	14

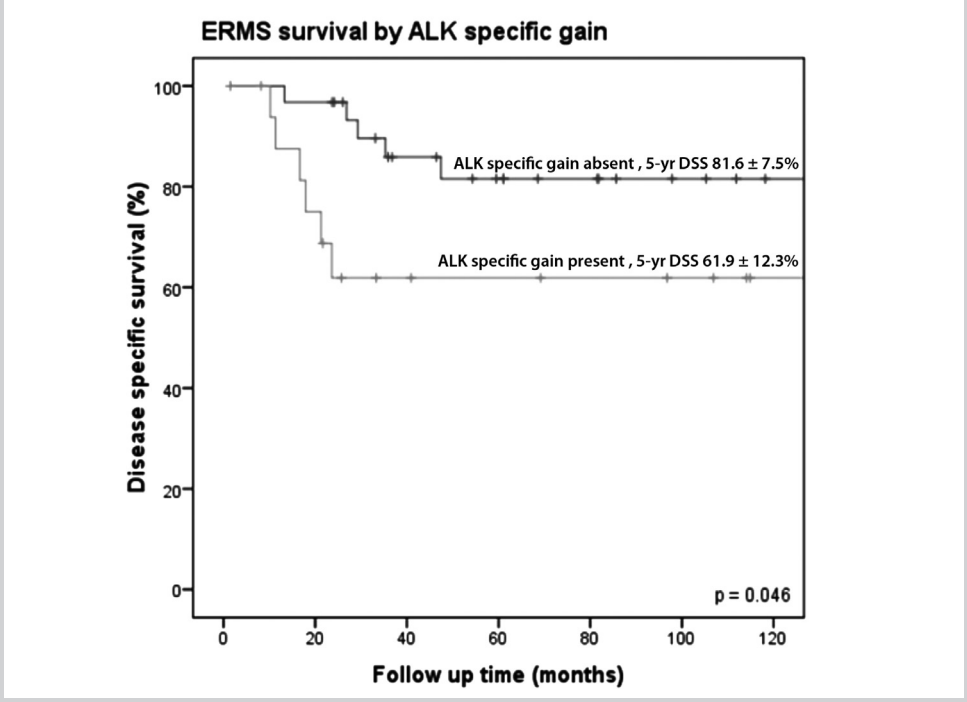
FAV= favorable location of the primary tumor, UNFAV= unfavorable location of the primary tumor. T= Tumor invasiveness; T1= non-invasive, T2= invasive to surrounding tissues, Tx= unknown. N= lymph node involvement; N0= no clinical/CT/histological evidence for lymph node involvement, N1= evidence for lymph node involvement. M= metastatic disease; M0= no distant metastases, M1= distant metastases present. IRS= pre-treatment staging system. Clinical group= post-surgical clinical group. CR= complete remission. DF= distant failure, LF= local failure. EFS= event free survival (months). FU= follow-up; NED= no evidence of disease, DOC = dead due to other cause, DOD= dead of disease.

Clinical and prognostic implications of ALK

The clinical characteristics investigated for a potential relation with ALK in primary tumors are summarized in table 1. We could retrieve the clinical follow-up data of 73 patients (median follow-up of 46.5 months). In all patients, a significant relation between ALK protein expression and ARMS histology ($p<0.001$), metastatic disease at diagnosis ($p=0.033$), and higher median age at diagnosis ($p=0.041$) was observed. *ALK* copy number gain/amplification was significantly related with ARMS histology ($p<0.001$), and the presence of metastatic disease at diagnosis ($p=0.022$).

In ERMS, we observed a significant relation between specific *ALK* copy number gain and metastatic disease (5/5 of metastatic versus 13/45 of non-metastatic patients displayed *ALK* copy number gain, $p=0.004$) and worse survival (n=49, 5-year DSS 61.9 ± 12.3% versus 81.6 ± 7.5%, $p=0.046$) (figure 2).

Figure 2
Effect of specific *ALK* gain on ERMS survival.



This figure shows a significant poorer survival in patients with ALK specific gain in the primary tumor (N=18, 5-year survival 61.9 ± 12.3%) versus non-specific gain or no gain (N=31, 5-yr DSS 81.6 ± 7.5% p=0.046 N at 5 yrs 17)

In ARMS, we did not observe any relations between ALK protein expression or copy number alterations and clinical presentation or outcome, due to the nearly absence of cases without protein expression or copy number gain. However, DSS for the whole group of ARMS was particularly low (n=17, 5-year DSS 31.2 ± 11.3%).

DISCUSSION

The identification of novel therapeutic targets in RMS is of major clinical importance. In this study, we provide a descriptive characterization of ALK protein expression and underlying genetic aberrations in RMS. Furthermore, the clinical and prognostic implications of ALK status were explored in a representative subset of patients with complete follow-up data.

We demonstrated ALK protein expression in the vast majority of ARMS (81%, n=58) and in a significant number of ERMS (32%, n=125), which is higher than previously described in smaller series; 45-52% in ARMS and 15-23% in ERMS (16, 17).

As reported earlier in a small number of cases (n=6) selected for their high ALK protein expression (17), a high frequency of *ALK* copy number gains was found in our extensive cohort (ARMS 88% n=51 and ERMS 52% n=116; respectively). True amplification of the *ALK* gene was observed in only 4% of RMS in the present study, which is lower than the single amplification (17%, n=6) reported by Corao *et al* (17).

Previous studies also identified *MYCN* (*ALK* neighboring gene; 2p24) overexpression in ARMS (mainly in *PAX3-FOXO1* positive) and to a lesser extent in ERMS (10, 15, 38-40). However, *ALK* copy number gain in our series was not associated with gain/amplification of a larger area on chromosome 2p (including both *ALK* and *MYCN*), as was previously reported in neuroblastoma (27). Of note, the number of cases with *MYCN* amplification was particularly low (n=3 ARMS). Interestingly, the presence of *ALK* translocations/rearrangements as previously found by 2p23 break-apart FISH analysis (2/19) (17, 26, 41), was not confirmed in our study population.

We detected a high rate of genetic aberrations on mRNA level in the RTK domain of *ALK*; in ERMS more often than in ARMS. Seven tumors harbored whole exon deletions and one missense mutation was detected. To our knowledge, exon deletions have not been described before and we found that these deletions potentially lead to variable ALK isoforms. A 140 kDa variant of the ALK protein was earlier reported in neuronal tissues as well as in two neuroblastoma cell lines (UKF-NB3 and IMR-32) (22, 42, 43). Although it was earlier proposed that proteolytic cleavage of the extracellular domain is responsible for the presence of a shorter variant of ALK (44), our data indicate that alternative mRNA splicing is the mechanism behind the existence of variable ALK isoforms in RMS, as was postulated by Morris *et al* (32). Although activation by phosphorylation of the shorter variant protein was observed in neuroblastoma cell lines (43), the consequences of the existence of ALK splice variants remain to be revealed. We furthermore postulate that the identified point mutation might alter protein function, but this should be further elucidated.

There is an ongoing debate with regard to the optimal ALK detection method in different malignancies (45). Although the current study underscores the feasibility of ALK protein detection (IHC) and copy number evaluation (ISH) in RMS, there are some issues that should be considered. Importantly, the variant ALK isoforms might not be detected using the ALK1 antibody since it binds distal to the end of the isoforms (bp 1359-1460), leading to a potential underestimation

of protein expression in the current series. Additionally, the interpretation of gene copy number gain/amplification is complex. *ALK* copy number cut-offs in the present study were arbitrarily defined. By our cut-off strategy (>4 copies per nucleus in ≥10% of tumor cells) we aimed to prevent for overestimation of ALK copy number caused simply by DNA duplication in highly proliferative tumor cells. However, this strategy might lead to underestimation of low level copy number gains and impairs the detection of specific *ALK* losses.

Although the functional role of the observed ALK alterations remains unknown, the high rate of both copy number changes and protein expression in mainly ARMS and also progressive ERMS suggest a potential role for ALK in the tumor biology of these tumors. This is further supported by previous data reporting *ALK* overexpression on mRNA level in (translocation positive) ARMS (10-12) as well as the potential of the chimeric PAX3-FOXO1 protein to influence *ALK* transcription (15).

Although it is too early to draw firm conclusions concerning the potential effect of ALK inhibitors in RMS, the observed relation between specific *ALK* copy number gain and metastatic disease at diagnosis and poor outcome in ERMS encourages the need for investigations concerning the potential effect of ALK inhibitors in RMS patients with progressive disease. As the clinical and outcome data concerned only a limited subpopulation of our cohort, the relation of ALK with progressive disease and outcome should be further validated in a future cohort of RMS patients. Small molecule inhibitors of ALK are currently under (pre)clinical investigation and recent data have shown impressive clinical responses in ALK overexpressing NSCLC, ALCL and IMT (35, 46, 47). Mutations and amplification of the *ALK* gene have previously been shown to modify the response to ALK inhibitors (29, 30, 48, 49). We suggest that the evaluation of genetic changes is therefore essential in the development of predictive and selective models for future ALK inhibitory treatment. The observation that *ALK* RTK deletions are also apparent in RMS cell lines, indicates that potential modifying effects of these alterations can be studied further *in vitro* and in xenograft models of these cell lines. As ALK expression was shown to decrease after chemotherapy, the optimal sequence of ALK-inhibitors in combination with chemotherapy should be further investigated in preclinical models.

In conclusion, our study shows that ALK protein expression as well as copy number gain is a common feature in ARMS and progressive ERMS. Future (pre)clinical research should aim at the functional role of ALK and the potential effect of ALK-inhibitors in RMS, with special attention to the observed genetic aberrations.

APPENDIX

ALK IHC supplementary data

ALK immunohistochemistry was performed on 4 µm thick formalin-fixed, paraffin-embedded TMAs using antigen retrieval by heating in a pressure cooker in a 10 mM citrate buffer (pH 6.0). The slides were blocked with 3% hydrogen peroxide and 20% normal horse serum (Vector Laboratories Inc., Burlingame, USA). Mouse monoclonal antibody CD246 (ALK protein, clone ALK1, Dako, Denmark) was applied overnight in a humidified chamber at 4°C at a dilution of 1:10 in 1% bovine serum albumin–phosphate buffered saline (BSA-PBS). The sections were incubated with a secondary biotinylated horse-anti-mouse IgG antibody (Vector Laboratories Inc., Burlingame, USA) followed by incubation with an avidin-biotinylated horseradish peroxidase complex (ABC) using Vectastain ABC kit (Vector Laboratories Inc., Burlingame, USA). The catalysed reporter deposition (CARD) technique was used to enhance staining results and antibody binding was visualized with PowerVision DAB (3,3'-diaminobenzide) (ImmunoLogic, Duiven, the Netherlands).

Sequencing additional data

Fresh-frozen tissue samples with an amount of tumor cells ≥60% (n=43 ;24 ERMS, 19 ARMS) and four cell lines (RD, Rh18, Rh30 and Rh41, kindly provided by Dr. Peter Houghton, pediatric preclinical testing programm) were analyzed by cDNA sequencing. Total RNA was prepared using the RNA-Bee isolation reagent according to the manufacturer's protocol (Tel-Test Inc., Friendswood, TX). cDNA was transcribed from 1µg RNA using SuperScriptII reverse transcriptase (Invitrogen Life Technologies, Breda, the Netherlands). A region of 1.7 kb including the RTK domain of the ALK gene was PCR amplified. PCR analysis was performed using AmpliTaq Gold DNA polymerase (Applied Biosystems), with the following program: 94°C 10 min; 92°C 1 min, 60°C 45 sec, 72°C 1 min, 35 cycles; 72°C 10 min. PCR primers are listed in supplementary table 1. The sequencing reaction was performed using the BigDye Terminator reaction mix and samples were analyzed on the 3730 DNA Analyzer (Applied Biosystems). Mutations were confirmed by repeat amplification/sequencing and by consensus among two investigators.

Supplementary table 1
Primers for PCR and sequencing analysis

Primer	Sequence (5'-3')	PCR product size (bp)
ALK-1F	TGTAAAACGACGGCCAGTCATCAGTCCACTGGGCATC	464
ALK-1R	CAGGAAACAGCTATGACCTGCCAGCAAAGCAGTAGTTG	
ALK-2F	TGTAAAACGACGGCCAGTGAGCCCTGAGTACAAGCTGAG	505
ALK-2R	CAGGAAACAGCTATGACCAGCCACGTGCAGAAGGTC	
ALK-3F	TGTAAAACGACGGCCAGTAGTCCTTCCTCCGAGAGACC	528
ALK-3R	CAGGAAACAGCTATGACCGATGTTGCCAGCACTGAGTC	
ALK-4F	TGTAAAACGACGGCCAGTGATGGACCCACCCAAGAAC	517
ALK-4R	CAGGAAACAGCTATGACCGTACGTTGGGTTCCACAAGC	

* All primers contain a M13 sequence (underlined) introduced as a site to initiate sequencing.

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CHAPTER 6

Simultaneous targeting of IGF-1R and ALK in embryonal and alveolar rhabdomyosarcoma: a rationale choice

Submitted

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ABSTRACT

Background: Rhabdomyosarcoma (RMS) is an aggressive soft tissue tumour mainly affecting children and adolescents. Since survival of high-risk patients remains poor, new treatment options are awaited. The aim of this study is to investigate anaplastic lymphoma kinase (ALK) and insulin-like growth factor-1 receptor (IGF-1R) as potential therapeutic targets in RMS.

Methods: One-hundred-and-twelve primary tumours (embryonal RMS (eRMS) 86; alveolar RMS (aRMS) 26) were collected. Expression of IGF-1R and ALK was evaluated by immunohistochemistry. The effect of ALK inhibitor NVP-TAE684 (Novartis), IGF-1R antibody R1507 (Roche) and combined treatment was investigated by MTT assays in cell lines (aRMS Rh30, Rh41; eRMS Rh18, RD).

Results: IGF-1R and ALK expression was observed in 72 and 92% of aRMS and 61 and 39% of eRMS, respectively. Co-expression was observed in 68% of aRMS and 32% of eRMS. Nuclear IGF-1R expression was an adverse prognostic factor in eRMS (5-yr survival 46.9±18.7% versus 84.4±5.9%, p=0.006). *In vitro*, R1507 showed diminished viability in Rh41. NVP-TAE684 showed diminished viability in Rh41 and Rh30, and to a lesser extent in Rh18 and RD. Simultaneous treatment revealed synergistic activity against Rh41.

Conclusion: Co-expression of IGF-1R and ALK is detected in eRMS and particularly in aRMS. As combined inhibition reveals synergistic cytotoxic effects, this combination seems promising and needs further investigation.

INTRODUCTION

Rhabdomyosarcoma (RMS) is an aggressive soft tissue sarcoma. Although it is relatively rare with an estimated incidence of 4.5 per million, it is the most common pediatric soft tissue sarcoma, and accounts for 3-7% of all malignancies in children (1). Its two most common forms are embryonal (eRMS) and alveolar RMS (aRMS). The Children's Oncology Group (COG) reported dramatic increases in 5-year survival on chemotherapeutic regimens between 1972 and 1997 (55% to 73%) (2-3). However, the prognosis for the high-risk subset of RMS patients (e.g. alveolar histology, lymph node involvement, distant metastases, recurrent disease, and higher age) remains poor, not exceeding a 5-year survival of 50% (4-7). Therefore, there is an urgent need for new therapeutic strategies.

The specific targeting of receptor tyrosine kinases is an upcoming treatment strategy for many tumour types, including sarcomas (8). The insulin-like growth factor (IGF) system is probably the most extensively studied treatment potential in sarcomas over the past decade (9). As IGF pathway signaling is believed to play an important role in oncogenesis and progression of RMS, this seems a potential treatment target (9-10). This is supported by overexpression of both IGF-1R and mainly IGFII in RMS tumours, cell lines and xenograft models (11-14). Furthermore, IGF pathway inhibition by antisense and small interfering RNA, monoclonal antibodies (mAbs) and small molecule tyrosine kinase inhibitors (TKIs) against IGF-1R were shown to result in decreased RMS growth *in vitro* and *in vivo* (15-18)

Despite promising preclinical evidence of an anti-tumour effect of IGF-1R inhibitors in RMS, the results of clinical trials remain unsatisfactory because of the modest and temporarily anti-tumour effect (19-21). Since altered activation of the same intracellular survival pathways via alternative receptors was observed upon IGF-1R directed treatment (22-25), simultaneous targeting of these receptors could be a potential strategy (26). We hypothesize that the anaplastic lymphoma kinase receptor (ALK) is a potential candidate for simultaneous therapy, as high expression rates were observed previously (27-28), and as ALK downstream activation overlaps that of IGF-1R, involving the PI3K and MAPK pathways (29).

The aim of the current study is to investigate the (co-)expression of IGF-1R and ALK in RMS tissue samples and correlate this with outcome in a representative multicenter cohort study. Furthermore we investigate the effect upon (simultaneous) targeting of IGF-1R and ALK in RMS *in vitro*.

PATIENTS AND METHODS

Patients and tumour samples

Tumour material consisted of 112 therapy-naïve biopsies (86 eRMS, 26 aRMS) retrieved from the authors' (referral) files (UEF, AJHS) and PALGA, the nationwide network and registry of histo- and cytopathology in the Netherlands. The current cohort largely overlaps the cohort as we described in our previous publication (27). Clinical characteristics are summarized in table 1. Tissues and follow-up data were retrieved according to the Dutch Code on Proper Use of tissue *(http://www.federa.org/gedragscodes-codes-conduct-en)*. RMS diagnosis was reviewed and reclassified by an expert pathologist (UEF), based on criteria according to the WHO classification (30).

Tumour specimens were collected on tissue micro-arrays (TMA), containing 1-3 cores of 1-3 mm diameter for each sample.

Table 1
Patient characteristics

	eRMS		aRMS		
	N	%	N	%	
All	**86**		26		P
Age median (range years)	7 (0-64)		15 (0-72)		**0.018**
Location					**0.001**
Favorable	51	60.0	6	23.1	
Unfavorable	34	40.0	20	76.9	
Size					0.698
≤5 cm	17	37.8	4	50.0	
>5 cm	28	62.2	4	50.0	
Lymph node involvement					0.106
N0	45	81.8	11	61.1	
N1	10	18.2	7	38.9	
Metastatic disease					**0.007**
M0	50	90.9	11	61.1	
M1	5	9.1	7	38.9	
IRS stage					**0.004**
1	28	57.1	3	17.6	
2	1	2.0	2	11.8	
3	15	30.6	5	29.4	
4	5	10.2	7	41.2	
Alive at last follow-up	39/54	72.2	5/18	27.8	**0.002**
Follow-up time (months)	60.3 (1.5-292.3)		24.7 (0.5-254.3)		

Favourable locations included: orbit, head and neck non parameningeal, urogenital and hepatobilliary tract tumours. All other primary locations were unfavourable. N0= no lymph node involvement N1 = lymph node involvement present; M0= no distant metastases M1= distant metastases present; IRS stage = pre-treatment staging system according to the Intergoup rhabdomyosarcoma Study Group (IRSG) study IV. Missing data is due to inavailability of clinical and follow-up data of the cohort of patients included via PALGA. Follow-up time is mentioned as median (ranges) for the different subtypes.

Cell lines

RMS cell lines (RD, Rh18, Rh30 and Rh41) were generously provided by Dr. Peter Houghton of the Pediatric Preclinical Testing Program (Columbus, OH). RD cells were cultured in DMEM medium (PAA Laboratories GmbH, Pasching, Austria), Rh18 cells in McCoy's 5A medium (Lonza Benelux BV, Breda, the Netherlands) and Rh30/Rh41 cells in RPMI 1640 medium (PAA Laboratories GmbH). All media were supplemented with 10% fetal bovine serum (PAA Laboratories GmbH) and 1% Pen-Strep (Lonza Benelux BV) and cells were cultured in a humidified atmosphere of 5% CO2/95% air at 37°C. For IHC analysis, the cells were fixed with Unifix (Klinipath, Duiven, The Netherlands) and processed into AgarCytos.

