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CORSO DI DOTTORATO DI RICERCA IN: ONCOLOGIA CLINICA E SPERIMENTALE E IMMUNOLOGIA

CICLO XXI

TREATMENT CHALLENGES AND RISK STRATIFICATION FOR MELANOMA AND SARCOMAS

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Contents

PhD thesis abstract (English)

Background

Patients with primary soft tissue sarcomas (STS) with negative prognostic features or primary cutaneous melanoma (CM) spreading to the regional lymph node (LN) are at risk of disease progression. This work is aimed at improving risk stratification for patients with high-risk STS and CM.

Material and Methods

In STS, we retrospectively investigated patients with primary tumours enrolled in two randomised controlled trials (RCT), one showing non-inferiority of three versus five perioperative chemotherapy cycles and one failing to demonstrate a survival advantage for adjuvant chemotherapy, using the prognostic nomogram Sarculator. Finally, tumour dedifferentiation, a prognostic feature embedded in this nomogram, was analysed in 20 patients with dedifferentiated (DD) retroperitoneal liposarcoma using RNA-sequencing.

In CM, we investigated the prognostic value of sentinel LN (SLN) and non-SLN in a retrospective study (N=1,538). We examined immunohistochemistry (IHC)-detected lymphangiogenesis in patients with scalp CM (N=156). Finally, we tested whether IHC-detected markers of lymphangiogenesis and endothelial cell proliferation were associated to SLN and non-SLN metastasis in a retrospective analysis of 122 primary CM and SLN specimens.

Results

In high-risk STS, the Sarculator stratified three distinct overall survival (OS) categories (P<0.001) in a RCT investigating two different perioperative chemotherapy schedules. Tumour response according to Choi criteria differed across these categories and was associated with survival (P<0.001). When we applied Sarculator and used the same prognostic categories to stratify prognosis of STS enrolled in a RCT that tested the prognostic value of adjuvant doxorubicin plus ifosfamide, we showed that patients with extremity and trunk wall STS that fell in high and intermediate predicted OS categories did not show a survival benefit when treated with the study adjuvant chemotherapy. Conversely, patients with low predicted OS who received adjuvant chemotherapy had longer disease-free survival and OS.

We also investigated tumour dedifferentiation showing that increased cell proliferation and reduced differentiation marked the transition from well differentiated (WD) to DD components. We investigated rhabdoid DD (R-DD) and myogenic DD (M-DD) and found suppression of genes related to inflammation and vasculature development in R-DD versus WD. Also, we identified an increase of genes related to immune and inflammatory response in the M-DD, a result that was validated using IHC markers CD4, CD34, CD163, and CD209.

In SLN-positive CM patients, presence of non-SLN metastasis was an adverse prognostic factor for survival (P<0.001). AJCC TNM N stages were further stratified by non-SLN metastasis showing similar risk between patients with 1 positive SLN and 2-3 positive SLN with negative non-SLN (P<0.001). In scalp melanoma the degree of peritumoral and intratumoral blood vessel density (BVD) was greater than lymphatic vessel density (LVD) and ulceration was the only factor independently associated with intratumoral and peritumoral BVD. We then investigated several features of lymphangiogenesis in primary melanoma and SLN and found that peritumoral LVD in primary melanoma was associated with SLN metastasis, while proliferation index of lymphatics in the SLN was associated with

metastatic spread to non-SLN. Also, intra/peritumoral blood/lymphatic vessel density in SLN metastasis was associated with patient survival.

Conclusions

In primary STS we showed the value of perioperative chemotherapy for higher risk patients and investigated the role of tumour differentiation. In CM, we demonstrated that SLN may act as a barrier for metastasis spreading through lymphatics a process that may be at least partially explain by lymphangiogenesis. These information may have implications for adjuvant/neoadjuvant treatments.

Riassunto tesi di dottorato (italiano)

Introduzione

I pazienti con sarcomi dei tessuti molli (STM) con caratteristiche prognostiche negative o melanoma cutaneo (MC) con metastasi ai linfonodi regionali (LR) sono a rischio di progressione di malattia. Lo scopo di questa tesi è di migliorare la stratificazione prognostica dei pazienti con STM e MC.

Materiali e metodi

Abbiamo studiato retrospettivamente con il nomogramma Sarculator due studi randomizzati (RCT) che hanno valutato pazienti con STM primitivi, uno ha dimostrato la non-inferiorità di 3 o 5 cicli di chemioterapia periopererատoria e uno l'inefficacia della chemioterapia adiuvante. Infine, la dedifferenziazione tumorale, un parametro incluso nel modello prognostico utilizzato, è stata analizzata in 20 pazienti con liposarcoma dedifferenziato (DD) retroperitoneale mediante RNA-seq. Per quanto riguarda il MC, abbiamo retrospettivamente studiato il valore prognostico del linfonodo sentinella (LS) e dei linfonodi non sentinella (LNS) in 1.538 pazienti. Abbiamo studiato in immunoistochimica la linfangiogenesi di MC prima in 156 pazienti con MC dello scalpo e poi in 122 pazienti con MC sottoposti a biopsia del LS (BLS).

Risultati

Nei pazienti con STM ad alto rischio, il Sarculator identifica tre categorie di sopravvivenza (P<0.001) nel RCT che ha confrontato due modalità di trattamento. La risposta tumorale secondo Choi si modificava nelle varie categorie e comunque si associava alla sopravvivenza (P<0.001). Il Sarculator e le stesse categorie prognostiche sono anche state applicate allo RCT che ha valutato la terapia adiuvante con doxorubicina e ifosfamide, dimostrando che i pazienti con STM degli arti e del tronco beneficiavano della chemioterapia solo quando appartenenti alla categoria con sopravvivenza inferiore e non a quelle a sopravvivenza intermedio-alta. Abbiamo anche studiato la dedifferenziazione tumorale, dimostrando che una aumentata proliferazione cellulare ed una ridotta differenziazione definivano una netta transizione dalla componente ben- a quella de-differenziata. Inoltre abbiamo identificato la soppressione dei geni associati all'infiammazione e all'angiogenesi nei tumori con DD rabdomiosarcomatosa. Infine, abbiamo trovato un aumento dei geni associati alla risposta immune ed infiammatoria nei tumori con DD miogenica, un risultato che abbiamo validato qualitativamente con i marcatori CD4, CD34, CD163 e CD209.

Nei pazienti con MC e LS positivo la presenza di metastasi ai LNS è un fattore prognostico (P<0.001). La stadiazione TNM AJCC N è stata ulteriormente stratificata considerando le metastasi ai LNS e dimostrando una sopravvivenza simile tra pazienti con 1 LS positivo e 2-3 LS positivi in presenza di LNS negativi (P<0.001). Nei melanomi dello scalpo abbiamo dimostrato che la densità vascolare peri- e intra-tumorale è più rappresentata della densità linfatica e che si associa alla presenza di ulcerazione. Più in generale, in 122 pazienti con MC che erano stati sottoposti a BLS abbiamo visto che la densità linfatica perivascolare era associata alle metastasi del LS e che l'indice di proliferazione linfatico nel LS si associava alle metastasi ai LNS. Inoltre, la densità vascolare e linfatica intra- e peri-tumorale nel LS si associava alla prognosi dei pazienti.

Conclusioni

Abbiamo dimostrato il valore della chemioterapia perioperatoria per i pazienti con STM a rischio maggiore e apportato indicazioni sulla differenziazione tumorale. Nel MC abbiamo dimostrato che il LS può fungere da barriera alla progressione delle metastasi, un processo che può essere governato dalla linfangiogenesi. Queste informazioni possono avere un ruolo per la selezione dei pazienti ad alto rischio con STM o MC per trattamenti adiuvanti e neoadiuvanti.

CHAPTER 1

Introduction

Soft tissue sarcomas (STS) are a rare family of tumours that can arise anywhere in the body. The majority originate in the extremities (60%), where they occur most often within the my miofascial compartment, followed by the abdomen and retroperitoneum (20%),abdominal/thoracic wall (15%), and head and neck (5%). Incidence has been estimated at approximately five to six new cases per100,000 population each year [1-4]. The five-year overall survival rate for soft tissue sarcomas (at all stages) is approximately 50% to 60% [5, 6]. The most important prognostic factors are tumour location, histology, grade and size. Deep-seated tumours, defined as tumours located underneath muscular fascia, generally exhibit more aggressive behaviour, whilst superficial tumours, defined as tumours located above the muscular fascia, can lead to significant physical impairment before and after treatment [5, 6]. Overall, sarcomas include more than 70 different histological entities with liposarcoma (20%), leiomyosarcoma (15%), and undifferentiated pleomorphicsarcoma (15%) the most commonly diagnosed [7]. Different sub-types can also display differential degrees of malignancy [8-10] For instance, low-grade liposarcomas classically recur locally after surgery but only occasionally metastasise [11, 12]. Conversely, patients with high-grade leiomyosarcomas often have systemic disease at presentation [13]. An-other example is synovial sarcoma, a lesser common histology, that accounts for approximately 5% of all sarcomas, which have a significantly better response to chemotherapy and

better survival rates compared to other sarcomas [14]. Tumour grade is another powerful predictor of outcome [15, 16]. For instance, soft tissue sarcomas localised in the extremities exhibiting low and high gradehave approximately 90% and 60% survival rates, respectively. Finally, smaller tumours (< 5 cm) in the extremities have a 10-yearsurvival rate of 80%, which is double th e survival rate of tumours larger than 10 cm. The clinical decision-making process is multifaceted and should always be conducted in a multidisciplinary fashion [17 18, 19]. Primary tumours are treated with surgery, which is usually coupled with radiotherapy when there is a significant risk of local tumour relapse [20]. Perioperative systemic therapies have been tested to reduce risk of metastatic spread after surgery with or without radiotherapy in several randomized controlled trial (RCTs) [21]. Anthracycline-based regimens have marginal survival benefit to patients, ranging between 5 and 10%, which has been considered unsatisfactory particularly when balanced against meaningful toxicity [22]. As a results current guidelines recommend perioperative chemotherapy as an option to be discussed with patients with sarcoma showing high/risk features in the context of a challenging evidence [17].

In the last years, growing evidence suggested that other agents in addition to anthracyclines and ifosfamide may have antitumour activity in patients with metastatic STS [23]. Trabectedin showed effectiveness in high-grade myxoid liposarcomas (HG-MLS) [24], gemcitabine with or without docetaxel and dacarbazine or their combination for leiomyosarcoma (LMS)[25], ifosfamide for synovial sarcoma (SS) [26], etoposide for malignant peripheral nerve sheath tumors (MPNST) [27], and gemcitabine plus docetaxel in undifferentiated pleomorphic sarcoma (UPS) [28]. In light of these findings, the ISG-STS-1001 study (ClinicalTrials.gov Identifier: NCT01710176) compared three cycles of the epirubicin plus ifosfamide regimen tested previously (standard arm) with three cycles of an histology-tailored regimen (experimental arm), in a homogeneous group of the same high-risk primary STS (i.e., UPS, HG-MLS, SS, MPNST and LMS) arising in the extremities and trunk wall [29]. This RCT was stopped after accruing 286 patients, following the recommendation of the Independent Data Monitoring Committee when the third futility analysis, as the previous ones, identified a clear disease-free (62%

vs 38%) and overall (89% vs 64%) survival benefit for patients treated with three cycles of epirubicin and ifosfamide over patients treated with the histology-driven chemotherapy schedules, at a median follow-up of 12 months. Pre-planned subgroup analysis revealed that HG-MLS was the only histology where the experimental treatment trabectedin was as effective as the standard regimen.

Cutaneous melanoma is one of the deadliest forms of skin cancer. According to epidemiological data provided by the International Agency for Research on Cancer (IARC), its worldwide incidence in 2008 was estimated to be 199,627 new cases, with 46,372 deaths [30]. In the USA, cutaneous melanoma ranked fifth in men (44,250 new cases per year, representing 5% of all cancers) and sixth in women (32,000 new cases per year, representing 4% of all cancers) among all tumour histotypes [31]. The highest incidence is observed in Australia and New Zealand where melanoma is the fourth most commonly diagnosed cancer [32]. Melanoma is potentially curable in the early stages with the surgical removal of the primary tumour [33-37].

Once melanoma metastasises (i.e. spreads to lymph nodes, distant organs or both) due to its intrinsic biological aggressiveness and its typical resistance to medical therapy (both chemotherapy and radiotherapy), survival is poor or very poor, with a median overall survival of 24 months for those with American Joint Committee on Cancer (AJCC) TNM stage IIIC disease (unresectable lymph node metastasis), and nine months for people with AJCC TNM stage IV disease (distant metastasis) [38-41]. Overall, fewer than 35% (AJCC TNM stage IIIC) and 12% (AJCC TNM stage IV) of these people are still alive five years after their diagnosis [38-41]. Metastatic cutaneous melanoma (unresectable AJCC TNM stage IIIC and stage IV) was usually treated with systemic medical therapy while in the last decade new targeted therapies and immune check point inhibitors have been introduced [42, 43]. Patient survival with chemotherapy was dismal (median overall survival usually ranges between 10 and 16 months [38]) and these new drugs have resulted in a significant proportion of long-term survivors which can reach 20% of metastatic patients [44-52]. Surgery is feasible only in very few select cases showing a very limited tumour burden [53, 54], and radiotherapy is considered for symptom

palliation [55]. New insights into the prognosis of people with metastatic melanoma come from molecular profiling of primary tumour and distant metastases [56, 57]. Molecular studies have identified aberrant activation of the mitogen-activated protein kinase (MAPK) pathway and mutations in proteins along the RAS-RAF-MEK-ERK pathway in cutaneous (50% BRAF-mutated, 15% NRAS-mutated, and up to 17% c-Kit-mutated in chronically sun damaged people) and mucosal melanoma (11% BRAF-mutated, 5% NRAS-mutated, 21% c-Kit-mutated) [58]. Determination of the mutational status of a melanoma enables identification of those who may be suitable for new treatments, such as BRAF and c-Kit inhibitors. These information together with efficacy of immune check point inhibitors let to the approval of MAPK and immune checkpoint for the treatment of metastatic melanoma as well as in the adjuvant setting. Similarly, the above mentioned immune checkpoint inhibitors, and specifically the first-in-class anti-CTLA4 monoclonocal antibody ipilimumab [45] and the anti-PD1 nivolumab [46, 47, 49, 59, 60] and pembrolizumab [48, 61] gained approval in the metastatic setting where they have replaced standard chemotherapeutics [42] and then as adjuvant therapies for patients with lymph node metastasis who underwent CLND [60, 62, 63], outdating the use of interferon alpha [64, 65] for these patients.

PhD project overall aim

This PhD project targets the population of patients with STS and skin melanoma characterised by high-risk features. Patients with primary high-risk sarcoma, that are currently identified as those having tumours showing worrisome histologic features (deep location, large size, and high grade) will be investigated. These analyses will be parallelized by those conducted on high-risk cutaneous melanoma, identified as those patients harbouring sentinel lymph node metastasis.

Analysed will be aimed at improving risk stratification for these patients who are characterised by wide variations in their prognosis despite being labelled as high-risk. The ultimate goal of these prognostic stratification is to identify the best treatment options for these patients.