Immunohistochemistry

Immunohistochemical staining was performed to evaluate the expression of ALK and the IGF1 receptor ß (IGF-1R). The specifications are listed in table 2. Immunohistochemical staining of ALK and IGF-1R on tumour samples was performed as described previously (27;31). For ALK staining in RMS cell lines, 4 µm sections were pretreated with EDTA buffer by heating in a microwave oven. Endogenous peroxidase was then blocked (3% hydrogen peroxide) and sections were incubated with the primary antibody overnight at 4°C. Next, sections were incubated with Poly-HRP-GAM/R/R IgG (ImmunoLogic, Duiven, the Netherlands) and antibody binding was visualized with PowerDAB (3,3'-diaminobenzidine; ImmunoLogic). Slides were counterstained with haematoxylin, dehydrated and coverslipped. Positive control tissues were used as listed (table 2). Substitution of the primary antibody by 1% BSA-PBS served as negative control.

Table 2
Immunohistochemistry methods

Antigen	Antibody	Dilution	Antigen retrieval	Control tissue
IGF-1R	pAb rabbit IgG, #3027 (Cell Signaling Technology, Beverly, USA)	1/250 in 1% BSA-PBS	Microwave 3 min MAX, 10 min 180W in 10 mM citrate buffer pH6.0	Human breast
ALK	mAb rabbit IgG, #3633 (Cell Signaling Technology, Beverly, USA)	1/50 in 1% BSA-PBS	Microwave 3 min MAX, 10 min 180W in EDTA buffer pH9.0	Anaplastic large cell lymphoma

Immunohistochemistry scoring system
Nuclear and cytoplasmic staining was scored separately by three independent investigators (UEF, YMHV, JCG). Staining intensity was compared to positive control and scored as negative (0), weak (1), strong (2) or very strong (3), with a cut-off at ≥10% of cells. Cases with discordant results were re-evaluated and given a mean final score. Staining scores were binary recoded based on overall staining intensity of the protein (0 and 1 negative, 2 and 3 positive) (27).

Inhibitors
The TKI NVP-TAE684 against ALK was provided by Novartis (Basel, Switzerland). The fully human mAb R1507 against IGF-1R was provided by Roche Diagnostics (Penzberg, Germany).

Cell viability assay
RMS cells were seeded into 96-well plates at 5000 cells (RD, Rh30 and Rh41) or 3000 cells (Rh18)/100 µl/well and allowed to adhere. After 24 hours, a series of NVP-TAE684 (range 0.01-100.000 nM) and R1507 (0.1-100.000 ng/ml) doses were added and cells were incubated for 72 (RD and Rh30), 120 (Rh41) or 144h (Rh18), based on estimated growth rates. All drug concentrations and controls were completed in quadruplicate. Subsequently, 20 µl of 5 mg/ml MTT (3-(4,5- dimethylthiazol-2-yl)-2 ,5-diphenyltetrazolium bromide, Sigma-Aldrich, Zwijndrecht, the Netherlands) in PBS was added to each well and cells were incubated for another 3.5h at 37°C. Afterwards, the medium was carefully removed and the formazan crystals were dissolved in 150 µl of acidified isopropanol solution. Absorbance was read at 560 nm using an ELISA reader. The experiments were repeated in triplicate and IC50 values were calculated with GraphPad Prism Version 4.00 software.

Combination indices
To assess drug synergy, the combination index (CI) method as described by Zhao *et al* was used (32). Cell viability was measured using the MTT assay after treatment of Rh41 cells with NVP-TAE684 at concentrations 10, 50 and 100 nM combined with R1507 at concentrations 1, 5 and 10 ng/ml. We next identified the concentrations of NVP-TAE684 and R1507 monotherapies, which resulted in a similar level of cell viability reduction to that observed with each of the combination treatments. Subsequently, CI for the combination treatments was calculated as follows: CI = [Ca,x/ICx,a] + [Cb,x/ICx,b]. Ca,x and Cb,x are the concentrations of drugs A and B used in combination to achieve x% drug effect, ICx,a and ICx,b are the concentrations for single agents to achieve the same effect. A CI <1 indicates synergy of the combination therapy. The CI method could not be applied to RD, Rh18 and Rh30 cell lines, since monotherapy treatment with R1507 showed too little inhibitory effect. Instead, the IC50 of NVP-TAE684 of all three cell lines was combined with the highest R1507 concentration of 100 µg/ml. Cell viability was again assessed by MTT assay and the inhibitory effect of the combination treatment was compared with no treatment, monotherapy of NVP-TAE684 (IC50) and monotherapy of R1507 (100 µg/ml).

Statistical methods
A potential relation between categorical parameters was assessed by Chi-square or Fisher's exact (FE) testing when appropriate. A potential relation between categorical and continuous data was assessed by Mann-Whitney U test. The influence of parameters of interest on relapse-free (RFS) and overall survival (OS) was tested by the Kaplan-Meier method with Log Rank test. The relation between ALK and outcome was described in our earlier publication (27). A p-value <0.05 was considered statistically significant. All analyses were performed with SPSS version 16.0.

RESULTS

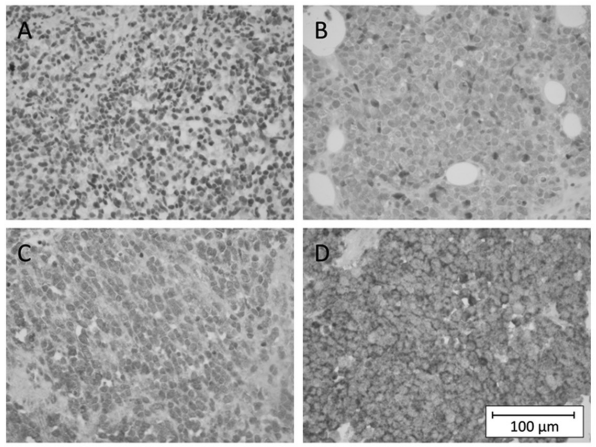
Immunohistochemical staining patterns
Staining frequencies for all (primary) tumours and subdivided by histological subtype are summarized in table 3. Immunohistochemical staining was reliably evaluable in the majority of samples, varying from 92.0 – 99.1%. Examples of staining patterns are displayed in figure 1. Cytoplasmic IGF-1R expression was seen in 72% of aRMS and 61% of eRMS (ns). Cytoplasmic ALK expression was more frequently observed in aRMS compared to eRMS (92% versus 39%, respectively, p<0.001). Nuclear expression was demonstrated for IGF-1R (eRMS 10%, aRMS 4%, ns) and ALK (eRMS 7%, aRMS 4%, ns).

Table 3
Frequency of ALK and IGF-1 receptor expression in RMS

IHC		IGF-1R		ALK	
		cytoplasm	nucleus	cytoplasm	nucleus
Overall	N	69/108	9/108	56/109	7/111
	%	63.9%	8.3%	51.4%	6.3%
eRMS	N	51/83	8/83	33/84*	6/85
	%	61.4%	9.6%	39.3%	7.1%
aRMS	N	18/25	1/25	23/25*	1/26.
	%	72.0%	4.0%	92.0%	3.8%

A siginificant difference (asterisk) in expression between eRMS and aRMS was seen for ALK (p<0.001). Percentages are presented as part of the reliably scored samples for each staining

Figure 1 *(color figure page 197)*
Immunohistochemistry of the ALK and IGF-1R receptor



Immunohistochemical staining (magnification 20x), A eRMS displaying very strong predominant nuclear IGF-1R staining; B aRMS displaying very strong cytoplasmic IGF-1R staining; C eRMS displaying strong cytoplasmic ALK staining; and D aRMS displaying very strong cytoplasmic ALK staining.

Relation between IGF-1R and ALK

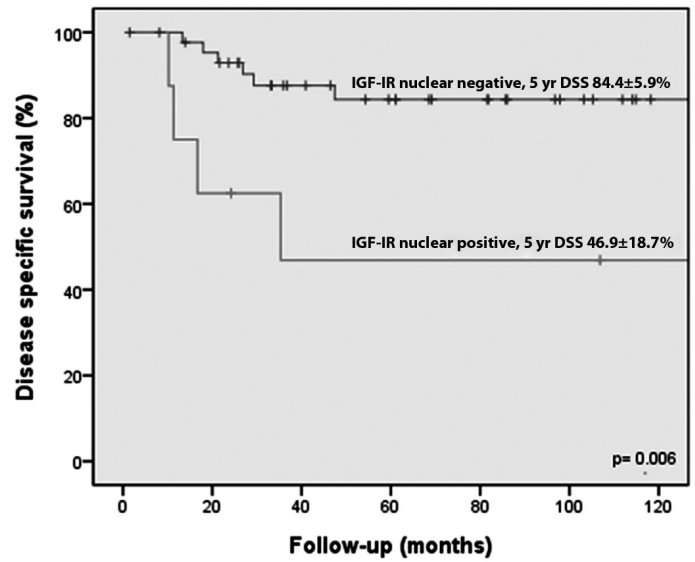
In eRMS, a significant co-expression of cytoplasmic IGF-1R and ALK was observed (n=81, 26/51 (51%) of IGF-1R positive samples versus 7/30 (23%) of IGF-1R negative samples display ALK expression, p= 0.019, co-expression in 32% of tumours). We furthermore observed a negative correlation between nuclear and cytoplasmic IGF-1R (n=83, nuclear IGF-1R expression present in 7/32 (22%) cytoplasm negative versus only 1/51 (2%) cytoplasm positive tumours, p=0.005). In aRMS, as the great majority of samples (92%) expressed ALK, no significant relation could be observed, although frequent co-expression of cytoplasmic ALK and IGF-1R (68%) was present.

Outcome

Follow-up data was complete for 72 patients (eRMS 54, aRMS 18), with a median follow-up of 46.9 months (range 0.5-292.3 months). For eRMS median follow-up time was 60.3 months (1.5-292.3), for aRMS 24.7 months (0.5-254.3).

The presence of nuclear IGF-1R was shown to be an adverse prognostic factor in eRMS (n= 53, 5-yr disease specific survival 46.9±18.7 versus 84.4±5.9%, p=0.006, figure 2). For aRMS, there was no significant prognostic effect of receptor expression, possibly due to small numbers (n=18).

Figure 2
Survival eRMS by nuclear IGF-1R expression



The presence of nuclear IGF-1R (red line) was shown to be an adverse prognostic factor in eRMS (n= 53, 5-yr disease specific survival 46.9±18.7 versus 84.4±5.9%, p=0.006

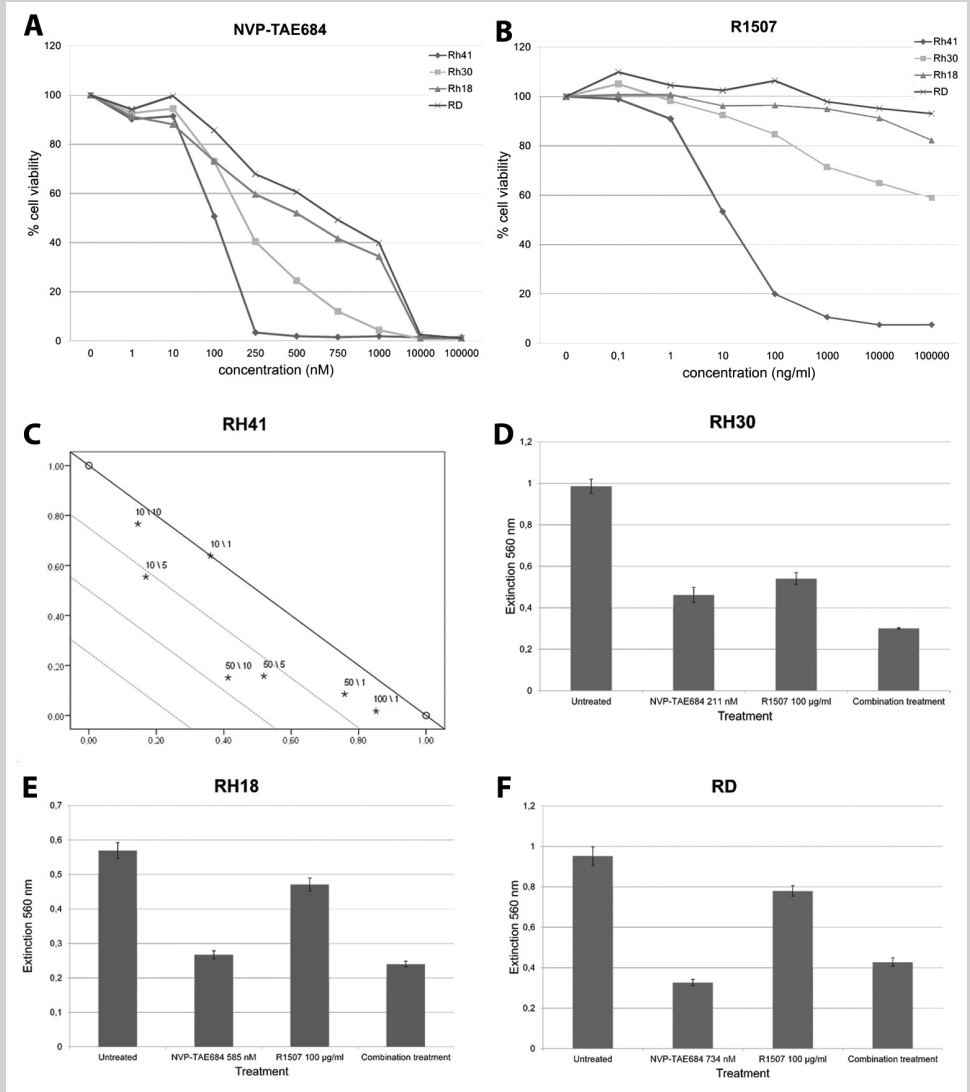
IGF-1R/ALK inhibition *in vitro*

Immunohistochemistry revealed ALK expression in aRMS cell lines Rh41 (++) and Rh30 (+) and to a lesser extent in eRMS cell lines Rh18 (±) and RD (±). The ALK TKI NVP-TAE-684 as monotherapy resulted in diminished cell growth in aRMS cell lines Rh41 (IC50 103 nM) and Rh30 (IC50 211 nM), and to a lesser extent in eRMS cell lines Rh18 (IC50 585 nM) and RD (IC50 734nM) (figure 3A).

IGF-1R expression was detected in Rh41 (+) and to a lesser extent in cell lines Rh30, Rh18 and RD (±). Inhibition of IGF-1R by mAb R1507 as monotherapy resulted in decreased cell growth only in aRMS cell line Rh41 (IC50 11 ng/ml). In the other cell lines the IC50 was not reached, and the maximum concentration of 100 µg/ml induced cell death in 41.2% of Rh30 (aRMS), in 17.5% of Rh18 (eRMS) and only in 6.9% of RD (eRMS) cells (figure 3B).

Simultaneous treatment in Rh41, indicates a synergistic effect with the combination of NVP-TAE684 and R1507 at all tested concentrations (combination index <1) (figure 3C). In the other cell lines, the maximum concentration of 100 µg/ml of R1507 and the IC50 of NVP-TAE684 was added simultaneously (figure 3D to F). In aRMS cell line Rh30, the bargraph indicates that a combination of both agents causes increased cell death (figure 3D).

Figure 3
In vitro experiments of NVP-TAE684 and R1507 in rhabdomyosarcoma cell lines (RD, Rh18, Rh30, Rh41)



A: Cell viability assays of NVP-TAE684 as monotherapy. On the X-axis the different concentrations of NVP-TAE684 in nM, on the Y-axis the percentage of viable cells; B: Cell viability assays of R1507 as monotherapy. On the X-axis the different concentrations of R1507 in ng/ml, on the Y-axis the percentage of viable cells; C: Result of synergy experiment for Rh41. The x- and y-axis, respectively, show the relative concentrations of R1507 and NVP-TAE684 in synergy compared to the concentrations required in monotherapy. If synergy is absent, the resulting asterisk will be located on the bold line. Asterisks located below this line represent synergy (Combination Index <1). Numbers next to the asterisks indicate the concentrations of R1507 (µg/ml) and NVP-TAE684 (nM); D-F display the bargraphs of cell viability assays of monotherapy and combinations of the IC50 of NVP-TAE684 and the maximum concentration of R1507 (100 µg/ml) in Rh30 (D), Rh18 (E) and RD (F).

DISCUSSION

We showed that co-expression of IGF-1R and ALK is detected in eRMS and particularly in aRMS and that combined inhibition reveals synergistic cytotoxic effects *in vitro* in aRMS. Furthermore, we detected nuclear IGF-1R expression to be an adverse prognostic factor in eRMS.

The current study adds relevant data to the clinical importance of the IGF-1R and ALK receptor pathway and its potential as a therapeutic target in RMS. The ALK expression data (>90% of poor prognostic aRMS) as well as the *in vitro* experiments of the ALK inhibitor NVP-TAE684 suggest that ALK represents a very interesting therapeutic target in these tumours. To our knowledge, we are the first to report that NVP-TAE684 is effective against RMS *in vitro* as monotherapy (aRMS>eRMS) and that the amount of responsiveness upon inhibition correlates with immunohistochemical expression of ALK in these cell lines. However, it should be considered that the presence of genetic alterations of the *ALK* gene (amplification, mutation and exon deletions) as we observed previously, might alter the sensitivity of RMS tumours (27).

Despite the promising preclinical results of IGF-1R directed treatment in xenograft models (16-18;33), clinical trials up till now have not shown optimal results in sarcoma patients. Only a limited number of dramatic clinical responses in recurrent/refractory sarcomas were observed, while the majority of patients showed only a modest and temporarily anti-tumour effect (10-40% response rate) (19-21). We still are convinced that IGF-1R as a therapeutic target deserves further clinical investigation, especially in combination studies (34).

Interestingly, we detected the presence of IGF-1R not exclusively in the cytoplasm but also in the nucleus in 10% of eRMS and 4% of aRMS. Recently, nuclear localization of IGF-1R was also identified in multiple malignant and non-malignant epithelial cell lines, in a substantial part of clear cell renal carcinoma (48%) and also in a small cohort of sarcomas (different liposarcomas, pleomorphic RMS, synovial sarcoma, desmoplastic small round cell tumor, Ewing sarcoma and osteosarcoma, total n=16, 75% nuclear staining) (35-36). Nuclear IGF-1R shows a predilection for localization to less dense DNA regions and it co-localizes with RNA polymerase II and binds to chromatin. It was therefore proposed that it is directly involved as a gene transcription factor (37), as was also observed for multiple other RTKs (38-39). The negative correlation between cytoplasmic and nuclear IGF-1R as observed in our cohort is in line with the hypothesis that nuclear translocation of IGF-1R takes place in certain conditions, which can be the result of import of the full-length receptor or by enzymatic release of the intracellular domains of the receptor, both initiated by ligand binding. This finding may be of great clinical importance, even more since nuclear IGF-1R was associated with adverse prognosis in eRMS.

A recent clinical study indicates that the exclusive presence of nuclear IGF-1R serves as a biomarker to predict increased sensitivity of sarcomas (osteosarcoma, Ewing sarcoma, liposarcoma, pleomorphic RMS, desmoplastic tumour, and synovial sarcoma) when treated with IGF-1R mAbs IMC-A12, SCH 717454 and CP-751.871 (36). Although, this represents a small (n=16) heterogeneous cohort with regard to histology and Ab treatment given - if these findings can

be confirmed in larger studies and extrapolated to RMS- nuclear expression of IGF-1R might predict a benefit of IGF-1R inhibition for eRMS patients with poor prognosis (18;40). Obviously, we need to increase our knowledge concerning the potential of combined targeted treatment. This is underlined by the synergistic effect we observed upon ALK/IGF-1R inhibition in Rh41 in the present study, and by previous studies indicating that the primary presence or upregulation of other RTKs upon IGF-1R inhibition facilitates resistance via alternative cell survival pathway activation, for example via the insulin receptor (IR) in Ewing sarcoma (24), platelet derived growth factor receptor a (PDGFRa) in a human RMS model (Rh41) (22), and human epidermal growth factor receptor 2 (HER2/EGFR) in RMS (RMS cell line Rh36 and a transgenic *PAX3-FKHR* aRMS mouse model) (25). A subsequent rising question is the optimal timing and combining strategy of targeted agents with conventional cytotoxic agents.

In conclusion, combined targeting of ALK and IGF-1R seems a rationale choice in (a)RMS and needs further investigation in xenograft models.

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CHAPTER 7

Building the bridge between rhabdomyosarcoma in children, adolescents and young adults: the road ahead

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ABSTRACT

Rhabdomyosarcoma (RMS) is a rare type of soft tissue sarcoma that mainly affects children, but also occurs in adolescents and (young) adults (AYA). Despite dramatic survival improvements reported by international study groups in children over the past decades, the awareness of a dismal outcome for older patients with RMS has grown. In contrast to the world-wide organization of care for children with RMS, standard care in adults lags behind. A step forward in RMS management for patients of all ages is urgently needed. Both paediatric oncologists and medical oncologists are essential players in development of a concept of RMS care, but bringing these two worlds together seems not so easy. This review provides an overview which highlights the similarities and differences in children and adults with RMS. Furthermore, it comes up with a novel concept to overcome the virtual gap between the treatment approach of children and AYA with RMS.

INTRODUCTION

Rhabdomyosarcoma (RMS) is an extremely rare type of soft tissue sarcoma (STS) that is thought to derive from mesenchymal stem cells and shows varying degrees of skeletal muscle differentiation (1). RMS occurs predominantly in children <7 years, has a second age peak in adolescence, and the incidence subsequently declines in older patients (2;3).

The two main distinguishable histological subtypes that affects both adults and children are embryonal RMS (ERMS) and alveolar RMS (ARMS) (2;4;5). A third subtype, pleomorphic RMS occurs almost exclusively in adults, and there is growing evidence that this tumour type should be biologically considered rather a distinct type of adulthood soft tissue sarcoma than a subtype of RMS (2;6). Therefore, this subtype is beyond the scope of this review considering age in relation to RMS.

Over the past decades, the awareness of a dismal outcome for RMS patients with increasing age has grown. Improvement of survival rates in children over the past decades resulted in a current 5-year survival rate of approximately 70-80% for children with RMS (7-9), while survival rates in adults are not exceeding 56% (range 21-56%) (2;4;5;10-18). Moreover, patients < 1 year and ≥ 10 years fare worse than patients 1-9 years in paediatric study populations (19;20). Whether this effect of age on outcome is attributable to differences in treatment approach or in tumour biology is unknown.

The centralization of cancer care in specialized childhood oncology centres, together with the standardized treatment of RMS within comprehensive trials, is considered the principal factor that is responsible for the gain in RMS survival in children in the western countries (7-9;21-23). In contrast to this centralization of RMS treatment in children, the relative rarity of RMS in (young) adults in the burden of all adult-type cancers led to dispersion of patients with RMS in adult oncology centres. Also, the relative lack of clinical trial participation in adolescents and (young) adults with sarcoma has been proposed as one of the major reasons of the consequential lack of survival improvement (24).

RMS requires aggressive multimodality treatment which -as in many childhood cancers- results in a significant rate of acute toxicities and long-term effects (25). The "traditional" VAC/VAI-based (vincristine, D-actinomycin and cyclophosphamide or ifosfamide) regimens developed in the early seventies underwent only minor modifications over time, primarily resulting in improvements for patients with low-risk disease. Unfortunately, survival for high-risk patients (e.g. patients with irresectable ARMS at unfavourable sites, distant metastatic disease, and recurrent disease) remains poor, not exceeding 50% (19;26-30). Along with the increase of survival rates in the young population of RMS patients, prevention of long-term 'costs' as late organ toxicity, infertility and second tumours becomes more important. Although there is an urgent need for new –less harmful- therapeutic options, an important limitation in childhood RMS trials conducted over the past decades is the relative lack of introduction of new (targeted) therapies.

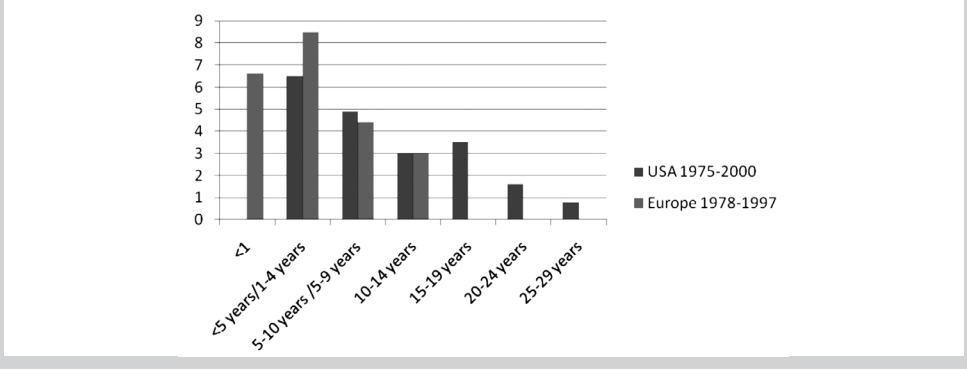
Although experts in paediatric RMS treatment have proposed that treatment of adults should be based on the current paediatric treatment protocols (5), bringing the two worlds together seems not as easy as that. This review provides an overview highlighting the similarities and differences in epidemiology, tumour biology, diagnosis, treatment approach, and accrual to clinical trials with new agents together with a concept to overcome the existing virtual separation line between treatment approach in children, adolescents and (young) adults (AYA) with RMS.

EPIDEMIOLOGY

Within the total cancer burden in the western countries which is almost 300/100,000 (Western Europe and United states of America (USA), 2008), RMS is an extremely rare tumour with an average incidence of 4.5-6.9/1,000,000 (age standardized incidence rate in children, and AYA) (31-34). RMS accounts for up to 57-70% of STS (corresponds to 3.4-3.7% of all cancers) in children 0-14 years, whereas it accounts for only 5.2-6.5% of STS (corresponds to 2.9% of all cancers) in AYA aged 15-29 years (35;36). The incidence subsequently declines, as within the overall burden of cancer incidence only 1.3% comprises STS and within this group only 6.3% is RMS (36).

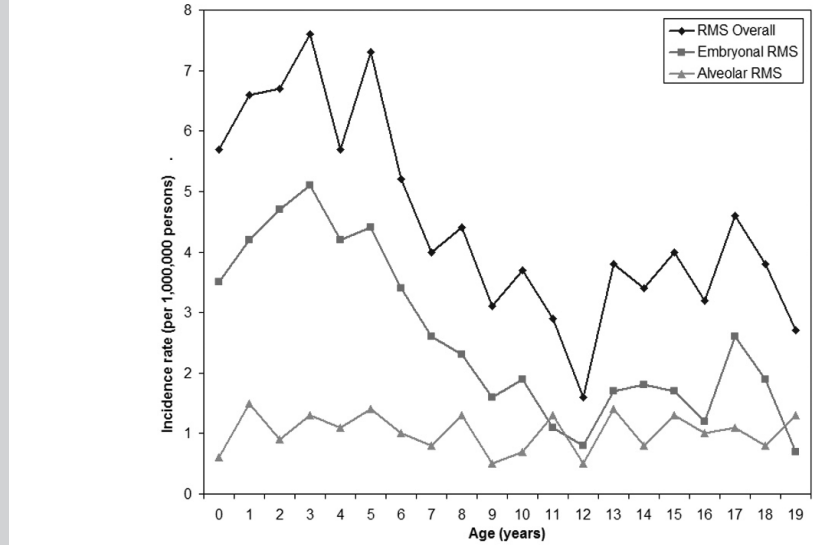
Figure 1 displays the incidence of RMS in patients 0-29 years in Europe (data retrieved from 1978-1997) and the USA (1975-2000) (35;37). Data concerning RMS incidence in patients >14 years in Europe could not be retrieved from literature. RMS incidence shows a characteristic age peak in young children 0-4 years after which the incidence declines, followed by a second smaller age peak in adolescents 15-19 years. There is a typical age-distribution of ERMS and ARMS in children (displayed in figure 1b). After the age of 19 the incidence declines rapidly to 1.6 per million in the age group 20-24 years and 0.8 per million in the age group 25-29 years. In contrast to the widely available information on incidence rates in children, the data on incidence rates in adults are scarce. Besides, an important limitation to consider is the inclusion of pleomorphic RMS in adult series. In a large series of patients retrieved from the SEER database from 1973-2005 (N=2,600 patients), ERMS (20.4%) and ARMS (14.5%) comprised approximately one third of RMS diagnoses in adults (>19 years) (2).

Figure 1a *(color figure page 197)*
Incidence rates of all subtypes of RMS in patients 0-29 years of age.



*Data regarding patients with RMS >14 years in Europe were not available

Figure 1b *(color figure page 198)*
Incidence of ERMS and ARMS in the childhood population.



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PREDISPOSITION AND ONCOGENESIS

Genetic syndromes

Genetic alterations seem to be a key player in the development of RMS. This is emphasized by the association between several genetic syndromes and early RMS development, including Li-Fraumeni syndrome (*p53* mutation), Costello syndrome (*HRAS* mutation), neurofibromatosis type 1 (*NF-1* gene mutation), and Beckwith-Wiedemann (mutation or deletion 11p15.5 chromosomal region) (38;39). Nevertheless, most RMS cases are considered sporadic, as only 9% of RMS cases are syndrome-related (40). Remarkably, an earlier study reported a much higher prevalence of minor or major congenital abnormalities (32%) in RMS patients on autopsy (involving mainly the central nervous system, genitourinary tract, gastrointestinal tract, and cardiovascular system) compared to 3% in the general population (41). Thus, it seems that additional underlying genetic conditions still remain to be revealed.

Oncogenesis

Oncogenesis in RMS is still not completely understood, but different mechanisms seem to be involved in ERMS and ARMS (42). In ERMS, oncogenesis is proposed to act via a mechanism in which myogenic progenitor cells of postnatal muscle, satellite cells, potentially give rise to ERMS (43-45). These myogenic progenitor cells are present in all muscle tissues throughout the body, and are supposed to be activated in a myogenic regulatory factor- regulated way for growth or remodelling after tissue injury (46;47).

In ARMS, oncogenesis is thought to be initiated in a mesenchymal stem cell undergoing a *PAX3/7-FOXO1* translocation as key event and subsequently (an) additional (second) hit(s) results in ARMS formation (48-51). These mesenchymal precursor cells are found throughout the body (predominantly in bone marrow), and the recent description of a "leukemic" variant of RMS in the bone marrow in the absence of a primary tumour strengthens this hypothesis (52;53). Contradictory, mature muscle cells also have shown the ability to give rise to ARMS after *PAX3-FOXO1* introduction by dedifferentiation in maturing myoblasts (54). The *PAX3-FOXO1* translocation is the main genetic feature in ARMS as it is present in the majority of ARMS (55%), whereas a *PAX7-FOXO1* translocation occurs in a lesser amount of ARMS cases (22%) (55). Translocation-negative ARMS (23%) is currently under discussion as it potentially shows favourable clinical outcome (56;57) and is genetically more identical to ERMS than to translocation-positive ARMS (56;58;59). These differential patterns of oncogenesis have led to numerous investigations concerning genetic events in RMS. Major genetic events are summarized in table 1a and b.

Age predisposition and oncogenesis

The complexity of mechanisms involved in RMS development together with the typical age-pattern observed, pose questions to age-related biological differences in ERMS and ARMS. Except for syndromes that are generally associated with young onset of disease, little is known concerning the aetiology of RMS at different ages. Biologically, the *PAX7-FOXO1* fusion is associated with younger onset of disease, extremity primaries and a lower rate of metastatic disease, whereas the *PAX3-FOXO1* positive RMS shows an association with higher age and represents a highly malignant phenotype which encompass bone marrow involvement and is therefore associated with adverse outcome (55;67). However, a recent study confirmed the relation of *PAX3-FOXO1* with metastatic disease and higher age at diagnosis, but did not find a relation with bone marrow involvement survival (57). The scarcity of data regarding age-dependent mechanisms of oncogenesis and tumour biology warrants further biological studies in RMS patients of all ages.

DIAGNOSIS

Histological diagnosis

Histological diagnosis is the cornerstone in establishing the diagnosis and making the consequential treatment decisions in RMS. The first well-developed classification was grounded in 1964 by Horn and Enterline, who distinguished embryonal, botryoid, pleomorphic and alveolar RMS ('conventional scheme') (68). Since then, refinements of the available techniques have increased accuracy of classification and the identification of new subtypes, i.e. the spindle cell variant of ERMS. The most recent 'prognosis-tailored' International classification of RMS (ICR) was developed in 1995 (table 2) (69).

Immunohistochemically, RMS shows intranuclear expression of the myoregulatory proteins myogenin and myoD1, with myogenin showing the highest specificity (up to almost 100%) and sensitivity (approximately 90%) (70;71). Myogenin expression is seen more extensively in ARMS. With a cut-off point at 50% of the cells showing nuclear staining of myogenin, a sensitivity of 0.82, and a specificity of 0.75 for distinguishing ARMS from ERMS was reported (72).

Table 1a Major genetic events in RMS. ERMS= embryonal RMS ARMS= alveolar RMSLOI= Loss of imprinting, LOH= Loss of heterozygosity

RMS GENETICS	ERMS	percentage	ARMS (all)	percentage	ARMS (translocation positive)	Percentage	ARMS (translocation negative)	percentage
Main genetic features [55;58;60-62]	LOH 11p15.5 (IGF2, H19, CDKN1C)	23/ 77%	t (2;13)(q35;q14) PAX3-FOXO1 translocation	55%	LOI 11p15.5	46%		
	9q22 loss (PTCH1 gene)	~30%	t(1;13)(p36;q14) PAX7-FOXO1 translocation	22%	LOH 11p15.5	24%		
	loss 1p35-36.3	~30%[3]	translocation negative	23%				
genetic gains [56;63;64]	MYCN (2p24.1)	16%			MYCN (2p24.1)	31%	MYCN (2p24.1)	26%
	CDK4 (12q13.3-14.1)	12%			CDK4 (12q13.3-14.1)	40%	CDK4 (12q13.3-14.1)	11%
	FGFR1 (8p11.2-p11.1)	39%						
	Chromosome 7	25%			Chromosome 7	2%	Chromosome 7	11%
	Chromosome 8	74%			Chromosome 8	0%	Chromosome 8	55%
	Chromosome 11	31%			Chromosome 11	10%	Chromosome 11	30%
	Chromosome 20	29%			Chromosome 20	20%	Chromosome 20	30%
genetic amplifications [56;64]	FGFR1 (8p11.2-p11.1)	6%	MYCN (2p24.1)	13%	MYCN (2p24.1)	19-20%	FGFR1 (8p11.2-p11.1)	11%
	MDM2 (12q14.3.3-q15)	6%	CDK4 (12q13.3-14.1)	12%	CDK4 (12q13.3-14.1)	16.7-24%	MDM2 (12q14.3.3-q15)	7%

Table 1b PAX3-FOXO1 target genes in RMS.

FUNCTION	Growth and oncogenesis	Migration, metastatic potential	Myogenic differentiation	Other
TARGET GENES PAX3-FOXO1	**ALK**	CXCR4	EYA2	**ABAT**
	FGFR4	**MET**	EYA4	ADRA1D
	FGFR2		MEOX1	ADRA2A
	IGF1R		MEOX2	ADRA2C
	KDR (VEGFR2)		MYF5/6	DHFR
	MEIS1		**MYOD1**	
	MYCN		PRRX1	
	SPRY1			

The genes in the table were found to harbour a binding site for the chimeric PAX3-FOXO1 protein by whole genome analysis by chromatin immunoprecipitation and DNA sequencing (ChIP-seq) by Cao *et al.* (65) and the genes in bold were additionally found previously to be over expressed in PAX-FOXO1 translocation positive tumours and cell lines by oligonucleotide expression profiling by Davicioni *et al*. (66).

Table 2
International Classification of RMS (1995) (69)

Subtype	*Percent*	*Age*	*Primary sites*
Superior prognosis			
Botryoid RMS	6%	"Infants and toddlers"	mucosa-lined hollow organs as nasopharynx, bile duct, bladder, vagina
Spindle cell RMS	3-4%	Median age 7 years	Paratesticular, head and neck, genitourinary, extremities
Intermediate prognosis			
Embryonal RMS	49%	Median age 8 years	head and neck (orbit and PM), genito-urinary tract, retroperitoneum/pelvis
Poor prognosis			
Alveolar RMS	30%	Age peak 10-25 years	extremities, perineum/anus, trunk
Undifferentiated sarcoma	-	-	-
Prognosis unknown			
RMS with rhabdoid features	-	-	-

Besides immunohistochemistry, optimization of molecular techniques in the past two decades led to optimization of diagnosis of ARMS, as 80% of the histomorphological diagnosed ARMS harbour detectable translocations (55).

Still, histopathological diagnosis of RMS is a challenge, in which age is an extremely important factor providing a rationale for the diagnostic procedures to be undertaken. For a detailed description of histomorphological features we refer to Weiss and Goldblum (1). The greatest diagnostic challenge in RMS is the distinction from other poorly differentiated small blue round-cell tumours, including neuroblastomas, Ewing's sarcoma, and primitive neuroectodermal tumours. But sporadically, poorly differentiated angiosarcomas, synovial sarcomas, malignant melanomas, melanocytic neuroectodermal tumours of infancy, granulocytic sarcomas, malignant lymphomas, and even bladder small cell carcinoma might mimic RMS (1;73;74). Furthermore, RMS in adults can occur as a heterologous component of epithelial, germ cell, sarcomatous or neuroectodermal derived tumours (75). The clinical relevance of this broad range of differential diagnostic options should be considered as this results in difficulties in clinical management of these tumours, especially in terms of (choice of) adjuvant systemic treatment.

Due to its rarity, an important complicating factor concerning histopathological diagnosis of RMS is that most pathologists are relatively unfamiliar with this diagnosis. In children, all pathological diagnoses are confirmed by central review of a specialized pathologist prior to inclusion into a clinical trial. Central pathological review for adult STS is also available in most developed countries, but its infrastructure could still be improved.

CLINICAL PRESENTATION AND STAGING

Clinical presentation

RMS can arise in a variety of sites throughout the body. The most common primary site includes the head and neck region (35%), followed by genitourinary (24%), and extremity primaries (19%)

(76). Generally, ARMS shows a clinically more aggressive phenotype with unfavourable clinical presentation (e.g. stage of disease) when compared to ERMS. Lymph node involvement (LNI) is seen in roughly 15-20% of RMS patients at diagnosis. However, ARMS histology as well as extremity and paratesticular primaries show a predilection for LNI, demonstrating rates of LNI in up to 40% (77-80). Approximately 20% of all RMS patients will present with distant metastatic disease at diagnosis, and the main metastatic sites involve the lungs and bone marrow but metastases may arise throughout the body (28). Children with ERMS seldom present with metastases (9 and 13% in IRSG II and IRSG III respectively), whereas children with ARMS present more frequently (27 and 28%) with metastatic disease (23).

Although clinical data on adults with RMS are relatively scarce, increasing age was found to be related to unfavourable clinical presentation, i.e. a relatively higher rate of invasive tumours, tumours at unfavourable sites and a higher metastatic potential (loco regional lymph nodes and distant metastases) (2;4;5;12;14). These clinical features can at least partly -but not fully- be explained by the different distribution of the histological subtypes with increasing age (table 2). Besides a higher rate of metastatic disease in older patients due to a relatively higher rate of ARMS, a higher rate of metastatic disease is observed with increasing age in ERMS as well (18).

Staging

Staging of RMS is based on the schemes developed by the paediatric study groups and should include evaluation of the local extent of the primary tumour (site, size, invasiveness in surrounding tissues), the presence of lymph node metastases and the presence of distant metastases. The most extensively used staging techniques in RMS comprise magnetic resonance imaging (MRI) to visualize size and local extension of the primary tumour, chest computed tomography (CT) scanning to evaluate the presence of lung metastasis or lymph node involvement, and bone scintigraphy for the detection of bone metastases. These techniques are extensively investigated and widely available, and therefore have taken a stable place into clinical practice. Optimal use and implementation of (novel) staging techniques in RMS is dependent on clinical appearance and thus should ideally be approached in an age-dependent manner. Therefore, we will discuss two existing age-related staging issues; lymph node detection and novel imaging techniques.