PhD project outline

The first section of this PhD thesis will focus on soft tissue sarcoma. Firstly, there will be an introduction to current definitions of high-risk patients, available therapies and opportunities for patient risk stratification (Chapter 2). Then, a risk assessment tool, the Sarculator, will be fitted with data of two randomised trials investigating chemotherapy for high-risk primary tumours identifying variations in prognosis and tumour response (Chapter 3) and patients more likely to benefit from treatment (Chapter 4). Tumour differentiation, a major determinant of patient outcomes in soft tissue sarcoma, will be analysed through in-depth genome sequencing analysis to characterise tumour with different malignant behaviours (Chapter 5).

The second section of this PhD thesis will then investigate patients with lymph node metastasis from cutaneous melanoma. Initially, an introduction will be provided to prognosis and therapies available for these patients (Chapter 6). Prognosis of patient affected by sentinel lymph node metastasis will be detailed focusing on presence of metastasis in lymph nodes beyond the sentinel lymph node, the so called non-sentinel lymph nodes (Chapter 7). Lymphangiogenesis will be hypothesized as a major player in the progression of melanoma through the lymphatics and to distant sites and will be analysed extensively in a group of patients with scalp melanoma (Chapter 8), a tumour location characterised by specifically high vascular infiltration, and in a broader group of patients were lymphangiogenesis has been assessed both in primary tumours and sentinel lymph node (Chapter 9).

A brief background, used methods, achieved results, and conclusions will be summarised in each chapter. Some of the chapters of this thesis have been already published in the literature (Chapters

2 [66], 3 [67], 6-8 [68-70]), another has been submitted for publication (Chapter 4), the remaining two are to be submitted in the future (Chapters 5 and 9). An overall conclusion of the PhD thesis with future perspectives have been presented in a final section (Chapter 10).

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CHAPTER 2

Neoadjuvant chemotherapy in soft tissue sarcomas: latest evidence and clinical implications

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Background

Soft tissue sarcomas (STS) are a group of rare tumours, accounting for more than 50 different subtypes, which can differ significantly in their disease presentation, response to currently available treatments, and risk of tumour progression.[1, 2] They account for about 1% of all solid tumours with 5 to 6 new cases every 100,000 people yearly.[3, 4] Sarcomas may develop at any age and in virtually all anatomic sites, making them a challenge for medical and surgical oncologists.[5]

Despite the complexity of these tumours, few treatment options are available.[6] Surgery is the standard treatment for primary STS and is aimed at reaching negative tumour excision margins.[7, 8] Radiotherapy is considered before or after surgery to lower the risk of local recurrence in tumours with worrisome features such as large size or high grade histology. In some cases it may be used to reduce the extent of surgery..[9]

Several efforts have been put in place over time to improve quality of surgery, optimise radiotherapy schedules, and refine selection of patients for systemic perioperative treatments, leading to an improvement of patient survival.[10, 11]

Adjuvant systemic therapies have been tested to reduce risk of metastatic spread after surgery with or without radiotherapy in several randomized controlled trial (RCTs).[12] Anthracycline-based regimens using doxorubicine as main chemotherapeutic agent were used in early study, while more recent trials tested anthracycline combined with ifosfamide.[13] These treatment strategies offer a survival benefit to patients ranging between 5 and 10%, which has been considered unsatisfactory particularly when balanced against high-grade toxicity.[13]

Neoadjuvant chemotherapy is increasingly used for patients with locally advanced and high-risk primary sarcomas[14, 15] and has several advantages over adjuvant systemic treatments. Firstly, when administered preoperatively chemotherapy can improve chances of performing conservative surgery, resulting in sparing nerves, vessels, and muscle groups with the ultimate aim of reducing the need for amputation and preserving muscles function, especially for extremity STS. In the

retroperitoneum, neoadjuvant therapy can reduce the need for extensive multivisceral resection. . Another potential benefit of neoadjuvant chemotherapy is an improvement in achievement of negative histologic margins, which are associated with a reduced risk of local recurrence and, to a lower extent, better survival. Remarkably, when neoadjuvant chemotherapy is delivered with radiotherapy these aims can be reached also indirectly, as chemotherapy acts as a radiosensitizer. Importantly, neoadjuvant chemotherapy can potentially improve patient survival directly through eradication of micrometastatic disease. In this regard, patients can experience significant post-operative complications and delays in starting on adjuvant systemic chemotherapy are common for sarcoma patients. Administering systemic treatments preoperatively can overcome this issue. Finally, pathological response to preoperative chemotherapy can inform decision of future therapeutic strategies.

Despite these theoretical advantages, the use of neoadjuvant chemotherapy for patients with sarcomas is limited by several issues, such as the well know heterogeneity of these tumours, patient old age and comorbidities, and challenges in identifying high-grade tumours at preoperative core biopsy.[16] Also a limited number of drugs, mainly cytotoxic agents, are available for patients with early stage STS.[17]

This review will present the latest evidence and clinical implications for neoadjuvant chemotherapy in high-risk STS, emphasising the importance of improving patient risk stratification for identifying those who are likely to benefit from available therapies through new prognostic tools, such as AJCC TNM staging system and nomograms. Also, this review will illustrate the limitations of applying neoadjuvant therapy to all patients with STS together with future perspectives.

Latest evidence and clinical implications

Patient risk stratification

Identification of patients who are at high-risk for relapse and may respond to currently available treatments can maximize effectiveness of preoperative chemotherapy and spare treatment-related adverse events to patients who are unlikely to respond. Staging of STSs, which is the most important tool to stratify patient prognosis, have been a long standing issue. The sixth and seventh edition of the AJCC staging manual account for only a limited number of prognostic information, including tumour size, grade, and location with respect to the superficial muscular fascia. The clinical value of this classification has been questioned particularly for retroperitoneal tumours[18] as these tumours are always deep seated and the 5cm size cut-off does not apply as these tumours are unlikely to be diagnosed when small. Experts agreed that prognostic tools need to account for differences across sarcoma histologies and primary tumour sites.[19, 20]

The just released eight edition of the AJCC TNM staging manual for STSs represent an unprecedented change in risk stratification of patients with sarcomas.[21] The manual includes at least two major changes: a specific staging for head and neck, limb and trunk, and retroperitoneal sarcomas, and inclusion of a nomogram for the prognostic assessment of patients with retroperitoneal sarcomas.[22] Size to define T stage categories are now tailored on the different primary tumour sites. Head and neck sarcomas are classified as T1, T2, T3, and T4 when their size is 2 cm or less cm, 2 to 4 cm, greater than 4 cm, and involving adjacent structures. Retroperitoneal, extremity and trunk tumours sarcomas are classified as T1, T2, T3, and T4 when their size is 5 cm or less cm, 5 to 10 cm, 10 to 15 cm, and greater than 15 cm. However, these cut-off values have some limitations. Despite acknowledging that size is a prognostic factor, they are arbitrary and most of patients with retroperitoneal STS have tumours larger than 15cm.

These issues are overcome by predictive and prognostic tools, which have advantages over standard AJCC TNM staging system as they inform physician choice on treatment to be performed on a single

patient, based on risk of disease progression and, ultimately, death.[23] Nomograms are becoming widely used among surgical and medical oncologists dealing with sarcomas.[24] The first nomogram, which was developed in 2002 by Kattan et al[25] and subsequently validated,[26-28] predicts the likelihood of being alive within 12 years from initial surgery. However, this tool included histological entities, such as malignant fibrous histiocytoma, that are no longer included in WHO sarcoma classification and inclusion of all tumour sites. Afterwards, several nomograms have been developed, including histology-specific nomograms for liposarcomas[29] and synovial sarcomas,[30] site-specific nomograms for both extremity[31, 32] and retroperitoneal sarcomas,[22, 33, 34] and uterine leiomyosarcomas.[35] Among these nomograms, the 'Sarculator', http://www.sarculator.com/, is a free available online resource that embedded nomograms for retroperitoneal[22, 36] and extremity[32] sarcomas. This prognostic tool predict distant metastasis free and overall survival at 5 and 10 years after surgery of primary tumour for extremity STS and at 7 years for retroperitoneal tumours. Remarkably, information not currently considered in the AJCC TNM staging manual were included in the Sarculator. Additional factors included in this prognostic tool for retroperitoneal tumours were: age, completeness of resection, histology, and multi-focality. Interestingly, there is a U-shaped association between tumour size and prognosis with very large tumours behaving as smaller sarcomas. This reflects the analysed population which included patients who underwent surgery and were distant metastasis free. Clearly, when retroperitoneal sarcomas are amenable to surgical resection despite being large, tumour biology is likely to be indolent as a more aggressive lesion will already have metastasised.[37] In the nomogram for extremity sarcomas, STS histology was an independent prognostic factors while completeness of surgical resection did not correlate with survival. Also, age was a predictor only for overall survival and was not included in the prediction for distant metastasis-free survival. Remarkably, there was a greater influence for tumour histology compared to the retroperitoneal sarcoma nomogram. Vascular sarcomas, leiomyosarcomas, and synovial sarcomas showed the highest risk of progression to distant sites and patient death.

Conversely, myxoid liposarcomas, dedifferentiated liposarcomas, undifferentiated pleomorphic sarcomas and myxofibrosarcomas were associated with better outcomes. Also, completeness of surgery was not relevant and a linear relationship between size and survival observed.

There are challenges for using these models when selecting patients for neoadjuvant therapies. Firstly, they are based on features available after the pathological examination of the whole tumour. A nomogram for synovial sarcomas, which are among the most chemo-sensitive sarcoma histologies, is accurate in identifying patients who may benefit from cytotoxic chemotherapy based on preoperative biopsy.[30] Also, the association between higher risk of metastasis and greater response to chemoradiation is still to be proven. It seems that nomograms can predict the pathological response after chemotherapy in patients with retroperitoneal sarcomas, although effectiveness of this treatment modalities is still unproven for these patients[38]. Although there are limitations in applying these models to neoadjuvant therapy, it can give prognostic information and identify patients that could benefit from adjuvant therapy and by extension, neoadjuvant therapy as well.

Neoadjuvant chemotherapy

The effectiveness of systemic perioperative chemotherapy for patients with high-risk STS has been widely debated.[12, 39] Major phase II-III trials are reported in Table 1. An American trial randomised patients with large, high-grade, extremity STS to a regimen of preoperative chemotherapy consisting of mesna, adriamycin (doxorubicin), ifosfamide, and dacarbazine (MAID), and followed by resection and postoperative chemotherapy with or without radiotherapy (44 Gy).[40] This trial included 340 patients with either metastatic or unresectable soft tissue and bone sarcomas and showed that an improved response rate may be relevant in high-grade, borderline resectable lesions or pulmonary metastases, particularly in younger patients.

Table 1. Phase II/III Randomised Controlled Trials (RCT) of neoadjuvant chemotherapy for soft tissue sarcomas. CR: complete response; PR: partial response; SD: stable disease; LRFS: local relapse-free survival; DRFS: distant relapse-free survival; RFS: relapse-free survival; OS: overall survival.

Study0	Inclusion criteria	Treatment arms	No. of pts	CR/PR (%)	SD (%)	LRFS (%)	DRFS (%)	RFS (%)	OS (%)
Antman 1993[40]	Measurable metastatic or unresectable sarcomas.	**ARM A:** Doxorubicin 60mg/m2 and Dacarbazine 1000 mg/m2 days 1-4 q3 weeks x 3 cycles +/- surgery.	170	29 (17%)	69 (41%)	-	-	2-yr: <5%	3-yr: 10%
		ARM B: Doxorubicin 60mg/m2 and Dacarbazine 1000 mg/m2 on days 1-4, Ifosfamide 7,500 mg/m2 on days 1-3 and mesna 10,000 mg/m2 on days 1-4, q3 weeks x 3 cycles +/- surgery.	170	55 (32%)	49 (29%)	-	-	2-yr: <5%	3-yr: 22%
Gortzak et al 2001 [41]	Size ≥8cm, Or Grade 2-3	**ARM A:** Doxorubicin 50mg/m2 day 1, Ifosfamide 5gm/m2 day 1 q3 weeks x 3 cycles followed by surgery.	67	14 (28%)	26 (53%)	-	-	5-yr: 56%	5-yr: 65%
		ARM B: Surgery alone.	67	-	-	-	-	5-yr: 52%	5-yr: 64%
Gronchi et al 2012 [42] (updated 2016 [43])	High grade Deep location ≥5cm Extremity or trunk tumours	**ARM A:** Epirubicin 120mg/m2, Ifosfamide 9gm/m2 x 3 cycles +/- XRT followed by surgery.	160	36 (23%)	77 (48%)	10-yr: 91%	10-yr: 66%	10-yr: 56%	10-yr: 64%
		ARM B: Epirubicin 120mg/m2, Ifosfamide 9gm/m2 x 5 cycles +/- XRT, surgery perfomed after 3 cycles.	161	30 (19%)	92 (57%)	10-yr: 94%	10-yr: 63%	10-yr: 58%	10-yr: 59%
Gronchi et al 2017[45]	High grade Deep location	**ARM A:** Epirubicin 120mg/m2, Ifosfamide 9gm/m2 x 3 cycles +/- XRT followed by surgery.	144	NA	NA	46-mo: 86%	46-mo: 74%	46-mo: 62%	46-mo: 89%

	≥5cm Extremity or trunk tumours (UPS, MPNST, SS, Myxoid liposarcomas, and leiomyosarcomas)	**ARM B:** histology driven chemotherapy*.	142	NA	NA	46-mo: 85%	46-mo: 45%	46-mo: 38%	46-mo: 64%

** High grade myxoid liposarcomas: Trabectedin 1.3 mg/m2, given in 24-hour continuous infusion day 1 q3 weeks x 3 cycles; Leiomyosarcoma: Gemcitabine 1800 mg/m2 on day 1 and Dacarbazine 500 mg/m2 on day 1 q2 weeks; Sinovial sarcoma: high-dose Ifosfamide 14 g/m2 on days 1-14 days by means of an external infusion pump q4 weeks; MPNST: Etoposide 150 mg/m2/day, days 1-3 and Ifosfamide 3g/m2/day, days 1-3 q3 weeks; Undifferentiated Pleomorphic Sarcoma: Gemcitabine 900 mg/m2 on days 1-8 and Docetaxel 75 mg/m2 on day 8.*

A European phase III RCT enrolled 134 patients with resectable high-risk primary and recurrent STS. Patients were randomised to either surgery alone or three cycles of doxorubicin (50 mg/m intravenous bolus) and ifosfamide (5 g/m 24 h infusion) before surgery.[41] Although this treatment regimen was feasible and did not compromise performance of subsequent surgery, chemotherapy followed by surgical excision was not proven being more effective than surgery alone (5-year disease-free survival: 56 and 52%, respectively). This trial was burdened by important limitations. Firstly, both primary and recurrent tumours were considered. Also, definition of high-risk has been argued as not only high grade tumours were considered but also FNCLCC grade 1 tumours were included when presenting as a large mass ($\geq$ 8cm). Also, dosages for both doxorubicine and ifosfamide are lower than those used in other studies. Importantly, this RCT closed early due to slow accruals underlying issues in referral bias to specialised sarcoma centres.