The awareness of the clinical importance of lymph node involvement in RMS has grown, as lymph node metastases were recently reported as predictor for poor outcome, especially in ARMS (81). Conventional staging for detecting lymph node involvement is unsatisfactory and thus of particular interest in certain RMS subgroups. For example, in clinical group 1 paratesticular RMS in patients >10 years (LNI 30-40%) abdominal CT scan instead of ipsilateral retroperitoneal lymph node dissection (RPLND) led to suboptimal treatment (no radiotherapy) and consequently a high rate of lymph node failure (7). Therefore, in paratesticular RMS in boys >10 years RPLND is part of the current diagnostic standard (82;83). However, less invasive strategies in order to reduce morbidity of radical lymph node dissection are preferable in these patients (84).

Although the value of sentinel lymph node sampling procedure (SLN) is established in adult oncology for a great variety of cancer types, in children with RMS this has not been performed

at a regular basis in the past. Since two recent studies, however, indicated that sentinel node procedure in childhood malignancies is technically feasible, safe, and reliable, the technique may obtain a standard place in the staging of RMS in the future in both children and adults (85;86). In extremity ARMS, SNL has already been proven beneficial on outcome in a retrospective study, and seems especially feasible in ARMS patients older than 10 years with a tumour size >5 cm (87-89). Contradictory, in-transit lymph node sampling followed by altered treatment resulted in prevention of LN failure, but had no effect on final outcome in extremity RMS (78). SNL also might be allocated routinely in head and neck as well as gynaecologic RMS in the future. The development of a SLN protocol might thus be of great benefit in order to reduce morbidity and optimize staging in particular RMS patients, as is currently under investigation in paratesticular RMS the IRS-V trial of the COG. Additional prospective evidence concerning an age-defined approach in lymph node staging in RMS subgroups is urgently needed.

Novel functional imaging techniques as Positron Emission Tomography (PET) are upcoming in oncology today. These new imaging techniques provide non-invasive information on the functional/metabolic status of a primary tumour, rather than only structural information. The most common used radiotracer over the past years is 18F-fluordeoxyglucose (18F-FDG), which has the capacity to monitor the glucose metabolism (90).

In adults with sarcoma, FDG-PET has been prospectively studied and resulted in evidence for its use in evaluation of tumour grade, in monitoring malignant transformation in patients with neurofibromatosis type I, for initial staging and re-staging, for treatment monitoring, as well as in predicting survival (91). In contrast to this widely obtained evidence for a successful role of PET in adults with soft tissue sarcoma, there is a scarcity of data in children. Although a number of PET studies in children consider PET as feasible and promising, all except one of these studies are retrospective and hold a maximum of 60 patients from a single institution including multiple paediatric tumour types (92-97). FDG-PET might also be optional for early detection of leptomeningeal involvement of parameningeal RMS (98).

Limitations to consider regarding PET/CT include its limited role in detection of lesions smaller than 5 mm, well-differentiated tumours and tumours with low metabolic rate. Furthermore, many infections and inflammatory processes throughout the body can lead to false-positive PET/CT results. Careful considerations regarding cumulative radiation dose in children should be made, but this should not outweigh the importance of appropriate monitoring of treatment effects potentially leading to clinical decision making consequences (99).

Furthermore, promising new tracers capable of monitoring angiogenesis (100), apoptosis (101), hypoxia (102), and other aspects of tumour metabolism (103) are being developed. The possibilities of advanced staging modalities to current standards hold a great opportunity in optimization of RMS diagnostics and for selecting patients for targeted treatments in the near future. However, there is still an urgent need for decent prospective clinical trials including patients -regardless of age- to establish the use of these staging modalities into RMS daily practice.

RISK STRATIFICATION

Investigations carried out over the past decades in large European and North American consecutive trials resulted in the development of two solitary risk-based staging systems for clinical use in children (summarized in table 3) (104;105). As this staging is closely related to outcome, patients are classified into low-, intermediate-, high- and very high-risk (the latter only in European scheme). The remarkable differences between these stratifications are relative "up-staging" of patients >10 years of age with embryonal tumours >5 cm with a postsurgical group I in the European schemes, as well as "down-staging" of metastatic ERMS in patients <10 years in the IRSG scheme, whereas all metastatic RMS in the European scheme are considered as identical risk group. Because of the lack of a risk-based staging system for adults with RMS, a TNM system is used in this respect (106).

Table 3 IRSG and EpSSG risk stratification

Risk group											
EUROPEAN COOPERATIVE GROUP (EpSSG)	Group	Histology	Site	Size	N	Metastases	Stage	Postsurgical group*	Age	Treatment	EFS/OS (%)
Low	A	ERMS /NOS	Any	*<5 cm*	N0	M0		I	*1<age≤10*	VA	90-95%
Standard	B	ERMS /NOS	Any	*>5 cm*	N0	M0		I	*age >10*	IVA +VA or IVA with or without RT	78-90%
	C	ERMS /NOS	Favourable		N0	M0		II-III	*<21*		72-88%
	D	ERMS/NOS	Unfavourable	*<5 cm*	N0	M0		II-III	*1<age≤10*		80-85%
High	E	ERMS /NOS	Unfavourable	*>5 cm*	N0	M0		II-III	*age >10*	IVA versus IVADo plus RT plus VC	55-60%
	F	ERMS /NOS	Any		N1	M0		II-III	*<21*		50-60%
	G	ARMS	Any		N0	M0		I-II-III	*<21*		50-60%
Very high	H	ARMS	Any		N1	M0		I-II-III	*<21*	IVADo plus RT plus VC	40-50%
Metastatic disease	*Not included in EpSSG protocol*					M1		IV			
IRS-V (COG) COOPERATIVE GROUP											
Low risk											88%
	Low-A	ERMS	Favourable	Any	N0	M0	1	I	<50	VA	
	D9602	ERMS	Favourable	Any	N0	M0	1	II	<50	VA+XRT	
		ERMS	Orbit only	Any	N0	M0	1	III	<50	VA+XRT	
		ERMS	Unfavourable	<5 cm	N0 or NX	M0	2	I	<50	VA	
	Low-B	ERMS	Favourable	Any	N1	M0	1	II	<50	VAC+XRT	
	D9602	ERMS	Orbit only	Any	N1	M0	1	III	<50	VAC+XRT	
		ERMS	Favourable	Any	Any	M0	1	III	<50	VAC+XRT	
						196					
		ERMS	Unfavourable	<5 cm	N0 or NX	M0	2	II	<50	VAC+XRT	
		ERMS	Unfavourable	<5 cm	N1	M0	3	I or II	<50	VAC (+XRT, GpII)	
		ERMS	Unfavourable	>5 cm	Any	M0	3	I or II	<50	VAC (+XRT, GpII)	
Intermediate	D9803	ERMS	Unfavourable	<5 cm	N0 or NX	M0	2	III	<50	VAC ± Topo + XRT	55-76%
		ERMS	Unfavourable	<5 cm	N1	M0	3	III	<50	VAC ± Topo + XRT	
		ERMS	Unfavourable	>5 cm	Any	M0	3	III	<50	VAC ± Topo + XRT	
		ARMS/NOS	Any	Any	Any	M0	1 or 2 or 3	I or II or III	<50	VAC ± Topo + XRT	
		ERMS	Any	Any	Any	M1	4	I or II or III or IV	<10	VAC ± Topo + XRT	
High	D9802	ERMS	Any	Any	Any	M1	4	IV	≥10 - <50	CPT-11, VAC + XRT	<30%
		ARMS/NOS	Any	Any	Any	M1	4	IV	<50	CPT-11, VAC + XRT	

IRSG (including patient up to 49 years) and EpSSG (including patients up to 20 years) risk stratification, with concurrent treatment and estimated survival rates. Survival rates are based on previous studies. ERMS= embryonal rhabdomyosarcoma, ARMS= Alveolar rhabdomyosarcoma NOS= not otherwise specified Favourable sites include: urogenital (non-bladder, non-prostate), head and neck (non-parameningeal), orbit, unfavourable includes all other sites. N0= lymph node involvement absent, N1= positive lymph nodes, Nx= unknown involvement lymph nodes. M0= No distant metastases, M1= distant metastases present. I= ifosfamide, Do= doxorubicin, V=Vincristine, A=D-actinomycin, C= Cyclophosphamide, Topo= topotecan, CPT-11 = irinotecan, XRT= radiotherapy RT= radiotherapy Asterix* post-surgical Clinical Grouping system.

An attempt for development of a uniform staging method should be undertaken to prospectively facilitate comparison. It is questionable whether the current childhood risk classification is suitable for guiding treatment decisions in adult patients because of the previously discussed differences in clinical presentation and tumour biology. As metastatic disease is seen more often in adult patients, combined studies could serve to enlarge the knowledge and to consequently optimize care for all patients with metastatic disease. This accentuates the need for inclusion of both children and adults in risk assessments, as this expanded population might provide new insights concerning risk stratification for all RMS patients.

TREATMENT

Children with RMS are treated within multidisciplinary, risk adapted, cooperative multi-institutional trials. This provides a world-wide infrastructure in which RMS treatment in children is embedded. The groups conducting these trials are the Soft tissue Sarcoma committee of the children's oncology group (COG, formerly intergroup RMS study group, IRSG) in the United States, and the recently formed European paediatric Soft tissue sarcoma study group (EpSSG). The latter was a result of joined forces of the International Society of Paediatric Oncology- Malignant Mesenchymal Tumor committee (SIOP-MMT), the German soft tissue sarcoma cooperative group (cooperative Weichteilsarkomen study, CWS), and the Italian Cooperative Group (ICG; associazione Italiana Ematologia Oncologia Pediatrica-Soft Tissue sarcoma committee, AIEOP-STSC).

Controversially, the rarity of RMS in adults has resulted in a dispersion of patients over different centres. Despite adult sarcoma treatment collaborations (e.g. European Organization for Research Treatment in Cancer, EORTC) do exist; there is a general assumption that there is a lack of clinical trial availability and inclusion for adult patients with ERMS/ARMS. Adolescents (15-19 years) with RMS might be treated either in a paediatric or an adult oncology unit, dependant on referral pattern. In children, treatment is coordinated by a paediatric oncologist. Adults will be referred to different departments for the different treatment modalities, and will thus be surrounded by multiple specialists without a central point of care.

Local treatment

Surgery

Although surgery is considered the mainstay of treatment for RMS in both adults and children, the optimal timing of local treatment (e.g. surgery and radiotherapy) has been under discussion. There was an essential difference in philosophy on optimal local treatment approach between the childhood study groups in Europe and the United states that reflect the current difficulties in treatment of RMS (107). The SIOP-MMT studies attempted to reduce aggressive local treatment, including avoidance of local treatment up to a time point after initial first-line or in the case of poor response even after second-line chemotherapy. On the contrary, the COG guidelines recommend aggressive local treatment (surgery and radiotherapy) at initial diagnosis, regardless of the potential side-effects. As a result, the MMT trial had to cope with an increased need for aggressive salvage treatment in the case of recurrence. Comparison of the COG IRS-IV study and the MMT-89 study shows an advantage of survival in the COG IRS-IV study (OS: 84 versus 71 % and EFS 78 versus 57%). The most striking differences in overall survival between the treatment strategies in the IRS-IV versus MMT-89 were observed in ARMS (5-year OS 71 versus 38%), limb- (5-year OS 71 versus 46%), and head and neck (head& neck non-parameningeal; 5-year OS 89 versus 64%; and orbital RMS 100 versus 85%) RMS. Survival was nearly identical for genitourinary primaries (5-year OS 86 versus 80% in bladder and prostate primaries and 90 versus 94% other primaries) and parameningeal RMS (5-year OS 64 versus 59% in patients <3 years and 78 versus 65% in patients ≥3 years). Although -based on these findings and cooperation between both groups- the current protocols more seem to converge, both strategies learned us much about local management of RMS. Long-term follow-up data are needed to evaluate the costs (e.g. acute and long-term morbidity and mortality)of both strategies.

Besides initial treatment, local treatment of recurrent disease might increase survival rates from <10% to 30-40% (108). Lung metastectomy is rarely performed in RMS patients, as there is very little clinical evidence for its benefit in RMS (109). It might be that the number of patients with metastatic disease in childhood studies is too small, and that addition of adult RMS patients enables more extensive evaluation of the value of metastectomy in RMS.

Another issue which might potentially compromise surgical management of RMS is decentralization of care. While most children presenting with an enlarging mass suspicious for a malignant disease are generally referred to a paediatric oncology unit in a specialized centre, adults may scatter out over a variety of surgical departments in sometimes even local hospitals. This may potentially result in an essential lack of experience, resulting in suboptimal surgical management by means of under- or over-treatment.

Radiotherapy

Radiotherapy (RT) plays a significant role in treatment of RMS patients with 1) irresectable tumours at diagnosis (e.g. parameningeal or abdominal primaries); 2) microscopic residual disease after resection, and 3) completely resected disease but alveolar histology or lymph node involvement (110). The evaluation of the benefit of RT is hard to reflect since RT has been incorporated for a long time in RMS treatment protocols. RT at an early time point (within 9 weeks in the IRS-IV study) seems preferable over delayed RT, but delayed RT may be feasible in particular subsets of patients without compromising overall survival (i.e. in orbit or bladder/prostate primaries) (107). In patients with intracranial extension of parameningeal RMS, early administration of RT within 2 weeks resulted in a significant survival benefit, showing a local failure rate of only 16% versus 37% in patients who received RT after >2 weeks from diagnosis (111). Furthermore, elimination of RT by the MMT studies was attempted in children <3 years with parameningeal RMS, resulting in a decreased OS from 62 to 44% (112). After radical surgical excision of RMS, ARMS seems to benefit modestly from RT with an increase in 10-year FFS from 25% to 30% (110). Furthermore, non-compliance to RT treatment schedules (i.e. omission/dose reduction or volume reduction) results in a higher rate of local recurrences in postsurgical group II disease (113). The most recent reported IRS V (D9620) trial, showed that RT dose reductions to 36 Gy (instead of 41.1 Gy in IRS-IV) do not result in lower local control rates in VA-treated stage 1/group IIA patients and group III orbital patients. Remarkably, RT reduction in patients treated with VAC

showed worse survival in D9602 when compared to IRS-IV, indicating that cyclophosphamide in combination with higher RT dose results in higher local control rates (114).

With regard to late toxicity, RT is one of the most harmful treatment modalities in cancer treatment resulting in a high rate of late morbidity such as development of fibrosis and secondary tumours (25;115). Because of its harmful effect on growing tissues, RT is generally administered with more caution in children. Besides, adults can also be very vulnerable to RT, for example in treatment for parameningeal RMS where older patients show an increased susceptibility for spinal cord injury following craniospinal irradiation and/or intrathecal treatment (116).

Promising techniques as intensity-modulated RT (IMRT), brachytherapy (BT) and proton beam RT are upcoming modalities for the local control of RMS. These techniques reduce loco-regional side effects and can thus reach a higher tumour dose without an increase of toxic side effects. The use of novel radiotherapeutic techniques has been investigated for various indications in adults but to a lesser extent in children. In RMS, these opportunities have been investigated in small cohorts and seem most promising in head and neck tumours, illustrated by the consecutive Ablative Surgery, MOld technique with afterloading brachytherapy and immediate surgical REconstruction protocol (AMORE-protocol, developed in 1993) (110;117-120). Importantly, a higher target dose coverage of IMRT when compared with conventional 3-dimensional conformal radiotherapy was recently reported in a decent cohort (N=375, COG-D9803) of intermediate risk childhood RMS, which did however not lead to an improved locoregional control or failure-free survival rate (121). Brachytherapy also serves as a way to obtain bladder preservation and in order to reduce morbidity (i.e. incontinence, sexual function) in prostate/bladder RMS and female genital tract RMS (122-125).

Although the newer techniques seem less harmful, no data regarding long term effects are available yet. IMRT for example leads to a higher scattered dose to the whole body. Therefore, considerations concerning the use of these technique in children compared to adults should be made; 1) a smaller relative body volume will be exposed to the high scattered dose of IMRT; 2) children are more sensitive to radiation-induced cancer compared to adults because of their longer life span; and 3) children with genetic susceptibility (for example germ line mutations) are also at higher risk for radiation-induced tumours (126). Although prospective information regarding long-term safety is widely unknown, IMRT, BT and PT are promising techniques in reducing acute toxicity of RT in RMS treatment and might be introduced as part of the standard treatment protocols in the near future.

Systemic treatment

Chemotherapy has been the main topic of discussion in RMS treatment controversies in adults versus children in the past years. With the introduction of chemotherapy in the seventies, a dramatic improve in RMS survival in children was seen. The current strategies of the European as well as the North American RMS study groups are shown in table 3. Although optimization of these regimens has been performed, no revolutionary changes in current regimens for children have taken place since all proposed treatment protocols (including high-dose-chemotherapy with stem cell rescue) failed to improve outcome (127). In adulthood soft tissue sarcoma treatment, controversial findings and opinions regarding the use of adjuvant chemotherapy still exist. As a result, aggressive local treatment is the mainstay of treatment in adult STS and information regarding the use of neo-adjuvant chemotherapy in adults with RMS is nearly absent. Additionally, when metastatic disease occurs, the adult patient is treated with palliative chemotherapy (either a single agent or a combination) when feasible.

Importantly, a retrospective analysis of adult RMS patients by Ferrari *et al* in 2003, pointed out that older patients with ERMS and ARMS could benefit equally from adjuvant treatment as children do. Polychemotherapy schedules resulted in nearly identical survival rates as had been reported in children (61% overall and 72% for ERMS) (5). Nevertheless, there is an evident heterogeneity in chemotherapeutic agents and dose intensities administered over the past years for adult RMS impairing true comparison with the protocols used in childhood RMS. Due to the rarity of RMS in adults; there are no prospective studies available considering the feasibility of childhood regimens in older patients up to now.

When an attempt is made to design a uniform protocol for children as well as AYA the potential differences in pharmacokinetics and pharmacodynamics should be considered, as this potentially results in different response rates, toxicity profiles and severity of toxicities with increasing age (128;129).

With regard to RMS pharmacology in particular, vincristine clearance was shown to be lower in adolescents compared to younger children (age range 0.2-18 years, N=54), whereas another study could not establish this relationship between pharmacokinetics and age (age range 1.2-17.3 N=98) (130-132). Furthermore, d-actinomycin shows a higher area under the curve in patients who were younger (<40 kg) when compared to adolescents potentially leading to a higher toxicity risk (133). However, another study of 33 patients (age 1.6-20.3 yrs) found that age had no discernible effects on D-actinomycin pharmacokinetics (134). Cytochrome p450, important for the metabolism of alkylating agents (cyclophosphamide, ifosfamide), has been shown to be increased in children when compared to adults, potentially resulting in modification of the therapeutic effect (135). Doxorubicinol, a toxic metabolite of doxorubicin has a decreased clearance if body fat is greater than 30% which might result in age-related cardiac toxicity risk as there is a change in body fat disposition from young childhood up to adult age (136).

In a large retrospective study concerning children with RMS, the acute toxicity profile of systemic treatment was shown to be age-related. Children aged <1 year are the most susceptible for D-actinomycin hepatotoxicity, whereas older patients are more susceptible to vincristine neurotoxicity. Consequently, a rate of over 50% grade 3 or 4 CTCAE v3.0 toxicity in patient > 12 years with RMS was observed (137). Although this was confirmed by a second study (138), still the number of patients is low and further research into this harmful effect of vincristine in AYA is needed. Surprisingly, the latter study found a lower rate of haematological side effects in patients >15 years than in their younger counterparts (138). Alkylating agents, particularly cyclophosphamide and to a lesser extent ifosfamide, have shown a dose-dependent harmful

effect on testicular function (FSH levels) and fertility (infertility occurring in up to 90% of male patients treated with cyclophosphamide for Ewing's sarcoma/STS or Hodgkin's lymphoma) (139-141), and it seems that prepubertal age is related to a lower rate of gonadal toxicity in males when compared to older age (142). In females, age- and dose-dependent effects of alkylating agents on infertility and premature ovarian failure are reported as well, i.e. increasing age (post menarche) at the time of treatment increases the risk (143-146).

The knowledge on age-related pharmacology needs to be expanded in order to develop appropriate protocols of chemotherapy in children, AYA and adult patients with RMS. Close collaboration with pharmacologists for monitoring of RMS patients providing information regarding pharmacokinetics and pharmacodynamics is therefore essential in future RMS treatment for monitoring and optimizing safety and efficacy. Furthermore, there is an urgent need for new therapeutic options in RMS patients of all ages in order to optimize the balance between costs of cure (e.g. acute and long-term morbidity and mortality) and potential benefit of RMS treatment. At the moment, there is a remarkably high incidence of treatment related (late) toxicity and a subsequent risk of a second tumour after RMS (observed-to-expected (O/E) ratio 7.7), caused mainly by radiation and chemotherapeutic treatment (O/E ratio of 15.2 in patient treated with chemo-RT versus 1.4 for surgery alone in all childhood STS) (147).

NEW TREATMENT POSSIBILITIES

Potential targets

Potential targets for RMS treatment imply the different tumorigenic mechanisms. There are currently many new drugs available against receptors/growth factors, intracellular signalling molecules, cell cycle apoptosis proteins, proteasome, Hsp90 and Histone deacetylase (HDAC), angiogenic proteins, as well as the fusion protein in ARMS *(PAX3-FOXO1)*. This was also reviewed by Wachtel and Schäfer in 2009 (table 4) (148).

Table 4
Possible treatment targets in RMS.

RMS TARGETS	Receptors/ growth factors	Intracellular signalling molecules	Angiogenesis	Cell cycle/ apoptosis	Other
Targets	**IGF1R**	**mTor**	**VEGF**	**CDK4/CDK6**	**Proteasome**
	c-Met	**PDK-1/AKT**	**VEGFR**	p53	
	PDGFR	**Src**		**Bcl-2**	**Hsp90**
	c-Kit	Mek/Erk		**TRAIL**	
	CTGF/CCN2	Estrogen receptor		Survivin	**Histone Deacetylases**
	Midkine	Mirk/Dyrk1B			
	CTL-4	Smad4			**Cancer-specific fusion proteins**
	ALK	**Retinoic acid**			

Modified from Wachtel *et al* (148). Bold= targets under investigation in phase I/II clinical trials.

Clinical trials

Trial availability

To get proper insight in the availability of clinical trials for RMS patients, we performed a search for clinical trials over a period of ten years on *www.clinicaltrials.gov* (search criteria "rhabdomyosarcoma" and "sarcoma" N=761). We selected trials which started from the 1st of January 2001- 1st of January 2011, had actually been open for inclusion, studied an "intervention", and indeed included RMS or soft tissue sarcoma patients. For the selected trials (N=221), specific data on ERMS and ARMS inclusion, minimum and maximum age at the moment of inclusion, and type of intervention (cytotoxic, targeted, immunotherapy, stem cell transplantation related, RT or other) were documented. An overview of trial availability by age and trial phase is summarized in figure 2a -2c.

A focus on phase I and I/II combined trials revealed a total of 106 trials available over a 10-year period which explicitly included sarcomas/RMS. A substantial part (N= 43; 40,6 %) of sarcoma/RMS phase I trials included children <18 years. Secondly, we looked in more detail to trials with targeted treatment (N=34), or tumour immunology related treatment (N=6) in patients <18 years (table 5a and table 5b). Obviously, an increase in availability of trials with targeted treatment is seen over time, as the number of trials conducted from 2006-2011 doubled when compared to 2001-2005 (N=23; 67.6 % versus N=11; 32.4%, respectively).

A focus on phase III availability revealed a total of 14 trials for the period 2001-2011 (displayed in table 5c). These included first-line treatment (10), maintenance treatment (1) and treatment of refractory/recurrent RMS (3). First-line studies focussed on image-guided IMRT (1), and chemotherapy (9). Six of the first-line chemotherapy trials represent the IRSG and European childhood study groups protocols. The other first-line chemotherapy studies (3) included one EORTC study which administered doxorubicin/ifosfamide including metastatic/advanced ARMS (age ≥18 years), one industry-sponsored study testing trabectedin versus doxorubicin including ARMS patients ≥18 years, and one study of doxorubicin with palifosfamide in patients ≥18 years (ERMS/ARMS inclusion was not exclusively mentioned).

It is remarkable that IRSG study protocols include patients up to age 50 years, whereas the European study protocols (EpSSG, CWS) have an upper age limit of 20 years. However, despite inclusion criteria up to 50 years, the recent D9602 (low risk) study only managed to include one patient aged 27, and all others were younger than 21 years (114). Importantly, ERMS was excluded in two out of three adult STS phase III trials involving chemotherapy. This clearly reflects the urgent need for cooperation between these groups.

Clinical trial design and infrastructure

In general, there is a lack of availability of new therapeutic agents in children when compared to adults. This is probably a result of the rarity of paediatric malignancies in general, which makes cooperation with pharmaceutical agencies a much greater challenge when compared to the large patient numbers available in adult oncology. At the moment, regulations of the European Medicines Agency (EMA) 'Paediatric committee' as well as the U.S. food and drug

Table 5a
Targeted treatment trials explicitly including sarcoma/RMS over the 10-yr period 2001-2011

No	NCT ID	Phase	N	Start year	SUBTYPE	RISK GROUP	Age	Interventions	TARGET	Funded by	Sponsors
1	NCT01204450	Phase I	20	2009	ERMS/ARMS	RELAPSED	2-17	Temsirolimus; valproic acid	mTor/HDAC	Other|NIH	UNC Lineberger Comprehensive Cancer Ce
2	NCT00187174	Phase I	0	2004	ERMS/ARMS	REFRACTORY/RECURRENT	3-21	Everolimus	mTor	Other|Industry	St. Jude Children's Research Hospital|Nova
3	NCT00093821	Phase I	70	2004	ERMS/ARMS	REFRACTORY/RECURRENT	0-21	tanespimycin	HSP90	Other|NIH	Memorial Sloan-Kettering Cancer Center|N
4	NCT00428272	Phase I	73	2006	ERMS/ARMS	REFRACTORY/RECURRENT	1-29	Lexatumumab; recombinant interferon gamma	TRAIL-R2	NIH	National Cancer Institute (NCI)
5	NCT00354848	Phase I	40	2005	ERMS/ARMS	REFRACTORY/RECURRENT	2-17	cediranib maleate	VEGFR2	NIH	National Cancer Institute (NCI)
6	NCT00609141	Phase I	48	2008	*	REFRACTORY/RECURRENT	1-21	cixutumumab	IGF1R	Other|NIH	Children's Oncology Group|National Cance
7	NCT00077454	Phase I		2004	ERMS/ARMS	REFRACTORY/RECURRENT	0-21	erlotinib hydrochloride; temozolomide	HER1/EGFR	Other|NIH	Children's Oncology Group|National Cance
8	NCT00012181	Phase I		2001	ERMS/ARMS	REFRACTORY/RECURRENT	0-21	alvocidib	CDK-inhibitor	Other|NIH	Children's Oncology Group|National Cance
9	NCT00929903	Phase I	46	2009	*	METASTATIC/RECURRENT	2-25	pazopanib hydrochloride	VEGFR	Other|NIH	Children's Oncology Group|National Cance
10	NCT01132911	Phase I	5	2010	*	REFRACTORY/RECURRENT	1-21	Vorinostat (SAHA); Velcade (PS-341, Bortezomib)	HDAC and proteasome	NIH	National Cancer Institute (NCI)
11	NCT01130623	Phase I	5	2010	*	REFRACTORY	1-21	Pazopanib (GW786034)	VEGFR	NIH	National Cancer Institute (NCI)
12	NCT00132158	Phase I	30	2005	ERMS/ARMS	REFRACTORY	0-21	Irinotecan (Camptosar), Gefitinib (Iressa)	EGFR	Other|Industry |NIH	St. Jude Children's Research Hospital|Astra
13	NCT00976508	Phase I	60	2009	*	REFRACTORY	≥10	Figitumumab; pegvisomant	IGF1R	Industry	Pfizer
14	NCT01236586	Phase I	5	2010	*	REFRACTORY/RECURRENT	1-21	RO4929097; Dexamethasone	gamma-secretase	NIH	National Cancer Institute (NCI)
15	NCT00704054	Phase I		2008	*	REFRACTORY/RECURRENT	1-17	ridaforolimus	mTor	Industry|Other	Merck|H. Lee Moffitt Cancer Center and R Center
16	NCT00678769	Phase I	89	2008	*	ADVANCED/METASTATIC	≥16	IMC-A12; Temsirolimus	IGF1R/mTor	Other|NIH	M.D. Anderson Cancer Center|National Ca
17	NCT00960063	Phase I	65	2009	*	ADVANCED	0-21	Temozolomide, Irinotecan, and SCH 717454; Vincristine, Doxorubicin, Cyclophosphamide (CAV), and SCH 717454; Ifosfamide, Etoposide (IE), and SCH 717454	IGF1R	Industry	Schering-Plough
18	NCT00303940	Phase I	26	2005	ERMS/ARMS	REFRACTORY/RECURRENT	2-17	Carboplatin; talabostat mesylate; temozolomide	dipeptidyl peptidase (DPP)	NIH	National Cancer Institute (NCI)
19	NCT00949325	Phase I| II	45	2009	ERMS/ARMS	REFRACTORY/RECURRENT	≥1	temsirolimus (Torisel) plus liposomal doxorubicin (Doxil)	mTor	Other|Industry	Sidney Kimmel Comprehensive Cancer Cen
20	NCT00788125	Phase I| II	143	2008	ERMS/ARMS	RECURRENT/METASTATIC	1-24	Carboplatin; dasatinib; etoposide phosphate; ifosfamide	ABL and Src	Other|NIH	City of Hope Medical Center|National Canc
21	NCT00996346	Phase I| II	30	2009	*	REFRACTORY	≥10	irinotecan, temsirolimus	mTor	Other|Industry	New Mexico Cancer Care Alliance|Wyeth
22	NCT01182896	Phase I| II	5	2010	ERMS/ARMS	REFRACTORY/RECURRENT	1-21	PF-02341066	c-Met/ALK	NIH	National Cancer Institute (NCI)
23	NCT00668148	Phase II	185	2008	ERMS/ARMS	REFRACTORY/METASTATIC /RECURRENT	≥12	IMC-A12 (cixutumumab)	IGF1R	Industry	ImClone LLC
24	NCT01222715	Phase II	100	2010	ERMS/ARMS	REFRACTORY/RECURRENT	0-29	Bevacizumab; cyclophosphamide; temsirolimus; vinorelbine	VEGF/mTor	Other|NIH	Children's Oncology Group|National Cance
25	NCT00642941	Phase II	191	2007	ERMS/ARMS	REFRACTORY/RECURRENT	≥2	RG1507	IGF1R	Industry|Other	Hoffmann-La Roche|Sarcoma Alliance for R
26	NCT00643565	Phase II	150	2008	ERMS/ARMS	METASTATIC	0-18	bevacizumab [Avastin]; Standard chemotherapy	VEGF	Industry	Hoffmann-La Roche
27	NCT00031915	Phase II		2002	ERMS/ARMS	METASTATIC/RECURRENT	≥10	imatinib mesylate	PDGFR, c-kit, ABL	NIH	National Cancer Institute (NCI)
28	NCT00148109	Phase II	45	2005	*	METASTATIC/ADVANCED	≥16	Cetuximab	EGFR	Other|Industry	University of Michigan Cancer Center|Brist
29	NCT00831844	Phase II	140	2009	ERMS/ARMS	REFRACTORY/RECURRENT	0-30	cixutumumab	IGF1R	Other|NIH	Children's Oncology Group|National Cance
30	NCT00093080	Phase II	212	2004	ERMS/ARMS	METASTATIC/ UNRESECTABLE	≥15	ridaforolimus	mTor	Industry	Merck|Ariad Pharmaceuticals
31	NCT01010672	Phase II	50	2009	*	METASTATIC	≥13	ridaforolimus	mTor	Industry	Merck|Ariad Pharmaceuticals
32	NCT00154388	Phase II	191	2001	*	UNKNOWN	15-69	Imatinib mesylate	ABL,PDGFR, c-kit	Industry	Novartis
33	NCT01112384	Phase II	32	2010	ARMS	RECURRENT/METASTATIC	≥16	SB939	HDAC	Other	NCIC Clinical Trials Group
34	NCT00538239	Phase III	650	2007	*	METASTATIC	≥13	ridaforolimus	mTor	Industry	Merck|Ariad Pharmaceuticals

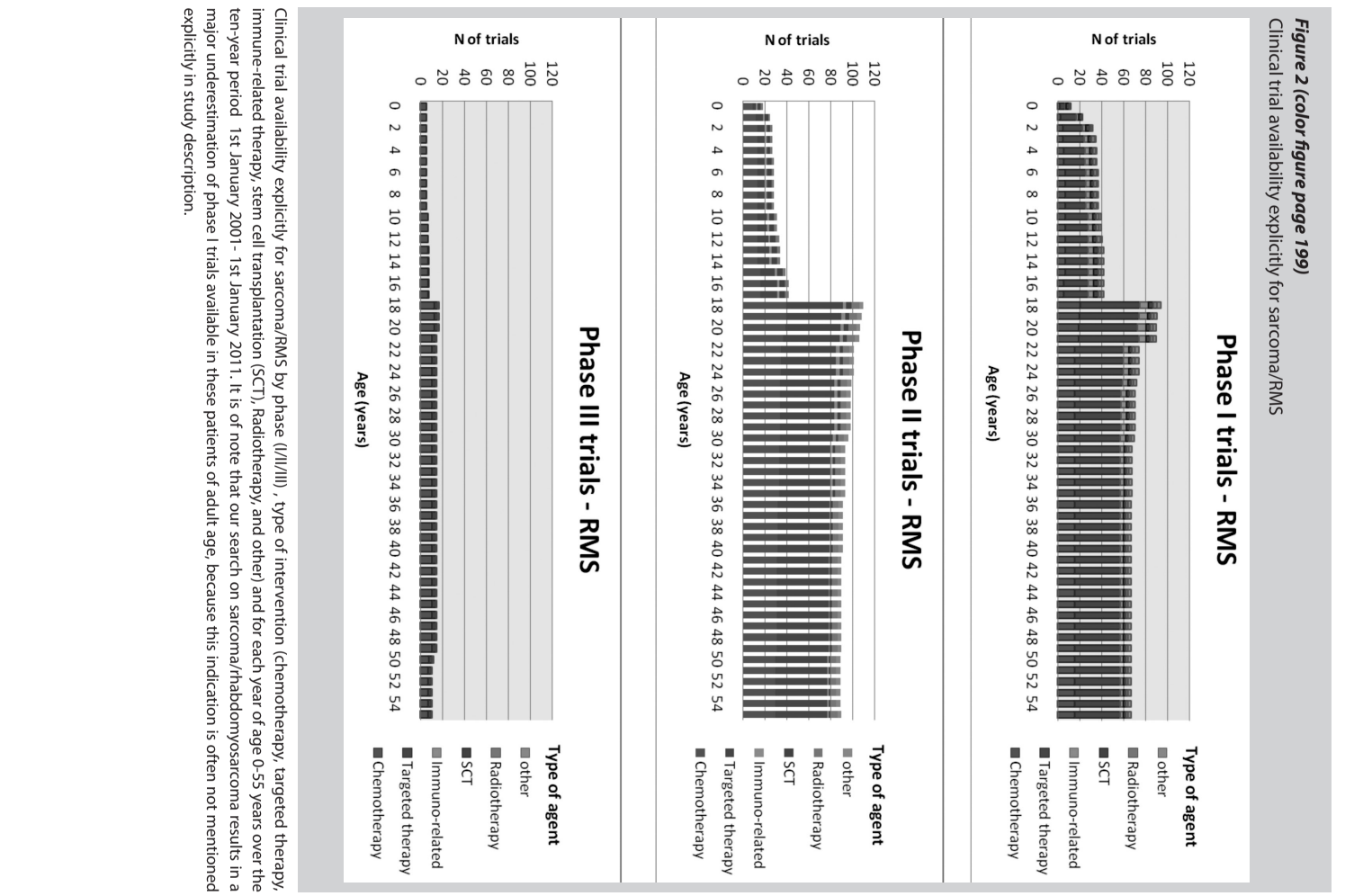
Targeted treatment trials explicitly including sarcoma/RMS over the 10-yr period 2001-2011. Asterix* means that sarcoma inclusion was present in the description of the study, but that ERMS/ARMS inclusion was not exclusively mentioned in inclusion/exclusion criteria

Table 5b
Immuno-targeted trials availability explicitly including sarcoma/RMS specifically over the 10-yr period 2001-2011

No	NCT ID	Phase	N	Start year	SUBTYPE	RISK GROUP	Age	Interventions	Funded by	Sponsors
1	NCT01169584	Phase I	15	2010	ERMS/ARMS	REFRACTORY	2-21	Recombinant Vaccinia GM-CSF; RAC VAC GM-CSF (JX-594)	Industry|Other	Jennerex Biotherapeutics|Solving Kids Cancer
2	NCT01048892	Phase I	34	2009	ERMS/ARMS	REFRACTORY/RECURRENT	3-20	Seneca Valley virus-001	Other|NIH	Children's Oncology Group|National Cancer Institute (NCI)
3	NCT00003750	Phase I	24	2001	*	REFRACTORY/RECURRENT	0-21	hu14.18-IL2 fusion protein	Other|NIH	Children's Oncology Group|National Cancer Institute (NCI)
4	NCT00931931	Phase I	18	2010	*	REFRACTORY	13-30	HSV1716	Other|U.S. Fed	Children's Hospital Medical Center, Cincinnati|FDA Office of Orphan Products Development
5	NCT00923351	Phase I| II	47	2007	ERMS/ARMS	METASTATIC/RELAPSED	1-35	tumor lysate-pulsed dendritic cell vaccine	NIH	National Cancer Institute (NCI)
6	NCT00405327	Phase II	30	2006	ERMS/ARMS	METASTATIC/REFRACTORY/RELAPSED	0-30	tumor lysate-pulsed dendritic cell vaccine	Other	University of Michigan Cancer Center

Immuno-targeted trials availability explicitly including sarcoma/RMS specifically over the 10-yr period 2001-2011 Asterix* means that sarcoma inclusion was present in the description of the study, but that ERMS/ARMS inclusion was not exclusively mentioned in inclusion/exclusion criteria

Figure 2 (color figure page 199)
Clinical trial availability explicitly for sarcoma/RMS



Clinical trial availability explicitly for sarcoma/RMS by phase (I/II/III) , type of intervention (chemotherapy, targeted therapy, immune-related therapy, stem cell transplantation (SCT), Radiotherapy, and other) and for each year of age 0-55 years over the ten-year period 1st January 2001- 1st January 2011. It is of note that our search on sarcoma/rhabdomyosarcoma results in a major underestimation of phase I trials available in these patients of adult age, because this indication is often not mentioned explicitly in study description.