Another study was performed by the Italian and the Spanish Sarcoma Groups (ISG and GEIS).[42, 43] The design of this study was based on results of a previous trial ran by the former group which investigated adjuvant epirubicin and ifosfamide, which were given using a 5 cycles schedule.[44] Although the trial was closed in advance because of an early major disease-free survival benefit for patients treated with adjuvant chemotherapy, analysis of data with longer follow-up did show a small non-significant disease-free and overall survival benefit. Again, patients with several different sarcoma histologies were considered together and pathology review was not consistently performed. Also, drugs dose in the last two cycles was significantly reduced. In light of these considerations, a new study was designed comparing three cycles of epirubicin (120 mg/m2) plus ifosfamide (9 g/m2) given preoperatively with five cycles of the same drugs given perioperatively (three neoadjuvant cycles followed by surgery and two further adjuvant cycles).[42, 43] This study did not identify any survival difference between these two treatment modalities and three chemotherapy cycles given preoperatively were deemed as effective as five cycles. These results were criticized for the lack of a control arm where patients would have been treated with surgery

alone. However, patients in the two treatment arms have similar prognosis to those treated with adjuvant chemotherapy in the first above mentioned study by Frustaci et al, suggesting, though indirectly, a potential superiority of combined chemotherapy and surgery over surgery alone. A possible effectiveness for a perioperative treatment is also supported by the observed association between complete response and prognosis, although this evidence could be burdened by a selection bias that may lead to greater tumour response and longer survival independently.

Despite these considerations, this trial did not resolve the long lasting issue of whether a perioperative treatment can improve survival of patients with high-risk sarcoma. The Italian and Spanish Sarcoma groups went on designing a further randomised trial (ISG-STS-1001, ClinicalTrials.gov Identifier: NCT01710176) which has compared epirubicin (60 mg/m2/day on days 1 and 2) and ifosfamide (3 mg/m2/day on days 1, 2, and 3) using the three cycles schedule which was tested in the previous study with histology-tailored therapeutic regimens for five different STS histologies. Three cycles of the following regimens were administered: 1) gemcitabine (900 mg/m2 on days 1 and 8) plus docetaxel (75 mg/m2 on day 8) in undifferentiated pleomorphic sarcoma; 2) trabectedin (1.3 mg/m2) in high-grade myxoid liposarcoma; 3) high-dose prolonged-infusion ifosfamide (14 g/m2, given in in 14 days) in synovial sarcoma; 4) etoposide (150 mg/m2/day on days 1, 2, and 3) plus ifosfamide (3 g/m2/day on days 1, 2, and 3) in malignant peripheral nerve sheath tumours; and 5) gemcitabine (1800 mg/m2 on day 1) plus dacarbazine (500 mg/m2 on day 1) in leiomyosarcoma.

This multi-centre study was conducted also with the support of the French and Polish Sarcoma Groups and enrolled 287 patients with high-risk STS of the trunk or extremities, from the five above mentioned histological subtypes, which represent approximately four-fifth of all STS arising in extremity and trunk wall.[45] The study was planned to enrol 350 patients, however it was stopped early following the recommendation of the external independent data monitoring committee when the third futility analysis identified a clear disease-free and overall survival benefit for patients

treated with three cycles of epirubicin and ifosfamide. The median follow-up was 12.3 months and patients treated with standard chemotherapy had statistically significant disease-free (62% vs 38%) and overall (89% vs 64%) survival benefits compared to those who received tailored chemotherapy. Subgroup analysis revealed that mixoyd liposarcomas was the only tumour histology were the histology-driven chemotherapy with trabectedin was as effective as standard chemotherapy. Importantly, disease-free and overall survival of patients in the histology-tailored arm were similar to those of the control arm in the first Italian Sarcoma Group trial comparing adjuvant chemotherapy and observation,[44] leading to the conclusion that the tailored treatment was likely not effective. Likewise, disease-free survival and overall survival of patients on the standard chemotherapy arm were similar to that of patients in the trial comparing three versus five cycles of cytotoxic chemotherapy.

Neoadjuvant chemoradiation

While neoadjuvant chemotherapy is still not widely accepted among clinicians, the role of radiotherapy in patients with high-risk STS of extremities and trunk is supported by findings from RCTs, making this treatment standard in high-rsik patients.[46-49] Radiotherapy and chemotherapy has been combined together for increasing the chances of a local response, decreasing the extent of resection and improve the limb salvage rate for STS of the extremities. Also, chemotherapy can enhance the anti-tumour effect of radiation.

Radiotherapy can be delivered either preoperatively, (cumulative dose: 50 Gy), or post-operatively (cumulative dose: 66 Gy). The optimal timing of radiotherapy is debated as preoperative radiation doubles the risk of a wound complication, while postoperative treatment increases the risk of late adverse effects, such as fibrosis, oedema, and joint stiffness.[49, 50] In the above mentioned RCT, which randomised patients to three cycles of preoperative chemotherapy with epirubicin (120 mg/m2) plus ifosfamide (9 g/m2) alone or in combination with two further postoperative cycles, radiotherapy

could be delivered either preoperatively or postoperatively.[42, 43] Patients treated with these schedules had a cumulative incidence of local recurrence of 17% and 3% in case of positive and negative margins, respectively, at five years.[51] Remarkably, in those patients who underwent preoperative chemoradiotherapy and had a postoperative positive surgical margin, no local recurrence were observed. These observations are in keeping with non-randomised studies showing a similar risk of developing a local recurrence in patients expected to have a positive margin after neoadjuvant radiotherapy plus surgery and in those who had negative margins after surgery.[52-56] Importantly, these studies showed also that tumours with a positive margin are likely more biologically aggressive than those with a negative margin and these patients are at greater risk of both local and distant relapse, irrespective of surgery extent.[54, 55] In these patients preoperative radiotherapy can reduce viable tumour cells at the resection margins. Also, these patients are at high-risk of metastatic spread and should be considered for preoperative chemotherapy. When a positive margin is reported after radiotherapy plus surgery, a further radiotherapy boost seems not to lower local recurrence in patients with microscopically-positive margins.[51, 53, 57] This is another indirect observation supporting preoperative over postoperative radiotherapy, especially in patients with large tumours where resection margins are likely not to be negative.

Chemotherapy can be given alternating with radiation therapy or concurrently. The concomitant administration is aimed at increasing chances of tumour response as well as performing conservative surgery without jeopardizing tumour local control. However, the simultaneous use of radio- and chemotherapy doubles the risk of high grade thrombocytopenia, which is observed in about one third of patients.[58] One in six patients also develop postoperative wound complications. The unclear effectiveness of simultaneous chemo- and radiotherapy balanced against toxicity has let to variations in practice and use of different treatment schedules. For instance, patients with large (8 cm or more) and intermediate to high grade sarcomas presenting at some US referral centres

undergo two courses of preoperative radiotherapy (22Gy in 11 fractions delivered in each course with a total of 44 Gy) with three cycles of neoadjuvant chemotherapy with mesna, adriamycin, ifosfamide, dacarbazine (MAID) in between.[59] Patients treated with this schedule have local control, distant recurrence-free and overall survival of 91%, 64%, and 86%, respectively, after five years.[60] This treatment modality was also tested in a multi-centre prospective phase II study (Radiation Therapy Oncology Group Trial [RTOG] 9514) which enrolled 66 patients.[61, 62] Grade three or higher morbidity was observed in the vast majority of patients (97%), including three treatment-related deaths. Long-term results showed 5-year distant disease-free and overall survival rates of 64% and 71%, respectively. Other combination have been tested to reduce toxicity compared to MAID, such as intra-arterial adriamycin, intravenous ifosfamide, and a combination of intravenous cisplatin plus adriamycin and ifosfamide, which were administered together with a reduced-dose radiotherapy (28 Gy).[63] Ifosfamide resulted the most effective drug to administer together with radiotherapy and patients developing tumour necrosis had less incidence of local recurrence and better survival. Another approach based on preoperative radiotherapy (50Gy) combined with concurrent escalating doses of gemcitabine plus ifosfamide, which was added for patients treated with definitive radiotherapy or when a positive post-operative margins can be anticipated, was studied in a phase I trial.[64] This schedule achieved 5-year local control, distant metastasis-free, and overall survival rates of 85%, 80%, and 86%, respectively.

Evidence for treatment of retroperitoneal sarcomas with concomitant chemoradiation is lacking. The Italian Sarcoma Group conducted a phase I-II study enrolling 86 patients who received three cycles of high-dose long infusion ifosfamide (14 g/m2) and radiotherapy which was started on the second chemotherapy cycle and administered up to a total dose of 50.4 Gy.[65] Local and distant recurrence occurred in 37% and 26% of patients, respectively, after five years leading to a disease-free and overall survival of 44% and 59%, respectively. Although results were encouraging, only two-third of

enrolled participants completed the preoperative treatment, likely reflecting the burden of such treatment modality in a population who often presented with significant comorbidities and low performance status. A retrospective study compared outcomes of patients treated with neoadjuvant chemotherapy and surgery alone for retroperitoneal sarcomas.[66] Length of hospital stay, rate of readmission, and rate of reoperation for complications were similar for patients treated with these two approaches. However, three postoperative deaths occurred in those patients treated with neoadjuvant chemotherapy. Overall, considering the risk associated with chemotherapy in these patients and the observed incidence of local recurrences, research is focusing on radiotherapy. A population-based study showed an association between performance of radiotherapy and better survival in patients with retroperitoneal sarcomas.[67] A RCT comparing preoperative radiotherapy followed by surgery and surgery alone (EORTC-STRASS, clinicaltrial.gov ID: NCT01344018) will offer more definitive data on the role of radiotherapy with these tumours.

Newly introduced effective drugs for treatment of metastatic tumours are going to be tested in the neoadjuvant setting concurrently with radiotherapy. For instance, pazopanib, an orally available tyrosine kinase inhibitor, is being tested in combination with radiotherapy in a phase II non-randomised study (clinicaltrial.gov ID: NCT02575066) and in a phase II/III study randomising patients preoperatively to radiation plus pazopanib or to radiation alone (clinicaltrial.gov ID: NCT02180867). Also, radiotherapy can enhance effectiveness of immunotherapy, particularly checkpoint inhibitors.[68] Some patients with metastatic tumours who undergo radiotherapy develop tumour responses not only at the site of treatment but also on other tumour deposits, generating the so-called 'abscopal effect'.[69] The immune mechanisms underlying these effects has been better described suggesting that combinations of radiotherapy and immune therapy could impact patient outcomes.[70]

Hypertermia and neodjuvant chemotherapy

Regional hyperthermia is another therapeutic strategy for improving loco-regional control in patients with several malignancies, such as recurrent breast cancer,[71]melanoma,[72] cervical cancer,[73] and malignant germ-cell tumours.[74] In STS, the effectiveness of neoadjuvant chemotherapy can be enhanced when patients are treated also with hyperthermia.[75, 76] Hyperthermia sensitizes tumour cell by ionizing radiation, which acts as a pleiotropic damaging agent altering protein structures and influencing the DNA damage response.[77] A phase III RCT, which compared neoadjuvant chemotherapy with etoposide, doxorubicin, and ifosfamide alone or in combination with hyperthermia, showed patients with high-risk primary sarcomas treated with both modalities at lower risk of disease progression (76% vs 61% after two years), which was the primary endpoint of this study, compared to those who underwent only chemotherapy.[76] Also, the hyperthermia doubled tumour response (29% vs 13%) However, overall survival did not differ between the two groups. A subgroup analysis of patients with retroperitoneal and abdominal sarcomas, confirmed the effectiveness for improving tumour local control (56% vs 45% after 5 years) and disease-free survival (34% vs 27% after 5 years) in patients who had macroscopically complete tumour resection.[78] These results were recently updated analysing 9-year follow-up data and presented in abstract form.[79] A significantly prolonged overall survival was observed in patients receiving regional hyperthermia compared with patients receiving only chemotherapy (63% vs 51% after 5 years). Despite these positive results, the advantages for combination of neoadjuvant chemotherapy with hyperthermia has not been yet confirmed by other trials.

Open issues

Preoperative histology characterization at core biopsy

Pathological examination of core biopsy can lead to accurate diagnosis for extremities and trunk sarcomas[80]. However, core biopsy seems not accurate for defining tumour differentiation and grade of lipomatous tumours seated in the retroperitoneum, which are characterised by large size and significant heterogeneity.[81]

Assessment of tumour response

The evaluation of tumour response after neoadjuvant treatments is another unresolved issue. Radiological imaging is needed after neoadjuvant therapies in order to formulate an adequate surgical plan. Also, imaging can offer significant information on effect of neoadjuvant treatments. RECIST criteria, which are based on unidimensional tumour measurement, selection of target lesions, and a threshold for assignment of objective progression, are the most widely used tool to evaluate tumour response.[82] However, they are not always accurate to evaluate tumour response when molecular target agents are used, such as the case of gastrointestinal stromal tumours (GIST).[83] In these soft tissue tumours the effect of targeted therapies can result in different modifications compared to standard cytotoxic chemotherapy. Standard chemotherapy results in tumour shrinkage, while targeted drugs generate also changes in tumour density. Importantly, these differences are seen also in some sarcoma histologies treated with cytotoxic chemotherapy (**Figures 1 and 2**).[84, 85]

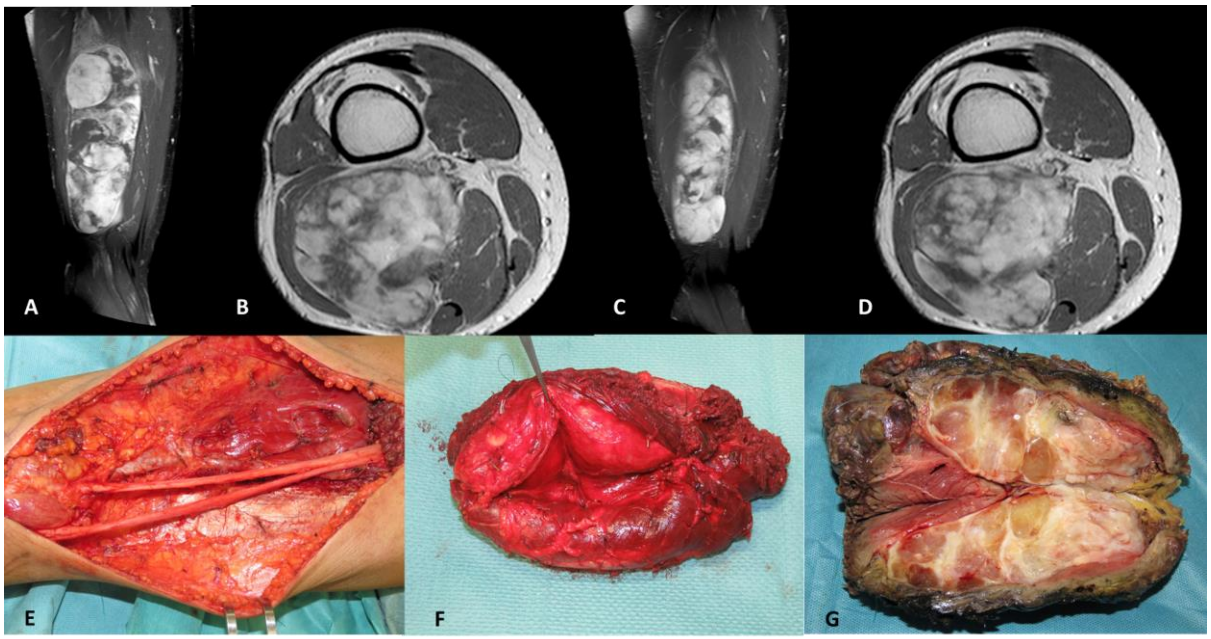

Figure 1. *A 38 years old male was diagnosed with a 8 x 6 x 21cm mass in his right posterior thigh (images A and B, contrast-enhanced MRI, TW1 weighted sequences). Percutaneous core needle biopsy revealed a high grade round cell myxoid liposarcoma (round cells component > 60%). This patient was treated with 3 cycles epirubicin (120 mg/m2) and ifosfamide (9000 mg/m2) and concomitant radiotherapy (50 Gy in 25 fractions). After neoadjuvant chemoradiation, contrast-enhanced MRI showed dimensional changes (8 x 2 x 16cm) and modification in pattern of contrast-enhancement suggesting a tissue response (images C and D). Surgery involved a wide excision of the posterior tight with the sciatic nerve dissected off the tumour (images E-G). Pathology report showed two small areas with hypercellularity (0.5 and 1.5 cm, respectively) and negative surgical margins.*

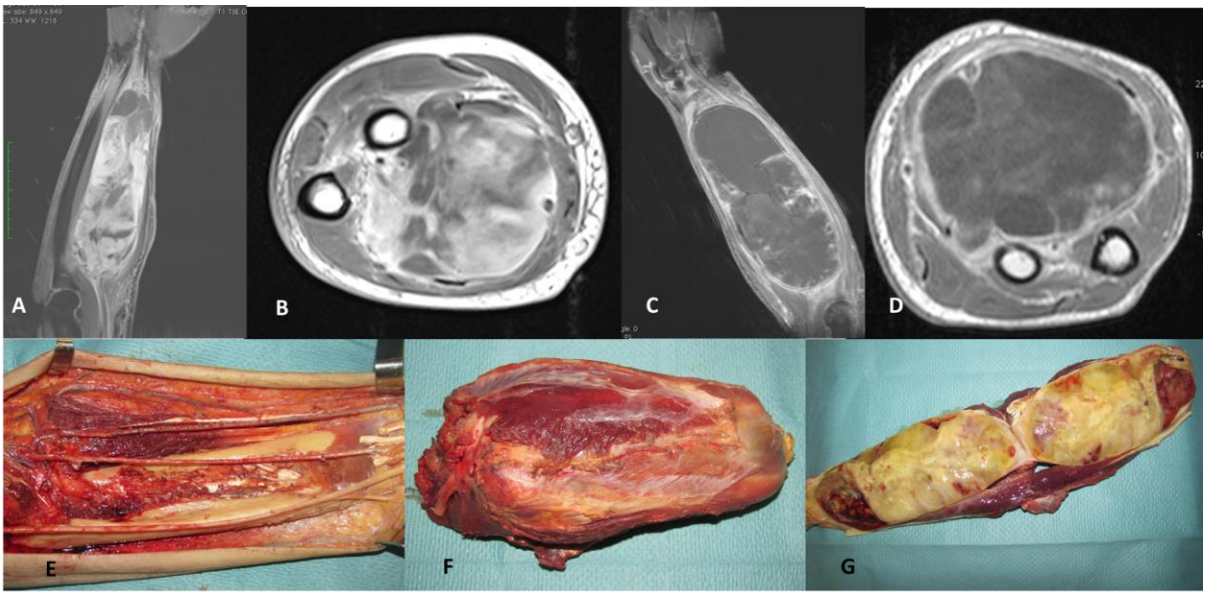

Figure 2. *A 58 years old man was diagnosed with a 6x20 cm mass in his left volar forearm. Percutaneous core needle biopsy revealed a high grade myxofibrosarcoma (images A and B, contrast-enhanced MRI , TW1 weighted sequences). This patient was treated with 3 cycles epirubicin (120 mg/m2) and ifosfamide (9000 mg/m2) and concomitant radiotherapy (50 Gy in 25 fractions). MRI showed an increased in tumour dimension and a strong reduction of tissue contrast enhancement suggested a tissue response (images C-D). Surgery involved a wide excision of the posterior forearm (Image E). The tumour was resected together with median nerve which was completely surrounded by the tumour (Images F-G). Pathology report showed significant presence of necrosis (70% of the tumour mass) and limited residual tumour (30%).*

For instance, in synovial sarcoma treated with epirubicin and ifosfamide, tumour attenuation at contrast-enhanced CT scan and tumour contrast enhancement at MR imaging adds predictive information to changes in tumour size.[84] Another useful tool to predict response to treatment for STS is positron emission tomography (PET)-CT. SUV values before and after neoadjuvant chemotherapy have been associated to the chance of developing a tumour response.[86, 87] However, FDG-uptake varies across sarcoma histologies and more research is needed to identify when PET-CT can significantly add to the management of these patients.[88]

Pathological examination provides definitive assessment of tumour response. However, guidance on how this evaluation should be performed are lacking and classification for characterising tumour necrosis and its patterns have not been established for STS. Existing data on the association between necrosis and survival are conflicting,[63, 89, 90] and further research is needed to improve prediction of prognosis of patients after neoadjuvant chemotherapy with or without radiation plus surgery. Secondary analyses of the STS-ISG-1001 are expected to shed lights on assessment of tumour response using imaging and pathology evaluation.

Histology-driven chemotherapy and patient selection

The effect of different chemotherapeutics across sarcoma histologies also needs further research. Several histotypes, such as alveolar soft part sarcoma,[91] clear cell sarcoma,[92] and classical-type epithelioid sarcoma[93] are among the most chemoresistant sarcomas. On the other hand, other sarcomas are considered more likely to respond to chemotherapy, such as synovial sarcoma and high grade myxoid liposarcoma (**Figure 1**), making them a candidate for neoadjuvant treatments. Synovial sarcomas are among most chemosensitive sarcomas,[94, 95] especially ifosfamide-containing regimens in the metastatic setting.[96] In the light of these findings, the ISG-STS 1001 trial randomised patients with synovial sarcoma to epirubicin plus ifosfamide or high dose ifosfamide. Patients treated with high dose ifosfamide did worse (HR 1.85, 95%CI 0.56 – 5.22), although this difference was not significant. Despite the observation of a better chemosensitivity for synovial sarcomas, studies showed that effect of cytotoxic chemotherapy may be relatively small. The above mentioned nomogram for patients with synovial sarcoma undergoing resection with curative intent showed that treatment with doxorubicin plus ifosfamide was associated with a statistically superior 3-year survival, although these improvements were lost over time.[30] An EORTC study pooled together data of 313 patients with synovial sarcomas treated in 15 different prospective trials showing these patients with a significantly higher chance of benefitting from chemotherapy compared to those having other sarcomas (28% and 19% response rate, respectively) which translated only in a small, although statistically significant, better survival (progression-free survival: 6 versus 4 months, respectively; overall survival: 15 and 12 months, respectively).[97]

Myxoid liposarcoma, which is defined by a DDIT3-FUS or DDIT3-EWSR1 gene fusion, is characterised by good outcomes, although high-grade tumours (i.e. round cell component >5%) showed more aggressive behaviour.[98] In a retrospective studies, virtually all patients with myxoid liposarcoma treated with chemotherapy survived 5 years after surgery.[99] Trabectedin, which blocks DNA binding of the oncogenic transcription factor FUS-CHOP,[100, 101] is an effective agent in this sarcoma subtype both in the metastatic[102] and neoadjuvant[103] setting. The above mentioned ISG-STS-1001 trial

compared standard epirubicin plus ifosfamide and trabectidine for these patients. Interestingly, the two regimens showed similar effectiveness (HR 1.03; 95%CI 0.24-4.39), which favours trabectedin for its more acceptable toxicity profile. These findings need to be confirmed in a larger prospective series and this study is going to be reopened enrolling only patients with high grade myxoid liposarcomas.

Undifferentiated pleomorphic sarcoma, has been considered a chemo-resistant histology with unfavourable prognosis, particularly when these tumours are located in the retroperitoneum.[104] In a recent population-based analysis, prognosis of patients with undifferentiated pleomorphic sarcoma was significantly better when adjuvant/neoadjuvant chemotherapy was used (median survival 78 and 49 months, respectively).[14] Also, these tumours harbours a significant genomic instability,[105, 106] suggesting they may be a candidate for newly introduced immune checkpoint inhibitors.[107] Overall, certain histologies lend themselves to tailored therapy such as MLS, however the ISG-STS-1001 study confirmed that an anthracyline with ifosfamide for all other subtypes is preferred.

Conclusions

Significant improvements in patient risk stratification through new AJCC TNM classification and nomograms can better stratify risk of patients with primary STS. This is of great importance since neoadjuvant epirubicin and ifosfamide showed effectiveness in locally advanced high-risk primary sarcomas of trunk and extremities. Better prognostic tools, wider array of chemotherapy options, and better predictive biomarkers are needed for patients with high-risk sarcomas.

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CHAPTER 3

High-risk soft tissue sarcomas treated with perioperative chemotherapy: improving prognostic classification in a randomised trial

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Background

Soft tissue sarcoma (STS) of extremity and trunk wall are considered at high risk of disease progression when harbouring adverse prognostic features, such as large size and high malignancy grade [1]. Standard treatment includes combination of surgery and radiotherapy [2-5]. Anthracycline-based neoadjuvant and adjuvant systemic therapies have been tested to reduce risk of metastatic spread after these local treatments in several randomized controlled trials (RCT) [6, 7]. These studies enrolled a mixed sarcoma patient population with variation in outcomes and showed a survival benefit ranging between 5 and 10%, which has been considered unsatisfactory particularly when balanced against meaningful high-grade toxicity [6]. Clinicians' opinions and clinical practice guidelines reflect these uncertainty suggesting that perioperative chemotherapy should be discussed with patients with high-risk tumours [4, 5]. Recently, a RCT which enrolled a more homogeneous population and compared optimal neoadjuvant epirubicin and ifosfamide with several histology-based chemotherapy schedules dosages failed to demonstrate superiority of this latter approach [8]. Participants with high-risk STS of extremities and trunk wall treated with anthracycline-based chemotherapy had a 24% improvement in disease-free survival and a 25% improvement in overall survival. These results, although needing further confirmation, underlined the importance of selecting a homogeneous sarcoma population when investigating perioperative treatment strategies.

Recent advances in patient staging further support efforts for improving selection of patients for perioperative treatments [9]. The 8th edition of the TMN staging system for sarcomas [10], which included prognostic nomograms [11], can improve patient risk stratification. In particular, prognostic tools potentially inform physician choice on treatment to be performed on a single patient [12]. Since Kattan et al tested the first nomogram for sarcoma [13], several other tools have been created and validated [11]. Among them, the Sarculator includes a nomogram for extremity STS that predicts

probability of overall survival and incidence of distant metastasis at five and ten years after surgery based on patient age and tumour histology, size, and grade [14].

In this study we stratified prognostic risk of currently defined high-risk STS patients, who have large and high-grade tumours and are considered for perioperative chemotherapy, and also investigated chances of developing a tumour response. The prognostic nomogram Sarculator [14] was used to predict overall survival (OS) probability and incidence of distant metastasis (DM) in high-risk patients who were enrolled in a RCT that tested neo-adjuvant chemotherapy [15, 16]. Finally, this study investigated association between nomogram-based prognostic stratification and radiological response to pre-operative chemotherapy.

Methods

Patients

This study analysed data from patients enrolled in a RCT conducted by the Italian and Spanish national sarcoma groups (ISG and GEIS) which has been described in details elsewhere (European Union Drug Regulating Authorities Clinical Trials No. 2004-003979-36) [15, 16]. Briefly, from January 2002 to March 2007 this study randomised 321 adult eligible patients affected by histologically proven localized adult-type STS located to the extremities or trunk wall that were seated deeply to the investing fascia, sized 5 cm or more, and with histologic malignancy grade equal to 3 according to the Federation Nationelle des Centres de Lutte Contre le Cancer (FNCLCC). Patients were assigned to receive either three preoperative cycles (arm A, N=160) or three preoperative and two postoperative cycles (arm B, N=161) of epirubicin (60 mg/m2/d, short infusion, on days 1 and 2) and ifosfamide (3 g/m2/d on days 1, 2, and 3). Radiotherapy was either given preoperatively (total dose: 44 to 50.4 Gy) or postoperatively (total dose: 60 to 66 Gy). The former group could also receive an

intraoperative (10 to 12 Gy) or postoperative boost (16 to 20 Gy) upon treating physician's discretion. All but seven patients went on having surgery. Follow-up cut-off date was April 2016.

Nomogram predictions

The prognostic nomogram for extremity STS included in Sarculator (http://www.sarculator.com) was fitted to data of participants included in the above mentioned RCT. This predictor tool generates individual probability of 5- and 10-year OS (pr-OS) and incidence of DM (inc-DM) at a pre-specified follow-up time by integrating patient age (continuous variable: 18-100 years), tumour size (continuous variable: 0·1-35 cm), tumour grade (categorical variable: I, II, and III), and tumour histology (categorical variable: leiomyosarcoma, dedifferentiated pleomorphic liposarcoma, myxoid liposarcoma, malignant peripheral nerve sheath tumours, myxofibrosarcoma, synovial sarcoma, sarcoma of vascular origin, undifferentiated pleomorphic sarcoma, and other). This nomogram was previously retrospectively tested and validated on 1,452 and 2,300 patients, respectively [14].

The 10-year pr-OS and the 10-year inc-DM, computed for each patients using Sarculator, were the variables of interest for this study. These were reported on percentage scale and grouped in three categories by using the 33-th and 66-th percentiles of the pertinent distributions.

Tumour response

Tumour response was evaluated centrally according to both RECIST and Choi criteria [17, 18]. Detailed description of assessment of tumour response is reported elsewhere [19, 20]. Briefly, baseline and preoperative MRI scans were collected from every participating centres and reviewed centrally. Turbo-spin-echo (TSE) T2-weighted images and TSE T1-weighted images followed by a contrast-enhanced TSE T1-weighted image MRI scans were considered. Contrast-enhanced TSE T1-weighted images were analysed before and after digital subtraction both qualitatively and semiquantitatively by drawing a region of interest around the margin of the whole lesion on sections taken every 5 mm

and measuring tumour contrast enhancement being muscles the reference tissue. Choi criteria, which were established for gastrointestinal stroma tumours [17, 18], were modified to other STS and magnetic resonance imaging (MRI) as follow: 1) partial response (PR) = tumour size decrease ≥10% or tumour density/contrast enhancement on CT/MRI studies decrease ≥ 15%; 2) progressive disease (PD) = new lesions or tumour size increase ≥ 10% without PR according to tumour density/contrast enhancement or tumour density/contrast enhancement decrease ≤ 15%.