Table 5c
Phase III trials explicitly including sarcoma/RMS over the 10-yr period 2001-2011

No	NCT ID	phase	N	Acronym	Start date	Subtype	Stage	Age	Agent type	Agent(s)	Type sponsor	Sponsor
1	NCT00796120	Phase III	80	CR015769	2008	ARMS	First-line METASTATIC	≥18	Chemotherapy	Trabectedin ; Doxorubicin	Industry	Johnson & Johnson Pharmaceutical Research & Development, L.L.C.|PharmaMar
2	NCT00061984	Phase III	450	EORTC-62012	2003	ARMS	First-line ADVANCED/METASTATIC	≥18	Chemotherapy	pegfilgrastim; doxorubicin hydrochloride; ifosfamide	Other	European Organization for Research and Treatment of Cancer
3	NCT01168791	Phase III	424	PICASSO III	2010	*	FRONT LINE METASTATIC	≥18	Chemotherapy	doxorubicin; palifosfamide-tris	Industry	ZIOPHARM
4	NCT00354744	Phase III	75	COG-ARST0431	2006	ERMS/ARMS	HIGH RISK	0-49	Chemotherapy	dactinomycin; cyclophosphamide; doxorubicin hydrochloride; etoposide; ifosfamide; irinotecan hydrochloride; vincristine sulfate	Other|NIH	Children's Oncology Group|National Cancer Institute (NCI)
5	NCT00876031	Phase III	320	CWS-2007	2009	ERMS/ARMS	HIGH RISK/VERY HIGH RISK	0-20	Chemotherapy	trofosfamide, idarubicin, etoposide	Other	University Hospital Tuebingen|Cooperative Weichteilsarkom Study Group|Deutsche Kinderkrebsstiftung|Gesellschaft fur Padiatrische Onkologie und Hamatologie - Germany|Gesellschaft fur Padiatrische Onkologie und Hamatologie - Austria|The Swedish Working Group for Pediatric Solid Tumours|Polish Paediatric Solid Tumour Study Group|Gesellschaft für Pädiatrische Onkologie und Hämatologie - Switzerland
6	NCT00379457	Phase III	600	EPSSG-RMS-2005	2006	ERMS/ARMS	LOCALIZED	0-20	Chemotherapy	dactinomycin; carboplatin; cyclophosphamide; doxorubicin hydrochloride; etoposide; ifosfamide; topotecan hydrochloride; vincristine sulfate; vinorelbine	Other	European Paediatric Soft Tissue Sarcoma Study Group|Italian Association for Pediatric Hematology Oncology|Children's Cancer and Leukaemia Group|Dutch Childhood Oncology Group
7	NCT00075582	Phase III	510	COG-ARST0331	2004	ERMS	LOW RISK	0-49	Chemotherapy	dactinomycin; cyclophosphamide; vincristine sulfate	Other|NIH	Children's Oncology Group|National Cancer Institute (NCI)
8	NCT01223248	Phase III	100		2010	*	First-line METASTATIC	≥18	Radiotherapy	Image-Guided (IG) IMRT 24 Gy verus IGIMRT 27 Gy in 3 fractions	Other	Memorial Sloan-Kettering Cancer Center|University of Pisa
9	NCT00538239	Phase III	650		2007	*	maintanance therapy META STATIC	≥13	Targeted	ridaforolimus	Industry	Merck|Ariad Pharmaceuticals
10	NCT00354835	Phase III	486	COG-ARST0531	2006	ERMS/ARMS	NON-METASTATIC	0-50	Chemotherapy	dactinomycin; cyclophosphamide; irinotecan hydrochloride; vincristine sulfate	Other|NIH	Children's Oncology Group|National Cancer Institute (NCI)
11	NCT00003958	Phase III	518	COG-D9803	2002	ERMS/ARMS	NON-METASTATIC ARMS, STAGE II,III (CGIII) ERMS, METASTATIC ERMS <10 years	0-49	Chemotherapy	dactinomycin; filgrastim; sargramostim; cyclophosphamide; topotecan hydrochloride; vincristine sulfate	Other|NIH	Children's Oncology Group|National Cancer Institute (NCI)
12	NCT00794521	Phase III	360	EORTC-62072	2008	ARMS	REFRACTORY/RECURRENT	≥18	Targeted	pazopanib hydrochloride	Other	European Organization for Research and Treatment of Cancer/GlaxoSmithKline
13	NCT00142571	Phase III	120		2003	ERMS/ARMS	REFRACTORY/RECURRENT	≥10	Chemotherapy	Gemcitabine; Docetaxel	Other	Memorial Sloan-Kettering Cancer Center|Connective Tissue Oncology Society|M.D. Anderson Cancer Center|Massachusetts General Hospital|Mayo Clinic|University of Michigan Cancer Center
14	NCT00210665	Phase III	3000		2005	*	REFRACTORY/RECURRENT	≥18	Chemotherapy	trabectedin	Industry	Johnson & Johnson Pharmaceutical Research & Development, L.L.C.

Asterix* means that sarcoma inclusion was present in the description of the study, but that ERMS/ARMS inclusion was not exclusively mentioned in inclusion/exclusion criteria

administrations''Best Pharmaceuticals for Children Act' (BPCA) and the 'Pediatric Research Equity Act' (PREA), encourages and forces pharmaceutical companies to test new agents in children as well (149;150). This hopefully provides a basis for a wider availability of novel treatment options in children with cancer as well as their older counterparts. However, the availability of multiple targeted treatments in phase I and II studies for a relatively low number of patients might controversially discourage enrolment, as selection of the appropriate trial for a patient becomes too complicated. Furthermore, due to national governments' regulations on drug administration in children and ethical complaints in many countries the enrolment of children in phase I trials is extremely difficult. Worldwide collaboration is needed to provide an infrastructure and regulate the optimal balance between trial availability and enrolment.

Table 6
Critical issues in RMS management

CRITICAL ISSUES IN RMS - Adults and children
EPIDEMIOLOGY
* Rare tumour – low incidence rates
* Incidence declines rapidly after age 20
* No data concerning true ERMS/ARMS incidence in adults
* Poor survival rates in adults (40%) versus children (70%)
BIOLOGY
* Scarcity of data regarding biological differences in adults versus children available
DIAGNOSIS
* Histological diagnosis - broad differential diagnosis
* Histological diagnosis – lack of standard central review in adults
STAGING
* Advanced clinical presentation in adults versus children
* relative scarcity of prospective trials with new staging techniques in (children with) RMS
RISK STRATIFICATION
* Translocation status implementation in risk stratification under discussion
* RMS-tailored risk stratification system in adults does not exist
TREATMENT
* Lack of centralization of RMS care in adults
* Absence of standardized protocols regarding RMS treatment in adults
* Age-related differences in pharmacology/ pharmacodynamics of systemic treatment
* Low trial inclusion/availability in Adolescent and Young Adults (AYA) patients
NEW TARGETS FOR TREATMENT
* Many biological targets identified in RMS
* Lack of interest of pharmaceutical agencies in a very rare tumour
* Phase I trial lack in children up to 5 years ago
* Accrual of children in phase I studies low
* Trial design and infrastructure optimization is needed

Besides trial availability issues, an extremely important issue to consider is the world-wide low accrual of AYA patients into clinical trials. Although an increase in inclusion rate is reported by recent studies, still, improvements have to be made for this group (151). The National Cancer Institute (NCI) network shows to be extremely important in providing clinical trials and providing the network for centres to participate in these trials in the United States (148). Furthermore, the NCI supports the multicenter drug development initiative "Pediatric preclinical testing program" (PPTP) which has provided important (preclinical) translational research aiming at the usefulness of the introduction of targeted agents in children (152).

CRITICAL ISSUES IN RMS PATIENT CARE

Table 6 summarizes the critical points in design of RMS care. In general, the rarity of RMS illustrated by its incidence rates clarifies the scope of the problem, given that the incidence declines very fast after the age of 20 years and true information regarding ERMS and ARMS incidence in adult populations is scarce. Furthermore, the etiological and biological diversity in RMS in adults versus children remains to be revealed. Therefore, epidemiologic and genetic studies in both children and adults are indispensable in order to increase knowledge about the different etiologic patterns and nature of RMS at different ages.

Histological diagnosis is the corner stone of diagnosis of RMS. The infrastructure for histological RMS diagnosis in children already exists, as this must be confirmed by a central review pathologist prior to inclusion into a clinical trial. Although boards for pathological review of RMS in adult do exist, probably not all diagnoses are truly confirmed and the differential diagnosis is challenging.

There is an essential deficit of prospective information concerning staging and risk stratification in adult versus children with RMS. As clinical presentation in adults seems more unfavourable than reported in children, it is questionable whether the current childhood risk classifications as well as the staging procedures are appropriate in adults as well. An attempt for the development of uniform staging methods as well as appropriate risk assessments should however be undertaken to facilitate comparison. Besides optimization of current standards, additional efforts should aim at trial availability for prospective testing of novel staging techniques in RMS patients of all ages.

Treatment in adults versus children with RMS has been incomparable up to now, and even within the childhood RMS study groups controversies do exist. Age is an important factor to consider as it greatly influences treatment decisions and possibilities. The major existing controversy in treatment of adult and childhood RMS is the use of chemotherapy. Increased knowledge concerning age-related pharmacology has to be obtained in order to develop feasible chemotherapy protocols in adults. Close collaboration of both paediatric and adult oncologists with pharmacologists for close monitoring to provide information regarding pharmacokinetics and –dynamics are therefore essential in future RMS treatment to monitor and optimize safety and efficacy.

Still, there is an urgent need for new therapeutic agents in RMS. Despite extensive preclinical research, the availability of clinical implementation of these targeted therapies is essentially compromised by the low incidence of RMS and pre-clinical research is performed scattered around the world which prohibits a quick move of new potential active drugs from bench to bedside.

FACILITATING THE ROAD AHEAD

Despite its rarity, there is no doubt that AYA patients with RMS do deserve equal attention as well as established standardized treatment as their younger counterparts currently do. The virtual distinction between RMS treatment in children and adults not only impairs optimization of treatment, but also of diagnosis, staging, new drugs, and organization of patient care. Therefore, bringing the knowledge obtained from both worlds together is essential to provide a basis for development of standardized treatment protocols, facilitation of introduction of new treatment possibilities and optimization of care for RMS patients of all ages in the near future.

Centralization and concentration

The number one challenge concerning RMS we have to overcome is the rarity of this tumour in children as well as adults. The solution from paediatric oncologists to this problem involves treatment concentration and global collaboration as fundamental strategy as reflected by the existing large childhood cooperative groups. RMS care in adults is seriously compromised by a relative lack of concentration and collaboration, resulting in the dispersion of patients treated at numerous adult oncology centres and departments. In general, it seems that RMS care should ideally take place in a specialized centre with experienced clinicians in order to obtain the best survival rates.

Cooperation and collaboration

The key players in RMS treatment are both paediatric and medical oncologists. Ideally, this cooperation will lead to an increase in knowledge by learning from each other's experiences and treatment concepts. An example of a project improving the awareness of clinicians with regard to treatment of rare tumours that are underrepresented in their daily practice population is the Tumori Rari in Eta` Pediatrica "TREP" project, which was grounded in Italy in 2000. The TREP project was initiated by paediatric oncologists to reach national collaboration of paediatric and medical oncologist for treatment of tumours which are very rare in children but common in adults. The intent for a call for guidelines came from paediatric oncologists not familiar with these tumours, which are rare-but not absent- in children, and were provided by medical oncologists. Such an approach might be feasible for RMS; however, one should add a critical side-note as the TREP project was embedded in the existing infrastructure and centralized care for children with cancer (153). In 2002, the COG followed with a committee for rare tumours at paediatric age. Their specific purposes were to develop an organizational framework to facilitate the study of infrequent tumours and create registries, biospecimen banks, and clinical trials (154). Centralization of informational support concerning rare diseases was facilitated by grounding of the National Organization of Rare Disorders Network (NORD; *http://www.rarediseases.org*). The global cooperation of medical oncologists in European, USA and Australian study groups in treatment of gastro-intestinal stromal tumour (GIST) is the example that collaboration in a rare adult-type tumours can also be very successful (155). These collaborations might serve as a starting point of RMS treatment design.

Key players in the "global RMS treatment group" should involve a panel of experts involved in RMS, i.e. paediatric and adult sarcoma pathologists, paediatric oncologists as well as medical oncologists, radiotherapists, and paediatric as well as adult surgeons of different specialties, as well as representatives from pharmaceutical companies. Furthermore, cooperation might highlight differences in needs for different age groups (RMS-specific as well as non-RMS-specific); for example fertility issues, long term follow-up methods, and the transition from paediatric to adult oncology (156). It could be very helpful to involve patient/parent advocacy groups in order to achieve global collaboration, since these groups often work in close international association. Moreover, to deliver age-adjusted healthcare for all patients with RMS, it is crucial letting the patients and their parents participate in this process and making us clear what they are in need of. Due to involvement and participation of patient and parent advocacy groups an impulse can be given to both cure and care.

Infrastructure and clinical trials

The currently available infrastructure for RMS treatment is mainly that of the childhood RMS treatment groups; the EpSSG and the COG. Preferably they should collaborate on RMS protocols within existing networks for adult sarcoma patients such as e.g. SARC and EORTC, and the World Sarcoma Network. This would enhance attractiveness for pharmaceutical partners to do studies in even this rare group of tumours and would increase the speed of accrual in clinical trials. Improvement of clinical trial availability and accrual is essential to make a step forward in the optimization of the current staging and treatment standards as well as the implementation of new diagnostic and therapeutic options for RMS patients of all ages. Nationwide as well as global awareness for the need of trial accrual of patients should be aimed at, in order to enlarge the trial accrual especially in young RMS patients. Although improvements concerning the availability and accrual to clinical trials in AYA have been made, within this respect AYA patients need ongoing attention.

Future perspectives

The outlined concept might also provide a rationale for the management of additional rare tumour types in which there is overlap between children, adolescents and (young) adults, i.e. Ewing's sarcoma, osteosarcoma and certain brain tumours. Some economy of scale can be reached by grouping the management of these tumours as happens already in certain network organisations. Although it probably will take quite a couple of years if we start from now, a steep increase in knowledge of RMS and other rare tumours of AYA age can be generated in order to develop world-wide standardized treatment, and to achieve the best quality of care and cure for patients of all ages.

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CHAPTER 8

Summary

Over the past decades, there has been a relative lack of awareness of adolescent and young adult (AYA) patients with cancer. This is reflected by the relatively low accrual and participation of this subpopulation in clinical trials, which is held as one of the factors responsible for a consequent lack in survival benefit when compared to their younger counterparts. Importantly, the AYA cancer population is unique in ways of a typical tumour type distribution, overlapping both child- and adulthood cancers. Furthermore, the awareness of the AYA population being distinct in terms of specific needs and concerns (e.g. fertility, insurance, employment, psychosocial issues) is rising. The basis to increase awareness and facilitate the development of tailored treatment and care of this young cancer population is a throughout description of the epidemiological features (e.g. incidence, distribution, survival) of cancers affecting this population. Another important feature to consider in both children and AYA with cancer is their generally long life-expectancy after surviving cancer, accompanied by an increased risk of development of long-term cancer related issues, including long term morbidity/mortality and second malignancies.

In **chapter 2** we characterized the population-based incidence and survival of 1,118 AYAs (12-24 years) with cancer diagnosed in the Northern Netherlands between 1989 and 2003. The main tumour types represented in AYA males were germ-cell tumours (predominantly non-seminomatous testicular cancer), lymphoma, and central nervous system tumours. In females, lymphoma showed the highest incidence, followed by carcinoma and melanoma. The total incidence of cancer in AYA increased (estimated annual percentage change, EAPC 2.2%). Survival did not change over the study period (overall 5-years survival 80.8%). Survival was high for lymphoma, germ cell tumours, carcinoma and melanoma, while survival was poor for leukaemia, soft tissue and bone sarcoma and central nervous system tumours. The Standardized Incidence Ratio (SIR) of a second primary tumour in this young cohort was high (30.6), corresponding to a cumulative incidence at ten years of 2.8%.

In **chapter 3** we reported an extended study concerning 23,161 AYAs (15-29 years) diagnosed with cancer in the Netherlands between 1989 and 2009. This study confirmed a significant increase in incidence over time, predominantly in males (EAPC 1.9%) when compared to females (EAPC 1.4%). The tumour type distribution as well as the overall survival rates (82.0 and 83.0% in males and females, respectively), were nearly identical compared to the study described in chapter two. With a median follow-up time of 6.8 years, 412 AYAs experienced a second primary tumour (1.8%). The SIR of a second primary tumour was lower than in the previous study in the Northern Netherlands; males 5.5 and females 5.3. The most striking result was the more than seven times increased risk of breast cancer after Hodgkin's lymphoma (n=26, SIR 7.4).

Rhabdomyosarcoma (RMS) is a rare type of soft tissue sarcoma that represents an ultimate example of a typical childhood malignancy which also occurs across the AYA age-spectrum and shows inferior survival rates in the older population. Despite the successive survival rates reported in children with RMS, the survival in particular subgroups of patients (e.g. those primarily diagnosed with alveolar histology, distant and/or lymph node metastases at diagnosis, diagnosed at older age, or with refractory/recurrent disease) remains disappointing. This urges the need for new therapeutic treatment strategies.

In **chapter 4** we reported that age is an independent prognostic factor of survival in rhabdomyosarcoma (RMS) patients based on a multivariate analysis in a multi-center retrospective cohort study of 169 patients (aged 0-73 years). As expected we observed a typical age-distribution of the two main histological subtypes of RMS; embryonal RMS (favourable prognosis, median age 7 years) and alveolar RMS (unfavourable prognosis, median age 15 years). Furthermore, older age was related to unfavourable clinical presentation (e.g. metastatic disease in embryonal RMS and parameningeal location of alveolar RMS). Survival for patients under 16 years of age was significantly better when compared to patients age 16 or older in the whole cohort, as well as in embryonal RMS, alveolar RMS, and non-metastatic embryonal RMS. Metastatic disease was also an important adverse prognostic factor for all patients with RMS (survival of patients diagnosed with metastatic disease did not exceed 10%). In multivariate analysis, age was a significant prognosticator of disease specific survival in all subgroups (all patients, embryonal, alveolar and non-metastatic embryonal RMS).

In **chapter 5** we reported that anaplastic lymphoma kinase (ALK) protein expression and *ALK* gene copy number gain was detected in the vast majority of alveolar RMS (80-90%), compared to 30-50% of embryonal RMS ($p<0.001$), thereby providing a potential treatment target. Furthermore, *ALK* copy number correlated with ALK protein expression in all primary tumours. *ALK* gene translocations were not observed. Importantly, we identified a novel missense mutation, as well as whole exon deletions in 7/43 patients and in one RMS cell line. Linkage of ALK status with clinical characteristics and outcome revealed that specific *ALK* gain was related to metastatic disease at diagnosis and consequently worse survival in embryonal RMS.

In **chapter 6** we reported a study on both insulin-like growth factor 1 receptor (IGF-1R) and ALK as potential targets for combined treatment in RMS. Expression of IGF-1R was seen in an equal amount of alveolar RMS (72%) and embryonal RMS (62%). Interestingly, we observed nuclear expression of IGF-1R in up to 10% of RMS, and this was related to worse survival in embryonal RMS. ALK expression was already described in chapter 5. Co-expression of IGF-1R and ALK was observed in a significant amount of alveolar RMS (68%) and embryonal RMS (32%). Therefore, these targets might be of major interest for simultaneous inhibitory treatment in RMS. We consequently tested the potential of IGF-1R-, ALK- and simultaneous inhibitory treatment *in vitro*. Inhibition of IGF-1R (R1507) resulted in diminished cell growth only in alveolar RMS cell line Rh41. The ALK inhibitor NVP-TAE-684 resulted in diminished cell growth in aRMS cell lines Rh41 and Rh30, and to a lesser extent in eRMS cell lines Rh18 and RD. Simultaneous treatment revealed a synergistic effect of R1507 and NVP-TAE684 in Rh41. Based on these results, we conclude that in RMS targeting of the ALK receptor, as well as simultaneous targeting of IGF-1R and ALK warrant further research, especially in patients with alveolar RMS and metastatic embryonal RMS.

In **chapter 7** we review the current data available concerning RMS treatment in children, adolescents and (young) adults. It provides a profound overview which highlights the similarities and differences in children and adults with RMS in terms of epidemiology, tumour biology, diagnosis, treatment approach, and accrual to clinical trials with promising new agents. The findings as provided in this review consequently lead to a concept for a global cooperation and centralization of treatment of patients of all ages, thereby facilitating progress in scientific knowledge and in the introduction of new agents in the future.