Statistical Analysis

Statistical analysis was performed using pr-OS as pivotal variable. OS was defined as the time from randomization to death from any cause or last follow-up. The pattern of OS was estimated by means of the Kaplan-Meier method [21]. The role of the variable pr-OS on OS was assessed by means of an univariate Cox regression model [22]. Additionally, the interaction between pr-OS and study treatment arm was assessed using a multivariate Cox regression model including the main effects and the first-order interaction. The association of pr-OS with the variables RECIST and Choi tumour response, was investigated by means of the Pearson chi-square statistic or Fisher's exact test whenever appropriate. The strength of association with pr-OS was assessed dichotomizing the tumour response variables as SD/PD versus PR and resorting to an univariate logistic regression model [23]. Furthermore, the interaction between the dichotomized response variables (SD/PD versus PR) and pr-OS was studied with the Cox regression model as above described for the treatment variable.

Analysis were conducted considering also the endpoint inc-DM to strengthen the association between Sarculator predictions and patient prognosis. The cumulative incidence (CI) of DM after randomization was assessed by processing data according to the competing risks approach [24]. The association of inc-DM with tumour response variables was evaluated by applying approaches described for the variable pr-OS.

All statistical analyses were carried out with the SAS software (version 9.4, SAS Institute Inc., Cary, NC).

Results

Randomised eligible patients were followed up for a median time of 114 months (interquartile range, IQR, 101 – 133 months) with a 10 year probability of OS equal to 0·61 (95% confidence interval, CI: 0·56–0·67) and a crude CI of DM of 0·34 (SE: 0·028). This study analysed data from 310 patients who underwent surgery and had all the clinical and pathological information required for the computation of the pr-OS and inc-DM (Table 1 and CONSORT diagram in Figure 1).

Table 1. Clinicopathological characteristics of the considered 310 patients overall and according to treatment arm. Asterisk indicates tumour histologies that were grouped in the 'Other' category of the Sarculator.

Variable	**Characteristics**	**ALL (N=310)**		**Arm A (N=153)**		**Arm B (N=157)**	
		N	**%**	**N**	**%**	**N**	**%**
Patient age (yeras)	median (range)	48 (15-79)		51 (15-79)		47 (16-74)	
Tumour size (cm)	median (range)	10 (2-30)		10 (2-30)		10 (3-30)	
Tumour grade	G2	45	14·52	26	16·99	19	12·10
	G3	265	85·48	127	83·01	138	87·90
Histology	ANGIOSARCOMA	1	0·32	0	0·00	1	0·64
	FIBROSARCOMA	3	0·97	1	0·65	2	1·27

	LEIOMYOSARCOMA	42	13·55	19	12·42	23	14·65
	UPS	75	24·19	42	27·45	33	21·02
	MPNST	21	6·77	13	8·50	8	5·10
	MYXOFIBROSARCOMA *	1	0·32	0	0·00	1	0·64
	OTHER *	10	3·23	5	3·27	5	3·18
	PLEOMORPHIC LIPOSARCOMA *	12	3·87	5	3·27	7	4·46
	PLEOMORPHIC RHABDOSARCOMA *	5	1·61	2	1·31	3	1·91
	ROUND CELL LIPOSARCOMA *	27	8·71	12	7·84	15	9·55
	SPINDLE CELL SARCOMA NOS	45	14·52	23	15·03	22	14·01
	SYNOVIAL SARCOMA	68	21·94	31	20·26	37	23·57
Pr-OS	median (range)	60 (9-92)		61 (12-92)		58 (9-88)	
Pr-OS categorized	Low (≤51)	107	34·52	40	26·14	67	42·68
	Intermediate (51 < pr-OS ≤ 66)	102	32·90	57	37·25	45	28·66
	High (>66)	101	32·58	56	36·60	45	28·66
Inc-DM	median (range)	44 (10-88)		42 (10-88)		47 (13-88)	
Inc-DM categorized	Low (≤38)	104	33·55	59	38·56	45	28·66
	Intermediate (38 < inc-DM ≤ 51)	103	33·23	51	33·33	52	33·12
	High (>51)	103	33·23	43	28·10	60	38·22

UPS: undifferentiated pleomorphic sarcoma; MPNST: malignant peripheral nerve sheath tumour.

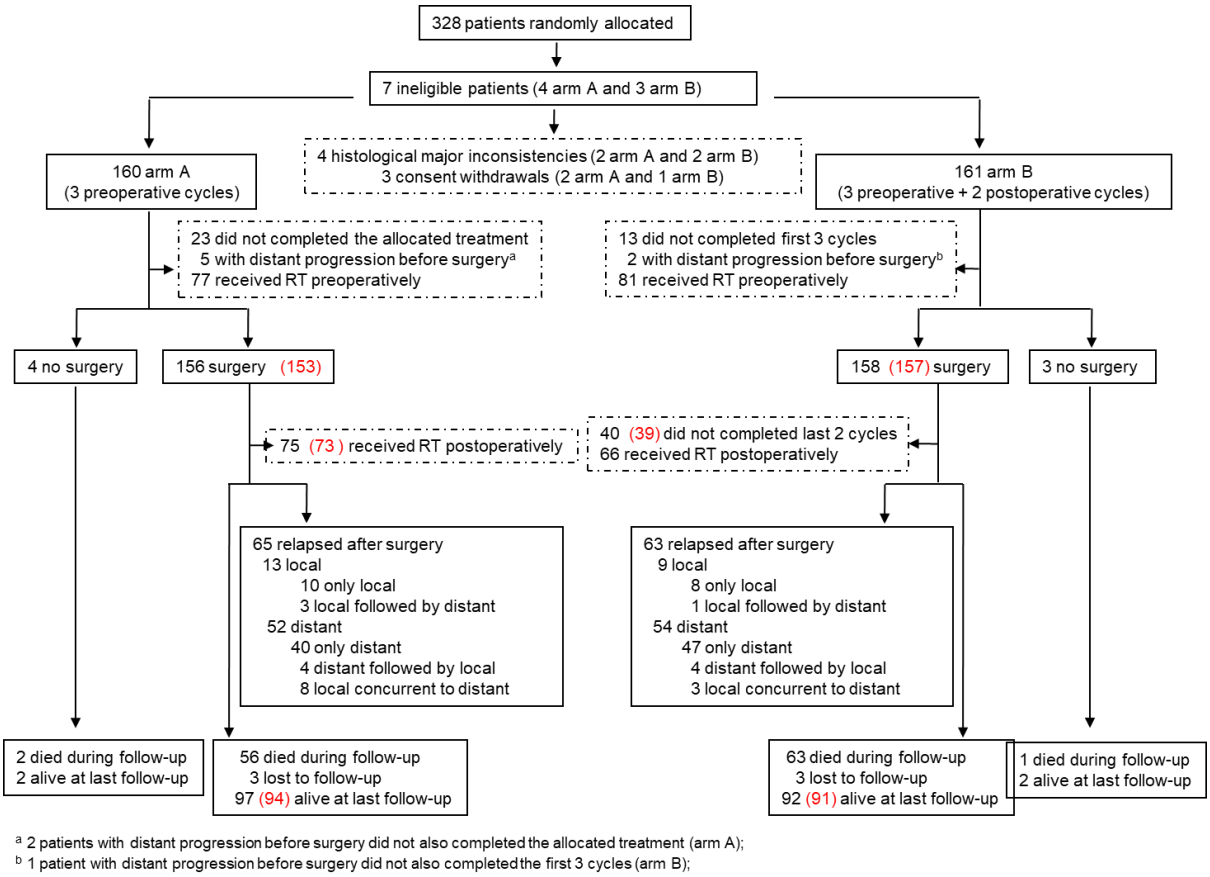

Figure 1. Consort diagram. There were 310 patients eligible for this study of 321 in the trial, which are reported in red and brackets in the diagram. All patients who did not undergo surgery were excluded from this analysis (N=7). Four more patients were excluded as they lack of information regarding tumour size (N=3) and grade (N=1), which were needed for Sarculator predictions.

<u>Nomogram-based probability of OS (pr-OS)</u>

Wide variations existed for pr-OS, which ranged between 9 and 92, with a median of 60 (Figure 2).

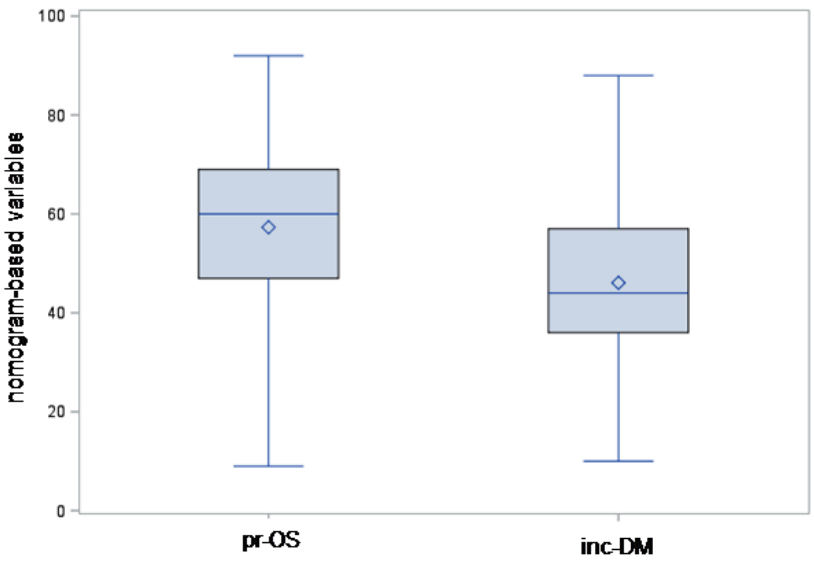

Figure 2. Variations in probablity of overall survilva (pr-OS) and incidence of distant metastasis 8inc-DM).

The cut-offs used to identify the three categories for pr-OS according to variable distribution were 51 (33-th percentile) and 66 (66-th percentile). The estimated 10-year OS corresponding to the low (pr-OS $\leq$ 51), intermediate (51 < pr-OS $\leq$ 66) and high (pr-OS > 66) pr-OS were 0·42 (95%CI 0·32-0·52), 0·63 (95%CI 0·53-0·72), and 0.78 (95%CI 0·68-0·85), respectively (Figure 3).

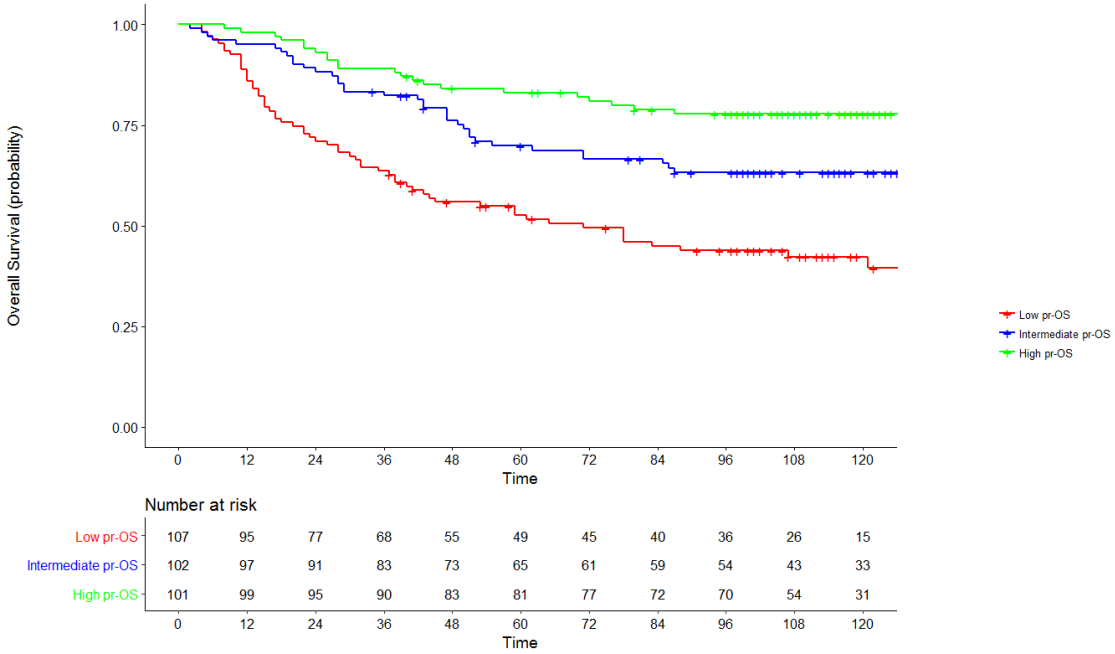

Figure 3. Survival according to three probability of overall survival (pr-OS categories).

Univariate Cox regression analysis showed that patients belonging to the intermediate (HR 0·51, 95%CI 0·34-0·78, P = 0·002) and high (HR 0·28, 95%CI 0·17-0·46, P<0·001) pr-OS category were at statistically significant lower risk of death compared to the patients classified in the low pr-OS category. The Figure 4 reports the pattern of OS by jointly considering the variables pr-OS and treatment arm of this RCT. The interaction term between pr-OS and treatment arm was not statistically significant in the Cox regression model, suggesting lack of differences in treatment effects within patient groups characterised by similar survival.

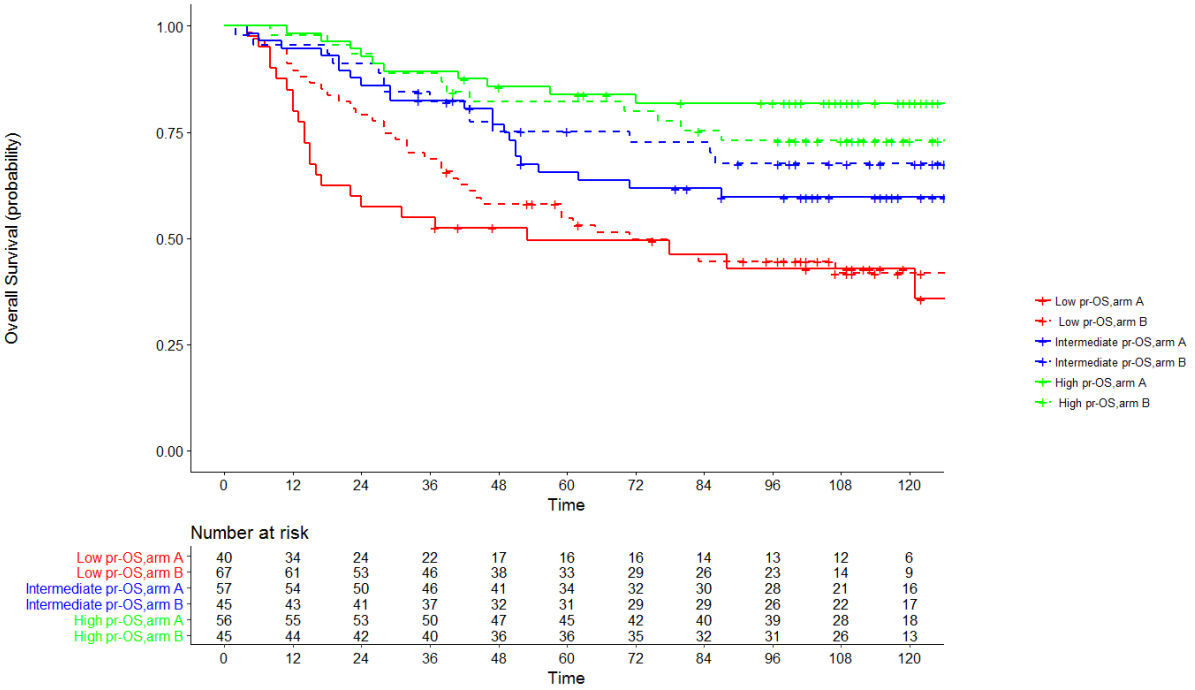

Figure 3. Survival according to three probability of overall survival (pr-OS categories) and study treatment arms (3 vs 5 cycles of perioperative chemotherapy).

Also, the association between nomogram predictions and response to chemotherapy was analysed to assess whether different patterns of tumour response according to RECIST and Choi criteria to neoadjuvant chemotherapy can be identified across Sarculator predicted pr-OS categories. Assessment of tumour response according to RECIST and Choi was available for 238 and 161 patients, respectively, as already reported [19]. By considering RECIST criteria, a statistically significant association with pr-OS was not observed (Table 2, chi-square p-value=0·22).

Table 2. Frequency distribution of tumour response according to RECIST and Choi criteria for pr-OS and inc-DM.

	Category	RECIST CRITERIA							Choi CRITERIA						
		PR		SD		PD		Tot	PR		SD		PD		Tot
Pr-OS	Low	18	18·9%	60	63·2%	17	17·9%	95	50	75·8%	6	9·1%	10	15·2%	66
	Intermediate	21	27·3%	45	58·4%	11	14·3%	77	49	90·7%	3	5·6%	2	3·7%	54
	High	13	19·7%	48	72·7%	5	7·6%	66	34	82·9%	6	14·6%	1	2·4%	41
	Total	*52*	*21·8%*	*153*	*64·3%*	*33*	*13·9%*	*238*	*133*	*82·6%*	*15*	*9·3%*	*13*	*8·1%*	*161*
Inc-DM	Low	14	22·2%	45	71·4%	4	6·3%	63	36	85·7%	6	14·3%	0	0·0%	42
	Intermediate	24	27·6%	50	57·5%	13	14·9%	87	52	91·2%	2	3·5%	3	5·3%	57
	High	14	15·9%	58	65·9%	16	18·2%	88	45	72·6%	7	11·3%	10	16·1%	62
	Total	*52*	*21·8%*	*153*	*64·3%*	*33*	*13·9%*	*238*	*133*	*82·6%*	*15*	*9·3%*	*13*	*8·1%*	*161*

Patients in the intermediate category showed the greatest chances of developing a RECIST PR with respect to the patients in the low category according to a univariate logistic regression model (Table 3). Tumour response according to Choi criteria achieved a borderline non-significant association with pr-OS (Fisher exact test p-value P=0·056). Logistic regression analysis showed that patients in the intermediate and high pr-OS categories had greater chances of developing a Choi PR than patients in the low pr-OS category, although only the former comparison was statistically significant (Table 3).

Table 3. Univariate logistic regression analysis for tumour response with RECIST and Choi criteria according to Sarculator predicted categories.

Prediction	**category**	**RECIST**		**CHOI**	
		OR	**95%CI**	**OR**	**95%CI**
Pr-OS	**Low**	1		1	
	Intermediate	1.60	0.78 – 3.29	*3.14*	*1.07 – 9.22*
	High	1.05	0.47 - 2.32	1.55	0.58 – 4.18
Inc-DM	**High**	1		1	
	Intermediate	2.01	0.96 – 4.22	*3.93*	*1.34 – 11.50*
	Low	1.51	0.66 – 3.44	2.27	0.81 – 6.34

Panels A and B of Figure 5 report the patter of OS according to the joint variable obtained by considering the three categories of pr-OS and the dichotomized tumour response for RECIST and Choi criteria, respectively. The first-order interaction term between pr-OS and tumour response resulted not statistically significant for both RECIST and Choi criteria in the Cox regression model. The main effect of tumour response (SD/PD vs PR) according to Choi criteria was significantly associated with OS. For explorative purposes, which were also supported by the OS pattern at survival curves (Figure 3, panel B), HRs were computed across the three categories of pr-OS by

combining beta coefficients of the above Cox model implemented for Choi criteria as appropriate. Patients who had Choi SD/PD showed a significant higher risk of death compared to those who developed a Choi PR in both intermediate (HR = 4·24, 95%CI 1·41 – 12·72) and low (HR = 2·65; 95%CI 1·32 – 5·3) pr-OS categories, while this trend was not statistically significant in the high pr-OS category (HR = 1·32; 95%CI 0·28 – 6·22).

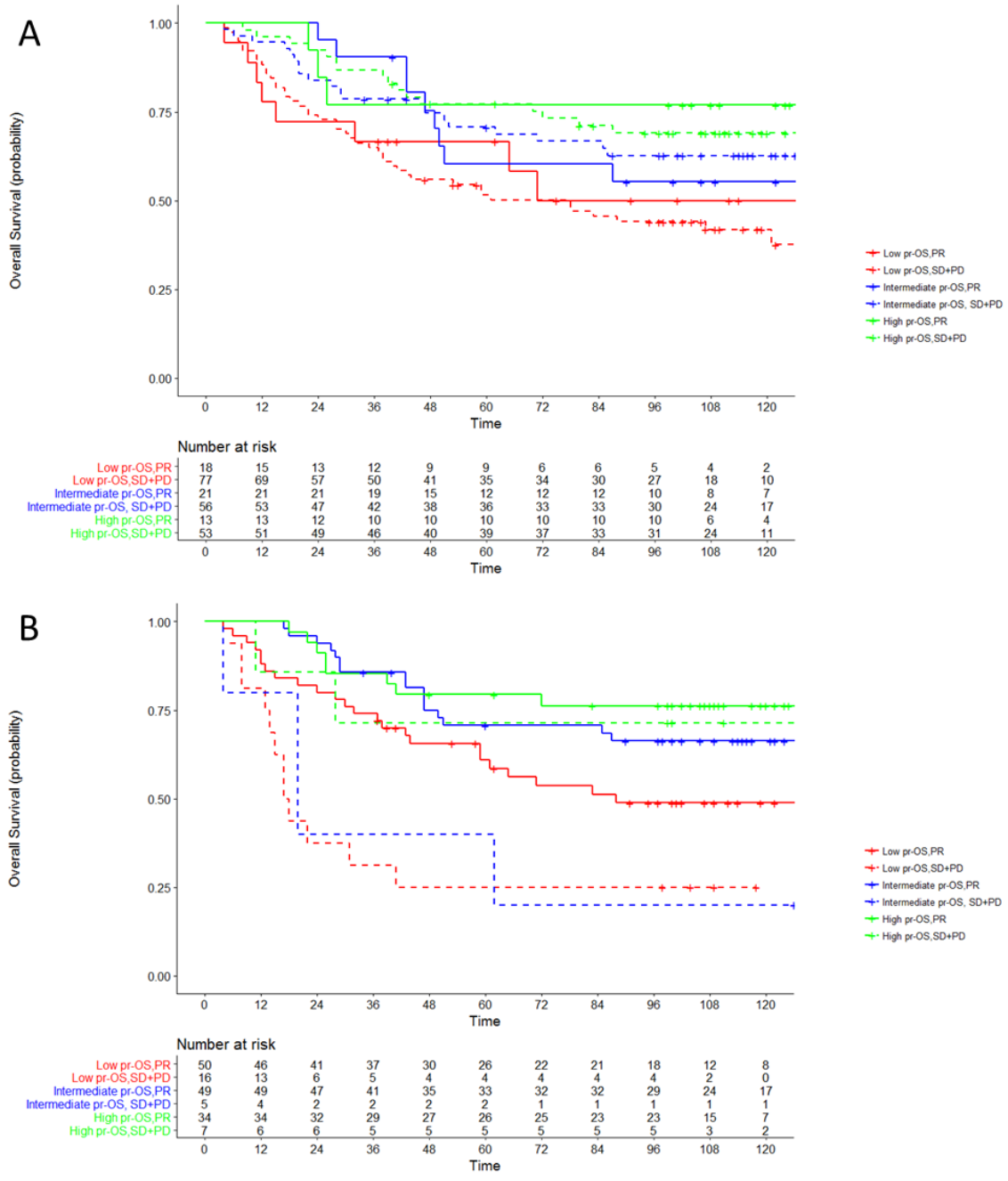

Figure 3. Survival according to three probability of overall survival (pr-OS categories) and radiological tumour response assessed with RECIST (A) or Choi (B) response criteria.

Nomogram-based incidence of DM (inc-DM)

Sarculator prediction for inc-DM was analysed to strengthen findings achieved investigating pr-OS. Variation in inc-DM was also detected with 10-year inc-DM ranging between 10 and 88 with a median value of 44 (Figure 1). The values of the two cut-offs used to identify the three considered inc-DM categories (i.e. low, intermediated, and high) were 38 (33-th percentile) and 51 (66-th percentile). The estimated 10-year cumulative incidence of DM corresponding to the low (inc-DM $\leq$ 38), intermediate (38 < inc-DM $\leq$ 51), and high (inc-DM > 51) category was 0·26 (SE: 0·04), 0·31 (SE: 0·05), and 0·48 (SE: 0·05) respectively (Figure 6).

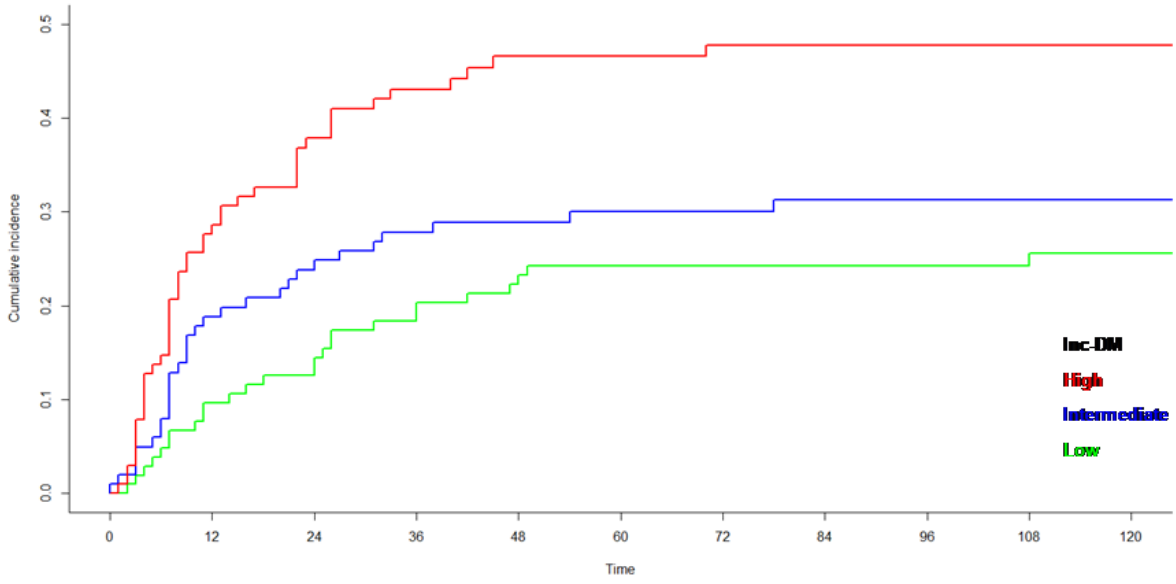

Figure 6. Ten-year cumulative incidence of distant metastases according to the three categories of inc-DM.

10-year inc-DM predicted by the Sarculator were higher than observed predictions of enrolled participants, which were 0.29 (SE: 0.69), 0.45 (SE: 0.38), and 0.65 (SE: 0.93) in low, intermediate, and high inc-DM categories, respectively. Again, study treatment effect was similar across inc-DM categories (Figure 7). Also, similarly to pr-OS, there was not a statistically significant association

considering tumour response measured according to RECIST criteria and inc-DM (chi-square p-value=0·10, Table 2). These findings were also confirmed by the univariate logistic regression model (Table 3) although, as for pr-OS, patients in the intermediate category had the greatest chances of developing a RECIST PR. Tumour response according to Choi criteria was significantly associated with inc-DM (Fisher exact test p-value P=0·006). Mirroring pr-OS findings, logistic regression analysis showed that patients in the intermediate category had statistically significant greater chances of developing a Choi PR than patients in the high category (Table 3).

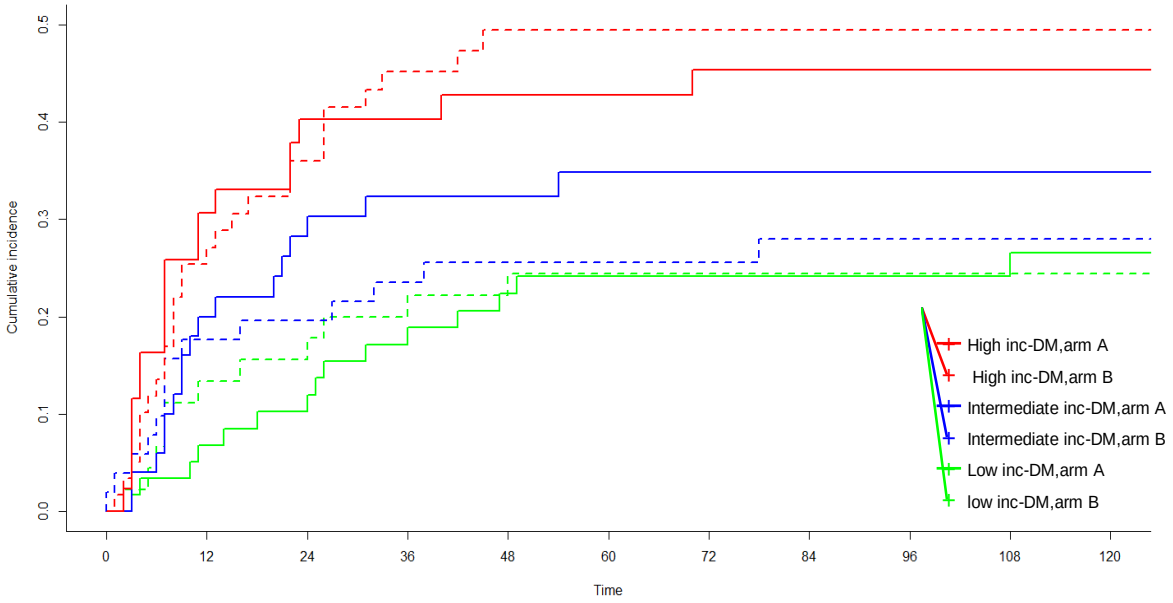

Figure7. Ten-year cumulative incidence of distant metastases (DM) according to incidence of DM (inc-DM) predicted by the Sarculator and RCT treatment arm (A vs B).

Discussion

The prognostic nomogram Sarculator identified prognostic variability in patients with high-risk STS undergoing neoadjuvant chemotherapy and three different categories characterised by low, intermediate, and high pr-OS were created. The prognosis of responsive patients was better when

Choi criteria were considered across the predicted categories, particularly for patients with intermediate and low pr-OS. These stratifications were paralleled by the inc-DM curves.