CHAPTER 9

General discussion and future perspectives

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Adolescents and young adults (AYA) suffering from cancer are a very heterogeneous group of patients with specific needs and tumourtype specific treatment-related care issues during and after treatment. Although the age group to which AYA refers is still a world-wide topic of discussion, it usually refers to patients aged 15-39 years or smaller intervals within this range (1). Although cancer in AYA is relatively rare (2-3% of all cancers), the incidence is nearly three times higher than in children (2). In contrast to the world-wide improvements of both pediatric and adult cancer care over the past decades, the awareness has grown that there has been a relative lack of attention for this age group resulting in the "adolescent and young adult gap in cancer care" (3).

Epidemiological knowledge concerning the AYA cancer population is of major importance for the development of tailored cancer care programs for this particular group of patients. Population-based studies (as described in **chapter 2 and 3** of this thesis) reveal that there is a typical age-related shift in incidence pattern of cancer types in AYA. Patients up to age 20 show the tail of childhood tumours (including acute lymphoblastic leukemia, Wilms tumours, certain subtypes of central nervous system tumours, and typical age-related sarcoma subtypes as rhabdomyosarcoma which will be discussed in more detail later on), while in older AYA patients adulthood tumours as breast carcinoma, thyroid carcinoma and melanoma are emerging. In between, the typical tumours of AYA age occur, such as testicular germ cell tumours and Hodgkin's disease. This unique distribution of tumour types causes them to be a heterogeneous group of patients which doesn't belong to specific childhood or adult cancer services.

The resulting effect is of major concern; although overall AYA show a relatively high survival rate of 80%, those diagnosed with typical childhood tumours show a fairly poor survival and there is an obvious deficit in survival improvement over the past decades when compared to children (2). Although a relatively low accrual to, and availability of, clinical trials in this subpopulation has been held responsible for this lack in survival, the most reasonable explanation is with certainty multifactorial and is supposedly tumour-related (differences in tumour biology), patient-related (delay in seeking medical help, worse tolerance of therapy, worse compliance), and health-care related (delay in diagnosis, treatment by professionals who are not familiar with the disease, decentralized care, and as earlier mentioned a lack of clinical trial availability and enrollment) (4).

"The cancer is over, now what?" (5). Late effects after suffering from AYA cancer are of major concern in these young cancer survivors with a generally long life expectancy. This is underlined by the high incidence of second tumours that we observed in both AYA studies, which probably even underestimates the real incidence due to relatively short follow-up. Besides second tumours, we should pay attention to other long-term morbidity/mortality as seen after cancer treatment in AYA, for example coronary artery disease after chest irradiation (6), heart failure in anthracycline treated patients (7;8), cardiovascular disease after cisplatin-based chemotherapy in testicular cancer (9), and multiple health issues in allogeneic hematopoietic stem cell transplant recipients (5). In children, major cancer survivor studies have been conducted with long-term follow-up data (10-14), while AYA cancer survivor studies are relatively scarce, with the exception

of a handful of studies mostly concerning late effects after a single type of malignancy at AYA age (5). Long-term follow-up studies focusing on the specific issues after surviving AYA cancers, should therefore be a fundamental part of improvement of AYA cancer care.

Last but not least, AYA with cancer are unique in ways of their specific needs including psychosocial care, fertility issues, work and employment, relationship and society related issues. Therefore, multiple initiatives have recently been taken world-wide to ensure specialized care for AYA cancer patients, including the development of AYA cancer departments/clinics/programs facilitating concentration of care and giving the opportunity to focus on their short- and long-term needs (1). The basis of tailored AYA cancer care is thus seeded, but the following years will point out whether we can harvest the effect of all effort.

Rhabdomyosarcoma (RMS) represents an ultimate example of a childhood tumour covering a wider age spectrum; children and AYA. Therefore, we performed a multi-center retrospective study which underlines the adverse outcome of this tumour type in AYA compared to children (**chapter 4**). This is in line with the observations in population-based studies concerning AYA (i.e. poor survival of childhood tumours in AYA compared to children, also highlighted in chapter 2 and 3) (15). We observed that higher age at diagnosis was related to advanced stage of disease at diagnosis (e.g. metastatic disease and lymph node involvement), which might be contributable to an age-dependent difference in tumour biology (more aggressive tumour phenotype) but also a potential delay in diagnosis in older patients, which is either patient-related, doctor-related or both. As treatment differences have been held responsible for the difference in survival, we made an effort to include treatment variables into a multivariate prognostic model. However, we were confronted with the compromising effect of the generally existing differences in treatment approach (especially in chemotherapeutic regimens) in children versus adults. The importance of these unsatisfactory findings is strengthened by an earlier study which indicated that survival rates in adults with RMS may be equal to children when 'appropriate' treatment according to childhood standards is given (16). Because it remains unknown to what extent biological and treatment effects play a role in the observed survival difference in children versus adults, this remains to be elucidated in prospective studies after introduction of uniform treatment schedules, which requests centralization of care for adults with these very rare tumours.

Molecular targeted compounds are upcoming in cancer treatment and also in sarcoma (17). During the development of targeted agents, many compounds fail to reach clinical application in sarcomas, due to preliminary ending of clinical trials after being unsuccessful in more common tumour types, and because pharmaceutical partners face more complexity in trial organization in studies in rare (subtypes of) sarcomas. Therefore, international collaboration is of utmost importance in the field of sarcoma, which is currently the case in Europe via the European Organization for Research and Treatment of Cancer (EORTC) and for which the World Sarcoma Network has been raised. In this way we hope to prevent that for sarcoma treatment new and interesting drugs are thrown out as babies with the bathwater (18).

In a search for new targets of treatment for rhabdomyosarcoma, two earlier studies (19;20) pointed towards a potential role of anaplastic lymphoma kinase (ALK) in RMS. This consequently formed the basis for **chapter 5**, where we describe anaplastic lymphoma kinase (ALK) as a potential target for treatment of RMS. We identified genetic alterations of the *ALK* gene (e.g. gain or amplification, whole exon deletions, and one novel mutation). The most important clinical finding was the observed relation between specific *ALK* gain (primary tumour) and metastatic disease as well as poor survival in embryonal RMS. Our observations and those previously reported by others, pose questions to a potential oncogenic role of ALK in RMS. Also, our study gives direction towards ALK as an important target for future RMS treatment (especially in patients with alveolar RMS and patients with advanced disease). We are therefore looking forward to the clinical outcomes of the planned phase II CREATE trial of the EORTC (NCT01524926), testing Crizotinib (ALK-MET inhibitor) in patients with advanced sarcoma, including locally advanced/metastatic aRMS.

The most extensively investigated target in RMS is probably IGF-1R (21). Although the first clinical results for IGF-1R inhibitors in sarcoma have been less promising than expected based on pre-clinical studies, we still feel that IGF-1R signalling is important in RMS. Therefore, we are convinced that IGF-1R inhibitors, maybe in combination with conventional chemotherapy or other targeted drugs, deserve further investigation.

In **chapter 6** we investigated combined inhibition of IGF-1R and ALK. We observed that co-expression of ALK and IGF-1R was frequently seen in RMS tumour samples and that simultaneous ALK and IGF-1R inhibition *in vitro* revealed a synergistic effect in one alveolar RMS cell line. Remarkably, we also identified a negative prognostic value of nuclear IGF-1R in embryonal RMS, however the functional effect of nuclear localization of the receptor is still unknown.

Besides the general obstacles to overcome in the development and implementation of targeted agents (clinical trial design, patient selection, biomarkers, development of potential combination strategies), the rarity of these tumours represents an additional compromising factor. Therefore, we recommend global cooperation for the introduction of standardized (comparable) treatment regimens for RMS in children and adults, as well as optimization of the prior conditions for the introduction of promising new treatment strategies, as was subject of the review in **chapter 7**. Moreover, this review also can serve as a basis for other rare pediatric solid tumours occurring in the AYA population such as for example Ewing sarcoma, osteosarcoma, and medulloblastoma.

General conclusion

In this thesis, we address two major issues in young cancer patients. First, we aim to provide a step forward to increase the worldwide awareness of the existence and unmet needs of the AYA cancer population. And we use RMS as a specific cancer type to discuss the position of AYA within this population. Second, we give insight into potential new treatment strategies for RMS patients and a concept to pass the river between care and treatment for rhabdomyosarcoma in children and adults. Optimization of AYA cancer care, as well as optimization of care for RMS patients of all ages can only be reached by efforts of all key players in the treatment of these rare groups of patients, by the introduction of standardized treatment and care, as well as collaboration at a global level.

"Because the people who are crazy enough to think they can change the world, are the ones who do."

Steve Jobs

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CHAPTER 10

Dutch summary

NEDERLANDSE SAMENVATTING

Gedurende de afgelopen decennia zijn adolescenten en jong volwassenen met kanker relatief onderbelicht. Dit wordt onder andere weerspiegeld door de lage inclusiecijfers in klinische trials, wat tevens wordt gezien als een belangrijke oorzaak voor de relatief slechte overleving vergeleken met kinderen met kanker. Er is een groeiend bewustzijn dat adolescenten en jongvolwassenen een unieke populatie zijn met specifieke behoeften en zorgen (bijvoorbeeld fertiliteit, verzekeringen, werk, psychosociale problematiek). De basis voor het onder de aandacht brengen en faciliteren van patiëntgerichte behandeling en zorg voor deze populatie, is het in kaart brengen van de epidemiologische paramaters van kanker in deze populatie (incidentie, verdeling van tumoren, overleving). Gezien de relatief lange levensverwachting na de overleving van kanker, is een ander aandachtspunt in deze jonge populatie het verhoogde risico op het ontwikkelen van lange termijn effecten (morbiditeit, mortaliteit en tweede tumoren).

In **hoofdstuk twee** van dit proefschrift beschrijven we de incidentie en overleving van 1118 adolescente en jong volwassen patiënten (leeftijdscategorie 12-24 jaar) met een maligniteit, gediagnosticeerd in de regio Noord-Nederland tussen 1989 en 2003. De meest frequente tumortypen bij mannen in deze populatie betroffen kiemceltumoren (vooral non-seminomen van de testis), gevolgd door lymfomen en tumoren van het centrale zenuwstelsel. Bij vrouwen werd de hoogste incidentie gezien voor lymfomen, gevolgd door carcinomen en melanomen. De totale incidentie van maligniteiten in adolescenten en jong volwassenen nam over de studieperiode toe met gemiddeld 2,2% per jaar. De 5-jaars overleving bleef gelijk gedurende de studieperiode (80,8%). De 5-jaarsoverleving was relatief gunstig voor patiënten met lymfomen, kiemceltumoren, carcinomen en melanomen, terwijl de overleving voor patiënten met leukemie, weke delen- en botsarcomen, en tumoren van het centrale zenuwstelsel ongunstiger was. De gestandaardiseerde incidentie ratio (SIR) van tweede tumoren in dit jonge cohort was hoog (30,6), wat overeenkomt met een absolute incidentie van 2,8% na 10 jaar.

In **hoofdstuk drie** beschrijven we een meer uitgebreide studie betreffende 23161 adolescenten en jong volwassenen (15-29 jaar) gediagnosticeerd met een maligniteit in heel Nederland tussen 1989 en 2009. Deze studie bevestigde de significante stijging in incidentie over de tijd zoals beschreven in hoofdstuk twee, waarbij deze stijging meer uitgesproken was bij het mannelijke geslacht (geschatte jaarlijkse incidentiestijging 1,9%) ten opzichte van het vrouwelijke geslacht (1,4%). De verdeling van tumortypen en de overlevingspercentages (82,0% en 83,0% in mannen en vrouwen respectievelijk) waren nagenoeg identiek ten opzichte van de studie beschreven in hoofdstuk twee. Met een mediane follow-up periode van 6,8 jaar, werd een tweede primaire tumor gevonden in 412 patiënten (1,8%). De SIR van tweede tumoren in dit cohort was echter lager dan in de voorgaande studie; 5,5 en 5,3 bij het mannelijke en het vrouwelijke geslacht respectievelijk. De belangrijkste bevinding was een meer dan 7 maal verhoogd risico op borstkanker na het Hodgkin lymfoom (26 patiënten, SIR 7,4).

Het rhabdomyosarcoom is een zeldzame weke delen tumor die een expliciet voorbeeld vormt van een kindertumor die tevens in adolescenten en (jong) volwassenen kan optreden en waarbij

in oudere patiënten een slechtere overleving wordt zien. Ondanks de toegenomen overleving in kinderen met een rhabdomysarcoom, is ook in de overleving in bepaalde subgroepen nog steeds slecht (patiënten met tumoren met alveolaire histologie, lymfeklier- of afstandsmetastasen bij diagnose, of patiënten met therapie-ongevoelige ziekte of recidief van de ziekte). Daarom is er een urgente noodzaak voor het ontwikkelen van nieuwe therapeutische opties.

In **hoofdstuk vier** beschrijven we dat leeftijd een onafhankelijke negatieve voorspeller van overleving is in een multivariate analyse van patiënten met een rhabdomyosarcoom in een retrospectieve cohort studie. De populatie in deze studie betrof 169 patiënten (leeftijdscategorie 0-73 jaar), die werden behandeld in meerdere universitaire medische centra in Nederland over de afgelopen decennia. Hierbij werd een typische leeftijdsverdeling van de twee belangrijkste subtypen gezien; het embryonale rhabdomyosarcoom (gunstige prognose, mediane leeftijd bij diagnose 7 jaar) en het alveolaire rhabdomyosarcoom (ongunstige prognose, mediane leeftijd bij diagnose 15 jaar). Bovendien bleek een hogere leeftijd bij diagnose geassocieerd te zijn met ongunstige klinische presentatie (zoals gemetastaseerde ziekte bij het embryonale subtype en parameningeale lokalisatie bij het alveolaire subtype). De overleving voor patiënten onder de leeftijd van 16 jaar was significant beter vergeleken met patiënten van 16 jaar en ouder in het hele cohort, maar ook in de subgroepen embryonaal rhabdomyosarcoom, alveolair rhabdomyosarcoom en niet-gemetastaseerd embryonaal rhabdomyosarcoom. Bovendien was gemetastaseerde ziekte een belangrijke ongunstige prognostische factor in alle patiënten met een rhabdomyosarcoom (5-jaars overleving minder dan 10%).
Ook in multivariate analyse bleek leeftijd een onafhankelijke voorspeller voor slechtere overleving in alle patiënten, evenals in patiënten met het alveolaire of embryonale subtype en in patiënten met een niet-gemetastaseerd embyonaal rhabdomyosarcoom.

In **hoofdstuk vijf** beschrijven we anaplastic lymphoma kinase (ALK) eiwitexpressie en winst van het aantal kopieën van het *ALK* gen in het overgrote merendeel van de alveolaire rhabdomyosarcomen (80-90%) en tevens in een aanzienlijk deel van de embryonale rhabdomyosarcomen (30-50%). Hiermee vormt het een potentieel doelwit voor nieuwe therapieën. Bovendien correleerde het aantal kopieën van het *ALK* gen met ALK eiwitexpressie in het hele cohort. Translocaties van het *ALK* gen werden niet gezien. Tevens identificeerden we één nooit eerder gevonden mutatie, en deleties van hele exonen in het messenger RNA (mRNA) van 7/43 tumoren evenals in één van de vier onderzochte cellijnen. Het koppelen van de *ALK* gen en eiwitexpressie status met de klinische parameters en overleving, toonde aan dat specifieke winst van het *ALK* gen gerelateerd was aan de aanwezigheid van metastasen op afstand en daarmee een slechtere overleving in het embryonale rhabdomyosarcoom.

Hoofdstuk zes beschrijft een studie die focust op zowel de insulin-like growth factor 1 receptor (IGF-1R) en ALK als potentiële doelwitten voor gecombineerde therapie in het rhabdomyosarcoom. Expressie van IGF-1R werd zowel gezien in het alveolaire (72%) als in het embryonale rhabdomyosarcoom (62%). Opmerkelijk hierbij was de observatie van nucleaire IGF-1R expressie in ongeveer 10% van de tumoren, waarbij dit bovendien gerelateerd was aan een slechtere overleving in het embryonale subtype. Co-expressie van beide receptoren werd

gevonden in 68% van het alveolaire en in 32% van het embryonale subtype. Gecombineerde remming van deze beide receptoren werd daarom onderzocht in rhabdomyosarcoom cellen *in vitro* (embryonale cellijnen Rh18 en RD, alveolaire cellijnen Rh30 en Rh41). Remming van de IGF-1R (met R1507) resulteerde in een evidente afname van groei in de alveolaire cellijn Rh41. De ALK remmer NVP-TAE-684 resulteerde in een afname van groei in de alveolaire cellijnen Rh30 en Rh41, en in mindere mate ook in de embryonale cellijnen Rh18 en RD. Combinatie behandeling kon worden getest in cellijn Rh41, waarbij een synergistisch effect van simultane remming van IGF-1R en ALK werd gezien. Al deze resultaten bij elkaar opgeteld, concluderen we dat ALK receptor remming, evenals gecombineerde remming van ALK en IGF1R verder moet worden onderzocht, en vooral veelbelovend kan zijn in patiënten met een alveolair rhabdomyosarcoom en in patiënten met een embryonaal rhabdomyosarcoom met ongunstige prognose.

In **hoofdstuk 7** beschrijven we de huidige kennis met betrekking tot de behandeling van kinderen, adolescenten en (jong) volwassenen met een rhabdomyosarcoom. We geven een gedetailleerd overzicht waarin we de overeenkomsten en verschillen beschrijven tussen kinderen en volwassenen met een rhabdomyosarcoom, waarbij we focussen op epidemiologie, tumor biologie, diagnostiek, behandeling en de inclusie in klinische trials met potentiële nieuwe behandelingen. De constateringen die hieruit voortkomen, leiden ons naar de beschrijving van een concept voor wereldwijde samenwerking en centralisatie van zorg voor patiënten met een rhabdomyosarcoom ongeacht de leeftijd bij diagnose. Hiermee beogen we de vooruitgang in wetenschappelijke inzichten en de vergemakkelijking van de introductie van nieuwe therapieën in de toekomst te bewerkstelligen.

APPENDICES

CURRICULUM VITAE

Carlijn van Gaal werd geboren op 21 oktober 1984 te Enschede. In 2002 behaalde zij het VWO-diploma aan het Twents Carmelcollege. Vanaf 2002 studeerde zij geneeskunde aan de Rijksuniversiteit van Groningen. In 2005 kreeg zij een proefproject met bijbehorend studentship op het onderzoek "leeftijd als prognostische factor in patiënten met een rhabdomyosarcoom", wat uiteindelijk leidde tot honorering van het MDPhD project (2006) 'Pathways and crossroads in adolescent and young adult ("AYA") cancer, with emphasis on rhabdomyosarcoma", beschreven in het huidige proefschrift onder leiding van promotoren prof. dr. W.T.A. van der Graaf en prof. dr. E.S.J.M. de Bont en co-promotor dr. Y. Versleijen-Jonkers. In 2007 behaalde zij haar doctoraal examen geneeskunde 'cum laude'. Zij liep het eerste jaar coschappen in het Universitair Medisch Centrum Groningen, waarna zij het jaar daaropvolgend coschappen liep in het Martini Ziekenhuis te Groningen. Het keuze-coschap werd gevolgd op de kinderafdeling van het Radboud Universiteit Nijmegen Medisch Centrum van januari tot juni 2009. Op 17 juni 2009 behaalde zij het artsexamen. Gedurende haar studie wist zij zich bovendien in te zetten voor vele zaken buiten haar opleiding zoals het geven van onderwijs in de bachelor fase geneeskunde (2006-2009), de International Federation of Medical Students Association (IFMSA, 2003-2005) en de organisatie van het International Student Congres Of Medical Sciences (ISCOMS 2005). Na de laatste coschappen vervolgde zij haar promotietraject aan het Radboud Universiteit Nijmegen Medisch Centrum van juni 2009 tot juli 2011 waarbij nauw contact met promotor prof. dr. E.S.J.M. de Bont in het Universitair Medisch Centrum Groningen bleef bestaan. Zij ontving in 2011 een Merit Award van de ASCO Conquer Cancer foundation voor het abstract 'Anaplastic lymphoma kinase (ALK) in rhabdomyosarcoma', ingediend voor de American Society of Clinical Oncology (ASCO) annual meeting 2011 in Chicago, Illinois. In 2012 kreeg zij een travel grant toegewezen voor het abstract "Simultaneous targeting of the Insulin-like Growth Factor 1 Receptor (IGF-1R) and Anaplastic Lymphoma Kinase (ALK) receptor in embryonal and alveolar rhabdomyosarcoma" ingediend voor de European Society for Medical Oncology (ESMO) meeting 2012 in Wenen. Vanaf juli 2011 tot juli 2012 werkte ze als arts-assistent op de afdeling kinderchirurgie van het Academisch Medisch Centrum (AMC) te Amsterdam. Vanaf september 2012 tot op heden werkt zij als arts-assistent algemene chirurgie in het Sint Antonius ziekenhuis te Nieuwegein. Zij ambieert een opleiding tot (kinder)chirurg.