The main limitation to the evidence generate in this study is the lack of a formal control group in the RCT trial used for this analysis [15, 16]. A direct comparison of patients treated with neoadjuvant chemotherapy plus surgery and those who underwent surgery with or without radiotherapy would offer chances to estimate the magnitude of survival benefit for chemotherapy across different predicted risk categories. The wide variation of prognosis detected for patients currently considered at high-risk of disease progression (i.e. high malignancy grade, size > 5cm) can be considered one of the main determinants of lack of evidence from several RCT for perioperative chemotherapy in high-risk STS [25, 26]. In this regard, findings of this study proves the principle that prognostic stratification through a prognostic tool can improve patient risk stratification and inform on the likelihood of response to chemotherapy according to predicted OS as well as incidence of DM.

Recent advances in perioperative chemotherapy for patients with high-risk STS underlie the value of these results [27]. A RCT has compared three cycles of epirubicin plus ifosfamide with three cycles of an histology-tailored regimen, in high-risk undifferentiated pleomorphic sarcoma, high-grade myxoid liposarcoma, synovial sarcoma, malignant peripheral nerve sheath tumours, and leiomyosarcoma arising in the extremities and trunk wall [8]. This RCT was stopped after accruing 286 patients when the third futility analysis identified a disease-free (62% vs 38%) and overall (89% vs 64%) survival benefit for patients treated with three cycles of epirubicin plus ifosfamide, which was the control arm of the study, at a median follow-up of 12 months. Although these findings need to be confirmed when longer follow-up will be analysed, they reinforce the recommendation of considering chemotherapy as a valuable option for early stage disease to be discussed with patients. A prognostic nomogram such as Sarculator could improve risk stratification for these patients in order to maximize treatment effectiveness and spare treatment adverse reactions to patients unlikely to achieve a survival advantage. For instance, some patients enrolled in the present RCT actually had a

10% risk of DM, so that one could hypothesize to spare these patients the medical treatment [8]. However, the activity of chemotherapy may be different across prognostic subgroups and this study evaluated also tumour response along with pr-OS and inc-DM. Patients predicted in the intermediate categories seem those who benefit more from neoadjuvant chemotherapy. Tumour responses are less frequently achieved in the low and high pr-OS and inc-DM group. The predictive value for tumour response improved by using Choi criteria over RECIST criteria (Figure 3). These findings may reflect a more homogeneous population in intermediate pr-OS category, which may include tumour histologies with greater chances of responding to anthracycline-based neoadjuvant chemotherapy (Supplementary Table S3). Since tumour response in this RCT was associated to patient outcome, tumour response may become an additional prognostic factor complementing Sarculator available at the end of patient treatment with perioperative therapies and surgery. Also the highest-risk patients (ie. low pr-OS category) responded to preoperative chemotherapy, although the magnitude of the association between response and OS was less pronounced as compared to the intermediate category. It is left to understand whether this may have therapeutic implications. For instance, patients with high-risk tumours would be expected to gain a higher absolute benefit from perioperative chemotherapy, although this might be offset by a lower sensitivity to it.

In this study predicted inc-DM with the Sarculator was higher compared to the observed incidence of DM, leading to the hypothesis that Sarculator may overestimate incidence of DM. However, Sarculator predictions are based on a multivariable model and should be considered more informative than the actuarial predictions, which calculated for enrolled patients with Kaplan-Meier method. Also, it could be hypothesised that the lower incidence of DM observed in the study participants compared to the predicted inc-DM by the Sarculator may reflect the efficacy of perioperative chemotherapy, considering that only a minority of patients used to build Sarculator have had perioperative chemotherapy (i.e. 26% and 2-52% in the training and three testing sets,

respectively). Although this hypothesis is intriguing and may provide further evidence favouring perioperative chemotherapy for high-risk STS it cannot be addressed properly in this study, which was not meant to investigate superiority of chemotherapy for high-risk STS, for the difference in statistical methodologies discussed above and is left for further research and development of Sarculator.

In conclusion, this study showed the wide variations in OS and risk of developing DM predicted by a nomogram within an apparently homogeneous high-risk STS treated with perioperative anthracycline-based chemotherapy. Three groups of patients with different pr-OS and risk of inc-DM were identified. In addition, Choi responsive patients did better, especially those with intermediate to low pr-OS. Further research is in progress to characterise chances of benefitting from neoadjuvant chemotherapy across prognostic risk categories defined by the Sarculator. Future trials investigating perioperative chemotherapy should value prognostic information of nomograms and Choi tumour response.

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CHAPTER 4

The impact of chemotherapy on survival of patients with extremity and trunk wall soft tissue sarcoma: revisiting the results of the EORTC-STBSG 62931 trial

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Introduction

Surgery and radiation therapy are the standard treatment options for high-risk soft tissue sarcoma (STS) of extremity and trunk wall with adverse prognostic features, such as large size and high tumour grade. [1, 2] Neoadjuvant and adjuvant systemic chemotherapy have been tested to reduce risk of metastatic spread leading to a 5 to 10% long-term overall survival (OS) benefit in meta-analyses, [3-5] which has been considered inconclusive mainly due to the conflicting results of individual trials. [2, 6-9] It has been suggested that several of the trials included patients of intermediate or low risk, which may have less benefit from adjuvant treatment. This lack of conclusive evidence has generated variations in treatment strategies. [10-13]. In particular, a large randomised controlled trial (RCT), the EORTC-STBSG 62931, which compared doxorubicin plus ifosfamide with observation after surgery for STS, failed to identify a survival benefit for adjuvant chemotherapy. [14] However, an updated meta-analysis of 2145 patients including those in EORTC 62931 showed an overall survival benefit for adjuvant chemotherapy (hazard ratio 0.86, 95%CI 0.75–0.97).[12] International guidelines suggest to discuss the option of chemotherapy with patients affected by a primary high-risk STS of extremity and trunk wall within a shared decision in the context of challenging evidence. [15, 16]

A recent RCT investigating a relatively homogenous group of adult high-risk patients affected by the five most common STS histology of extremity and trunk wall (i.e., high-grade myxoid liposarcoma, undifferentiated pleomorphic sarcoma, leiomyosarcoma, malignant peripheral nerve sheath tumours, synovial sarcoma, and leiomyosarcoma) showed an improvement in 4-year disease-free survival (DFS) and OS of approximately 20% for anthracycline-based chemotherapy compared to an histology-tailored schedule not including anthracycline. [17]

The prognostic nomogram Sarculator, [18, 19] which was developed using predictive information of tumour histology, patient age, and AJCC TNM prognostic features tumour grade and size, [20, 21] was

tested and validated on large patient series. [18] This tool was used to stratify prognosis of patients with high-risk STS enrolled in a randomised trial from Italian and Spanish Sarcoma Groups (ISG and GEIS) testing different perioperative anthracycline-ifosfamide chemotherapy duration [22, 23] and identified a wide range of predicted 10-year OS of enrolled patients ranging between 9 and 92%. [24]

We hypothesised that these variations in survival of patients with STS of extremity and trunk in studies conducted to date may have diluted a potential survival benefit for chemotherapy in higher risk patients thus offering an explanation for conflicting results between different RCTs, such as the EORTC-STBSG 62931 [14] and the ISG-1001. [17] In this study we fitted the Sarculator [19] to the individual patient data of the negative EORTC-STBSG 62931 RCT [14] to identify whether or not there were patients who had a survival benefit after adjuvant chemotherapy.

Methods

Patients

This study analysed data from patients enrolled in the EORTC-STBSG 62931, an unblinded RCT conducted by the EORTC in 36 sarcoma centres in Europe and Canada (ClinicalTrials.gov, ID number NCT00002641). [14] Briefly, this study randomised 351 adult patients (allocation ratio 1:1) affected by histologically proven localized adult-type STS located at any site with intermediate and high histologic malignancy grade (Trojani grade II and III). Patients were assigned to receive either five adjuvant chemotherapy cycles (treatment arm, N=175) or observation (control arm, N=176). The chemotherapy regimen was doxorubicin (75 mg/m²) plus ifosfamide (5 g/m²) with mesna and lenograstim (3 μg/kg) given every 21 days. Radiotherapy was delivered postoperatively at a total dose of 60 to 66 Gy when surgical excision was marginal or in case of previous incomplete surgery. In this RCT five postoperative chemotherapy cycles (treatment arm) were expected to increase 5-year survival from 50% to 65% with 95% significance and 80% power (α=0.05, β=0.2). The protocol of the

EORTC-STBSG 62931study was approved by the EORTC Protocol Review Committee and institutional review boards. The patients gave informed consent according to applicable laws in all participating countries. The current study was approved by EORTC through the 'Request for data platform' (http://www.eortc.org/request-for-data/).

Nomogram predictions

The prognostic nomogram for extremity STS included in the Sarculator (http://www.sarculator.com) was fitted to individual participant data from the EORTC-STBSG 62931 trial. This nomogram considers patient age (continuous variable: 18-100 years), tumour size (continuous variable: 0.1-35 cm), tumour grade (categorical variable: I, II, and III), and tumour histology (categorical variable: leiomyosarcoma, dedifferentiated or pleomorphic liposarcoma, myxoid liposarcoma, malignant peripheral nerve sheath tumour, myxofibrosarcoma, synovial sarcoma, sarcoma of vascular origin, undifferentiated pleomorphic sarcoma, and other) of a single patient to estimate probability of OS (pr-OS) at 5 and 10 years after surgery. The Sarculator was previously retrospectively tested and validated on 1,452 and 2,300 patients, respectively [18] as well as further validated in a prospective randomised trial investigating perioperative anthracycline-based chemotherapy for high-risk STS of extremities and trunk wall. [22-24]

Ten-year pr-OS was predicted with Sarculator for each study participant and reported as a percentage. Participants were stratified according to three pr-OS categories as follow: high (pr-OS > 66%), intermediate (51< pr-OS ≤ 66) and low (pr-OS ≤ 51%). These survival groups were identified in a previous analysis that fitted Sarculator to data of patients with high-risk primary STS of extremities and trunk wall enrolled in a RCT comparing three and five cycles of perioperative chemotherapy. [24] The median pr-OS of this study (60%) was also used to generate two groups of patients in order to strengthen findings of previous analysis.

Statistical Analysis

Participants of the EORTC-STBSG 62931 were included in this analysis if their STS was located in an extremity or trunk wall. Patients with tumours located in other sites, including head and neck, abdominal wall, abdomen, retroperitoneum, and uterus were excluded. Also, patients were excluded when data needed for computing pr-OS with Sarculator were unavailable.

Statistical analysis has been conducted considering pr-OS as the variable of interest. OS and disease-free survival (DFS) were the outcome variables. OS was defined as the time from randomisation to last follow up or death for any cause. OS was estimated by means of the Kaplan-Meier method. [25] A Cox regression model was fitted to assess the association between the variable pr-OS (categorical variable) and OS. [26] Additionally, a multivariate Cox regression model including the main effects and the first-order interaction was used to study the interaction between pr-OS and treatment arm (i.e. chemotherapy vs observation). The same analyses were conducted considering disease-free survival (DFS), calculated as the time between randomisation to the first recurrence or last follow-up for non-recurring participants. Relative risk reduction and number needed to treat were calculated for both OS and DFS. [27]

All statistical analyses were carried out with the SAS software (version 9.4, SAS Institute Inc., Cary, NC).

Results

Participants randomised in the EORTC-STBSG 62931 trial were followed up for a median time of 96 months [interquartile range (IQR) 70 – 118 months]. The 8 year probability of OS and DFS was 0.58 [95% confidence interval (CI): 0.52–0.63] and 0.51 (95% CI: 0.46–0.57), respectively.

The EORTC-STBSG 62931 trial randomised 351 participants who underwent surgery for STS either to adjuvant chemotherapy (N=175) or to observation (N=176). For the purpose of this analysis, 61 participants were excluded for having their STS located in the head and neck (N=7), abdominal wall

(N=7), gastro-intestinal tract (N=6), retroperitoneum (N=15), uterus (N=11), and other sites (N=13). One patient did not have all the data variables recorded that are needed to calculate Sarculator predictions and one patient had a tumour other than a STS.

The clinical and pathologic data of the remaining 290 patients who were deemed eligible for this analysis are reported in Table 1 and the CONSORT diagram is reported in Figure 1. There were 142 participants in the chemotherapy arm and 148 in the observation arm of the study.

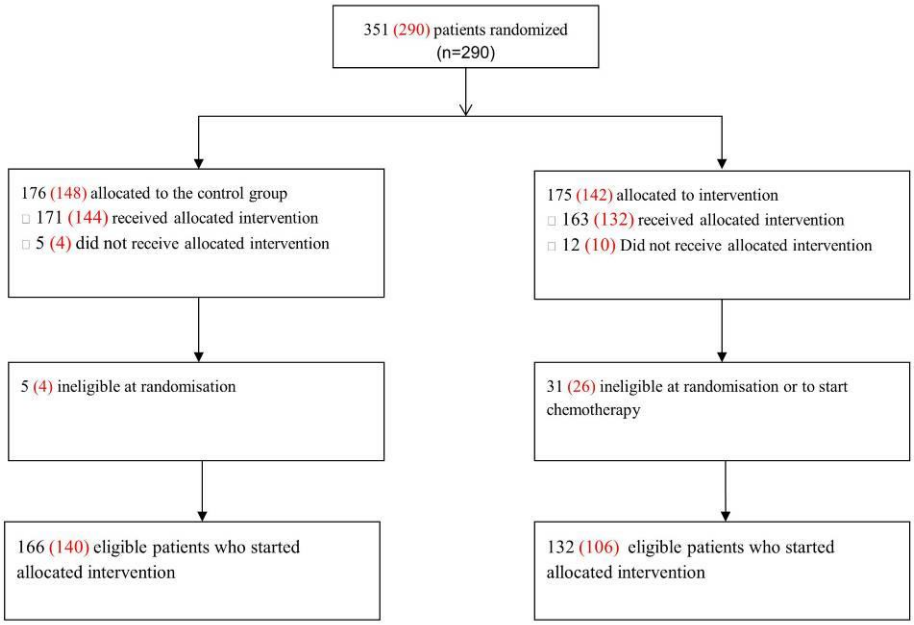
Table 1. Clinicopathological characteristics of 290 patients enrolled in EORTC-STBSG 62931 RCT and eligible for this analysis. MFH: malignant fibrous histiocitoma; UPS: undifferentiated pleomorphic sarcoma; MPNST: malignant peripheral nerve sheath tumour.