LIST OF PUBLICATIONS

The impact of age on outcome of embryonal and alveolar rhabdomyosarcoma patients. A multicenter study.
J.C. van Gaal, W.T. van der Graaf, B. Rikhof, Q.G. van Hoesel, S. Teerenstra, A.J. Suurmeijer, U.E. Flucke, J.L. Loeffen, S. Sleijfer, E.S. de Bont.
Anticancer Res. 2012 Oct;32(10):4485-97.

Cancer in adolescents and young adults (15-29 years): a population-based study in the Netherlands 1989-2009.
K.K. Aben, J.C. van Gaal, N.A. van Gils, W.T. van der Graaf, G.A. Zielhuis.
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J.C. van Gaal, E.S. de Bont, S.E. Kaal, Y.M. Versleijen-Jonkers, W.T. van der Graaf.
Crit Rev Oncol Hematol. 2012 Jun;82(3):259-79.

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J.C. van Gaal, U.E. Flucke, M.H. Roeffen, E.S. de Bont, S. Sleijfer, A.M. Mavinkurve-Groothuis, A.J. Suurmeijer, W.T. van der Graaf, Y.M. Versleijen-Jonkers.
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An infant with unexplained epilepsy.
J.C. van Gaal, R. Petru, L.T. Sie
Nederlands Tijdschrift voor geneeskunde. 2010;154:A2420

Behandeling van sarcomen bij adolescenten en jongvolwassenen: de prijs op termijn.
A.C. van de Luijtgaarden, S. E. Kaal, H.W. Schreuder, J.C. van Gaal, W.T. van der Graaf,
Nederlands Tijdschrift voor Oncologie, 2009, 6, 211 - 217.

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J.C. van Gaal, E. Bastiaannet, M. Schaapveld, R. Otter, J.C. Kluin-Nelemans, E.S. de Bont, W.T. van der Graaf.
Ann Oncol. 2009 Feb;20(2):365-73.

ABSTRACTS & PRESENTATIONS

Simultaneous targeting of the Insulin-like Growth Factor 1 Receptor (IGF-1R) and Anaplastic Lymphoma Kinase (ALK) receptor in embryonal and alveolar rhabdomyosarcoma. Abstract. Poster session ESMO 2012, Vienna, Austria. Travel grant winning abstract.

Anaplastic lymphoma kinase (ALK) in Rhabdomyosarcoma. Abstract. Poster Discussion Session ASCO annual meeting 2011, Chicago, Illinios. Merit award winning abstract.

Een zuigeling met onbegrepen epilepsie -Bewust bewusteloos-. Oral presentation, annual meeting Dutch association for children's neurology, 23rd Arpil 2010, De Hague, The Netherlands.

Cancer in adolescents and young adults 1989-2006 in the Netherlands. Poster presentation. Werkgroep epidemiologisch onderzoek Nederland (WEON), 10-11 June 2010, Nijmegen, the Netherlands.

Age as a prognostic factor for outcome in rhabdomyosarcoma (RMS) patients (pts).
Poster Discussion ASCO annual meeting 2008, Chicago, Illinios.

Cancer in adolescents and young adults (AYA) in the Northern Netherlands 1989-2003.
Poster Presentation Symposium for young research students/PhD's Training Upcoming Leaders in Pediatric Sciences (TULIPS), Dutch association of Paediatrics, 4th of October 2008, Veldhoven, The Netherlands.

Cancer in adolescents and young adults (AYA) in the Northern Netherlands 1989-2003.
Poster Presentation ASCO 2007, Chicago, Illinios.

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Vrienden en vriendinnen, in het bijzonder mijn roeiploeg Scylla, de clubacht 2005 der AGSR Gyas, ISCOMS Groningen, mijn hockeyteams bij GCHC, NMHC en Fletiomare, zonder jullie (sportieve) steun en afleiding op het veld en op het water was het nooit tot dit boekje gekomen. Helaas kan ik niet iedereen persoonlijk noemen, maar Judith van de poedersuiker ("is dat werkstukje van je nou al af""JA eindelijk"), samen de vierdaagse lopen, er werden heel wat trainingsuurjes samen doorgebracht voor die mooie prestatie in 2010. Kitty, al heel wat jaartjes vriendinnen, soms wat intensiever, soms wat minder intensief maar altijd goed. Clare en Naomi, geneeskunde buddies vanaf dag 1, en still going strong. Marloes, nu dokter in Afrika, we zien elkaar wat minder maar dat deert niet. Menno, mooie zeilweekenden! Korries (Nine, BJ, Wouter), mijn oude huisgenoten uit de Korreweg in Groningen, en huisgenoten en vrienden Bart en Marieke van de van Broeckhuysenstraat, wat een geweldige tijd heb ik daar met jullie gehad en jullie hebben me altijd gesteund, dankjulliewel!

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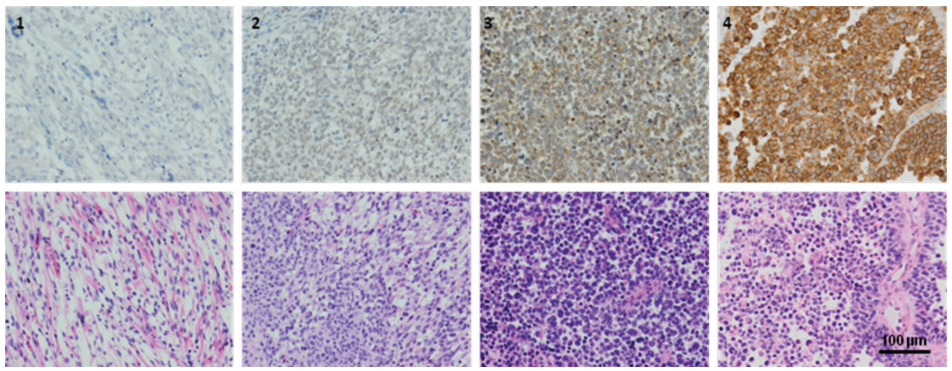
Lieve Michiel, bedankt voor je respect en vooral voor al je liefde die je me geeft. Je bent een rustpunt voor me en mijn rots in de branding. Je bent (een beetje) speciaal.
Met jou wil ik nog duizend dromen verwezenlijken!

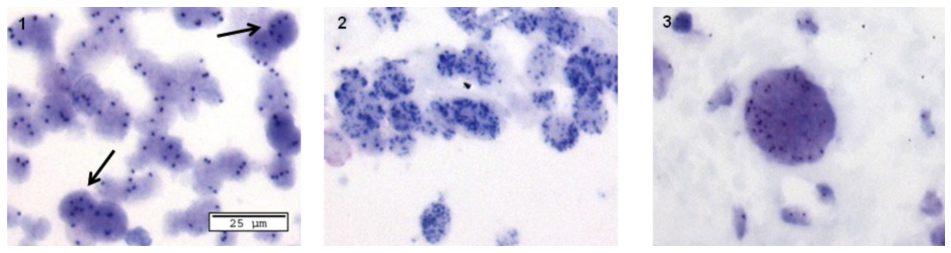
ADDITIONAL COLOR FIGURES

Chapter 5 Figure 1A
ALK protein expression by IHC with the ALK1 antibody (DAKO) in RMS.



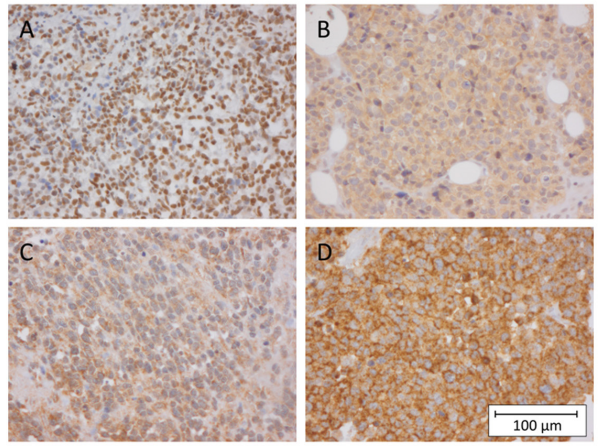
Upper row: 1. ALK negative staining in ERMS, 2. ALK weak staining inERMS, 3. ALK moderately strong staining in ARMS, 4. ALK strong staining in ARMS. Lower row: corresponding heamatoxylin and eosin (H&E) staining. Magnification: 20x

Chapter 5 Figure 1B
Chromogenic *in situ* hybridization (CISH) of the ALK gene in RMS.



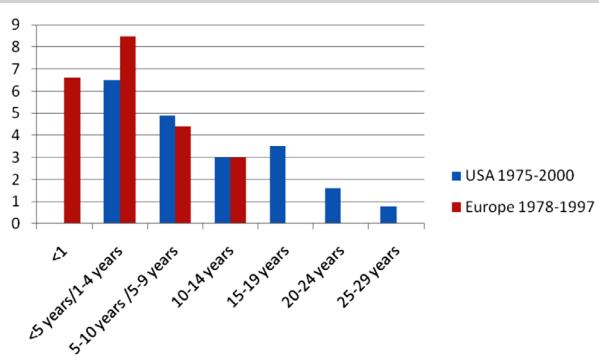
1. low level ALK gain up to 6 copies per nucleus (marked by black arrows) 2. Primary ARMS showing ALK amplification in all cells 3. Amplification of the ALK gene with >30 ALK copies in an ERMS lymph node metastasis during treatment. Magnification: 40x.

Chapter 6 Figure 1
Immunohistochemistry of the ALK and IGF-1R receptor



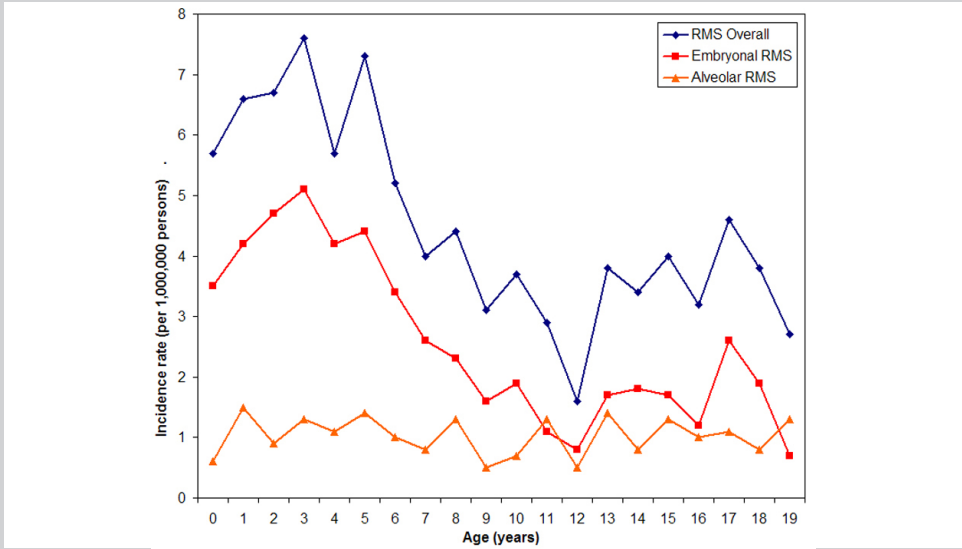
Immunohistochemical staining (magnification 20x), A eRMS displaying very strong predominant nuclear IGF-1R staining; B aRMS displaying very strong cytoplasmic IGF-1R staining; C eRMS displaying strong cytoplasmic ALK staining; and D aRMS displaying very strong cytoplasmic ALK staining.

Chapter 7 Figure 1a
Incidence rates of all subtypes of RMS in patients 0-29 years of age.



*Data regarding patients with RMS >14 years in Europe were not available

Chapter 7 Figure 1b
Incidence of ERMS and ARMS in the childhood population.



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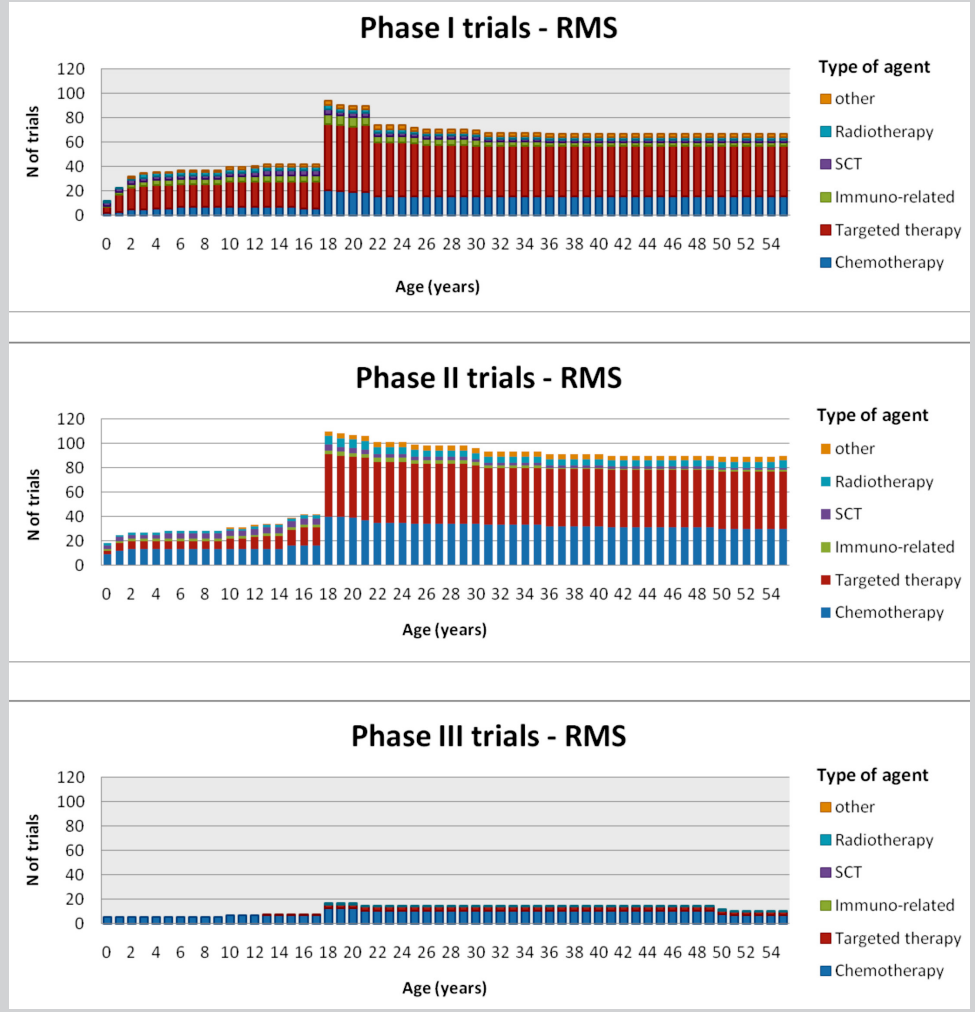
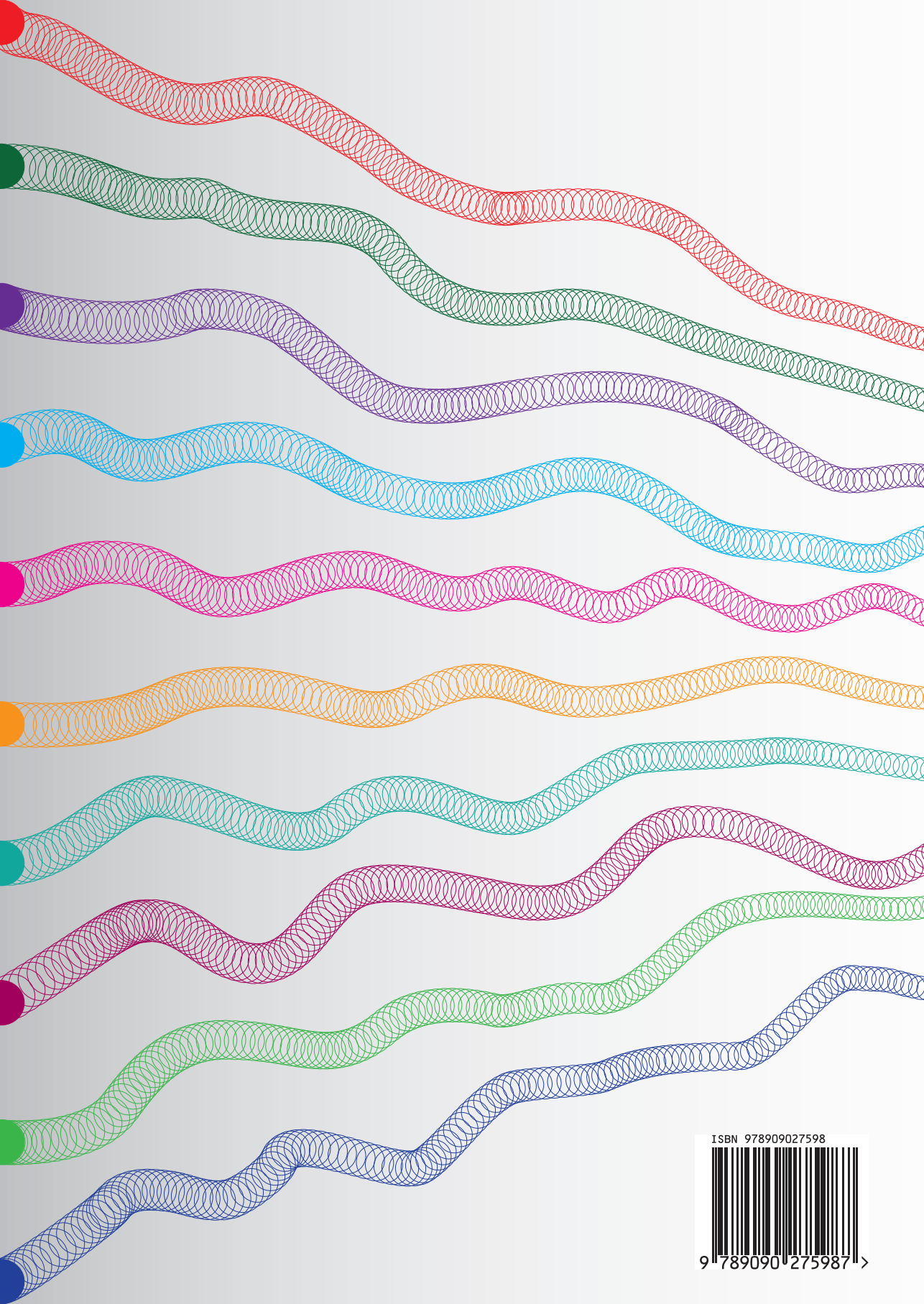
Chapter 7 Figure 2
Clinical trial availability explicitly for sarcoma/RMS



Clinical trial availability explicitly for sarcoma/RMS by phase (I/II/III) , type of intervention (chemotherapy, targeted therapy, immune-related therapy, stem cell transplantation (SCT), Radiotherapy, and other) and for each year of age 0-55 years over the ten-year period 1st January 2001- 1st January 2011. It is of note that our search on sarcoma/rhabdomyosarcoma results in a major underestimation of phase I trials available in these patients of adult age, because this indication is often not mentioned explicitly in study description.



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