Variables	**ALL N=290**		**Observation N=148**		**Adjuvant chemotherapy, N=142**	
	N	**%**	**N**	**%**	**N**	**%**
Age, median (range) in years	49	(18-71)	49	(18-71)	49	(18-69)
Sex						
Male	167	57.6	85	57.4	82	57.8
Female	123	42.4	63	42.6	60	42.3
Tumour size, median cm(range)	12	(0.3-35)	8.25	(0.3-35)	7.2	(1.2-30)
Tumour grade						
GRADE I	12	4.1	6	4.1	6	4.2
GRADE II	115	39.7	59	39.9	56	39.4
GRADE III	163	56.2	83	56.1	80	56.4
Primary tumour site						
Lower limb	183	63.1	91	61.5	92	64.8
Upper limb	51	17.6	27	18.2	24	16.9
Pelvic girdle	24	8.3	13	8.8	11	7.8

Scapular girdle	18	6.2	10	6.8	8	5.6
Thoracic wall	10	3.5	5	3.4	5	3.5
Paraspinal muscles	4	1.4	2	1.4	2	1.4
Tumour histology						
MFH/UPS	81	28.0	49	33.2	32	22.5
Fibrosarcoma	12	4.1	4	2.7	8	5.6
Liposarcoma	50	17.2	30	20.3	20	14.1
Leiomyosarcoma	35	12.1	11	7.4	24	16.9
Rhabdomyosarcoma	6	2.1	3	2.0	3	2.1
Angiosarcoma	3	1.0	3	2.0	0	0.0
Synovial sarcoma	48	16.6	21	14.2	27	19.0
MPNST	14	4.8	7	4.7	7	4.9
Unclassified sarcoma	31	10.7	16	10.8	15	10.6
Other	10	3.4	4	2.7	6	4.2
Type of surgery						
Exarticulation	13	4.5	5	3.4	8	5.6
Compartimental	48	16.6	21	14.2	27	19.0
Wide	137	47.2	73	49.3	64	45.1
Marginal	79	27.2	41	27.7	38	26.8
Intralesional	1	0.3	1	0.7	0	0.0
Missing	12	4.1	7	4.7	5	3.5
Protocol radiotherapy						
Not performed	42	14.5	24	16.2	18	12.7
Performed	225	77.6	114	77.0	111	78.2

Not delivered	23	7.9	10	6.8	13	9.2

Figure 1. CONSORT diagram. Consort diagram. There were 290 patients eligible for this study of 351 in the trial, which are reported in red and brackets in the diagram. Numbers of included patients are in red.

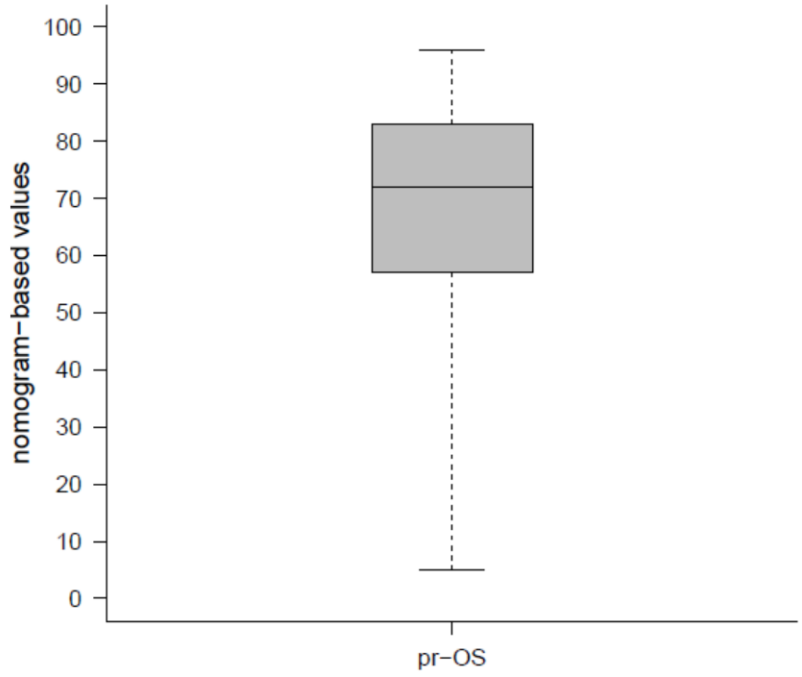


Overall survival

In the group of patients with extremity and trunk wall STS enrolled in this study administration of adjuvant chemotherapy was not associated with a OS benefit [Hazard ratio (HR) = 0.91, 95%CI 0.63–1.31], a finding that is consistent with the lack of survival benefit for chemotherapy observed in all patients enrolled in the EORTC-STBSG 62931 trial.

Data from each patient was fitted to the prognostic nomogram Sarculator. Predicted pr-OS ranged between 5% and 96%, with a median value of 72% (IQR 57-83%, Figure 2), showing wide variation across trial participants and highlighting the prognostic heterogeneity of patients enrolled in this trial.

Figure 2. Distribution of nomogram-based probability of overall survival (pr-OS). Box indicates the 25th and 75th percentiles of the distribution. The horizontal line inside the box indicates the median, and the whiskers indicate the extreme values.



Participants were then stratified into the three previously defined categories. [24] The majority of study participants (N=170 [58.6%], 90 Obs/80 Adj) were included in the high pr-OS group (pr-OS > 66). The remaining 68 (23.5%, 34 Obs/34 Adj) and 52 (17.9%, 24 Obs/28 Adj) fell into the intermediate (51 < pr-OS ≤ 66) and low (pr-OS ≤ 51) pr-OS category, respectively. Interestingly, distribution of patient and tumour characteristics used for calculated pr-OS (i.e. patient age, tumour grade, size, histology) did not differ between study treatment arm within each of the three pr-OS category (Table 2).

Table 2. Clinicopathological characteristics of 290 patients enrolled in EORTC-STBSG 62931 RCT and eligible for this analysis according to the study treatment arm and the three following Sarculator categories of probability of overall survival (pr-OS): high (pr-OS > 66%), intermediate (51< pr-OS ≤ 66), and low (pr-OS ≤ 51%). MFH: malignant fibrous histiocitoma; UPS: undifferentiated pleomorphic sarcoma; MPNST: malignant peripheral nerve sheath tumour.

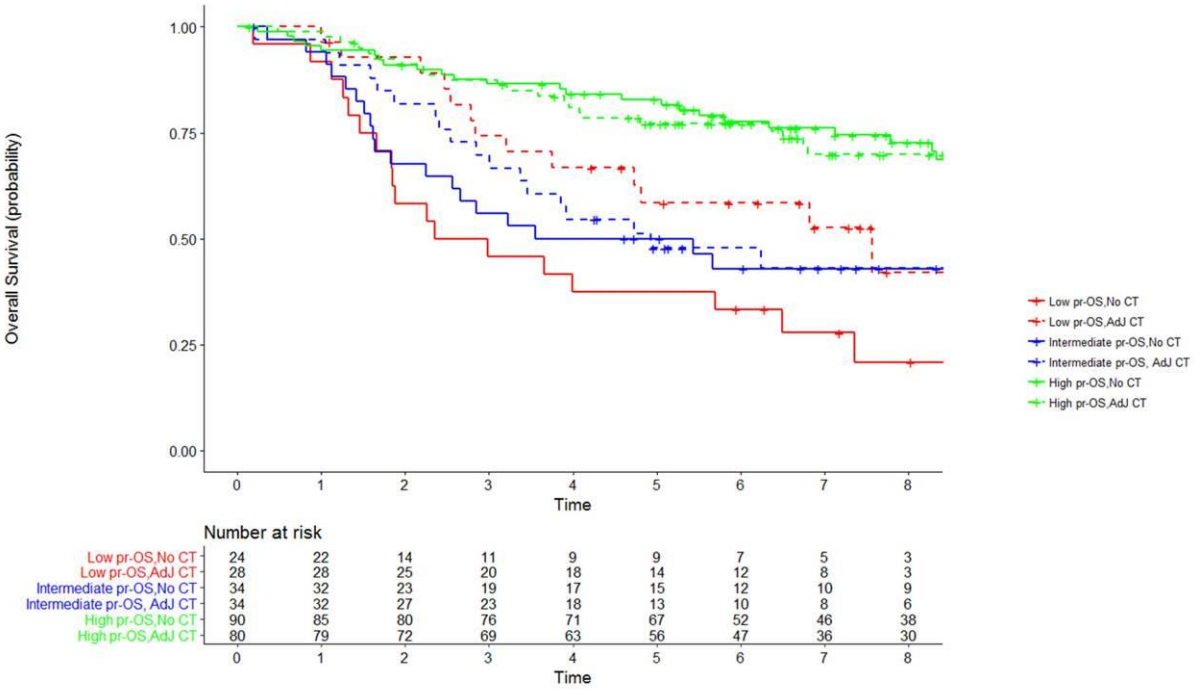
Variables	High pr-OS (>66%)				Intermediate pr-OS (51-66%)				Low pr-OS (≤51%)			
	Observation		Adjuvant chemotherapy		Observation		Adjuvant chemotherapy		Observation		Adjuvant chemotherapy	
	N	%	N	%	N	%	N	%	N	%	N	%
Age, median (range) in years	44 (37-57)		46 (34-54)		52 (39—58)		50 (40-58)		53 (46-60)		55 (43-63)	
Tumour size, median (range) in cm	6 (4-9)		5 (4-7)		11 (8-15)		10 (7-12)		14 (12-16)		14 (10-18)	
Sex												
Female	44	48.9	35	43.7	14	41.2	11	32.4	5	20.8	14	50.0
Male	46	51.1	45	56.3	20	58.8	23	67.6	19	79.2	14	50.0
Tumour grade												
GRADE I	6	6.7	6	7.5	0	0.0	0	0.0	0	0.0	0	0.0
GRADE II	46	51.1	46	56.3	7	20.6	8	23.5	6	25.0	3	10.7
GRADE III	38	42.2	29	36.2	27	79.4	26	76.5	18	75.0	25	89.3

Tumour histology												
MFH/UPS	32	35.6	15	19.7	8	23.5	4	11.8	1	4.2	3	10.7
Fibrosarcoma	0	0.0	2	2.6	0	0.0	2	5.9	1	4.2	0	0.0
Liposarcoma	21	23.3	12	15.8	6	17.6	6	17.6	1	4.2	1	3.6
Leiomyosarcoma	6	6.7	14	18.4	4	11.8	5	14.7	6	25.0	13	46.4
Rhabdomyosarcoma	1	1.1	2	2.6	0	0.0	0	0.0	1	4.2	1	3.6
Angiosarcoma	1	1.1	0	0.0	1	2.9	0	0.0	1	4.2	0	0.0
Synovial sarcoma	14	15.6	13	17.1	5	14.7	6	17.6	4	16.7	6	21.4
MPNST	2	2.2	7	9.2	4	11.8	2	5.9	1	4.2	3	10.7
Unclassified sarcoma	3	3.3	9	11.8	4	11.8	7	20.6	5	20.8	1	3.6
Other	10	11.1	2	2.6	2	5.9	2	5.9	3	12.5	0	0.0

The estimated probability of OS at the median follow-up time corresponding to the low (pr-OS $\leq$ 51), intermediate (51 < pr-OS $\leq$ 66) and high (pr-OS > 66) pr-OS were 0.33 (95%CI 0.18-0.48), 0.43 (95%CI 0.30-0.55), and 0.71 (95%CI 0.63-0.78), respectively. Cox regression analysis showed that patients belonging to the low (HR 2.90, 95%CI 1.84-4.57) and intermediate (HR 2.69, 95%CI 1.75-4.12, P<0.001) pr-OS category were at statistically significant higher risk of death compared to the patients classified in the high pr-OS category.

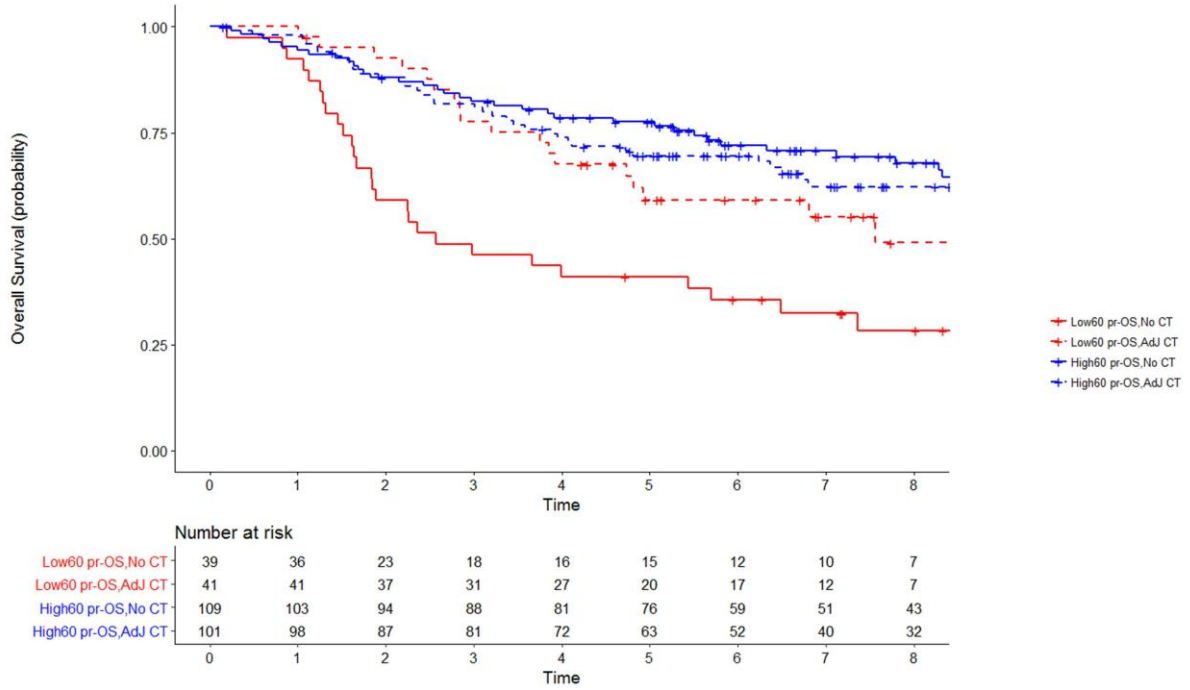
Study treatment arm was then factored in the analysis. Figure 3 reports the pattern of OS by jointly considering the variables pr-OS and treatment arm of this RCT. Adjuvant chemotherapy halved the risk of death in patients with low Pr-OS (HR=0.46, 95%CI 0.23-0.94). This effect was not detected in the intermediate (HR=1.00, 95%CI 0.53-1.88) and high Pr-OS categories (HR=1.08, 95%CI 0.61-1.90). Interestingly, the estimated 8-yr in the low pr-OS group resulted in a 21.3% 8-yr absolute risk reduction of death (8-yr OS: 42.1% and 20.8% for Adj and Obs, respectively) and in a number needed to treat (NND) of 4.69.

Figure 3.OS according to three pr-OS categories established in a previous study [24] and the EORTC-STBSG 62931study treatment arms.



In order to strengthen findings achieved investigating the three pr-OS groups and identify patients who might benefit from adjuvant chemotherapy, a further analysis was conducted categorising patients in two groups, low (pr-OS < 60%) and high (pr-OS ≥ 60%) predicted survival (Table 3). The value 60% represents the median predicted 10-year OS of the previous above mentioned study. 24 As expected, patients with a low predicted OS (N=80) were at greater risk of death compared to patients with higher predicted OS (N=210, HR=2.13, 95%CI 1.47-3.09). Consistently with the analysis conducted using three categories, there was a statistically significant reduction of the risk of death when adjuvant chemotherapy was used in the group at low predicted survival (HR=0.50, 95%CI 0.30-0.90) while this difference was not detected in patients with high OS (HR=1.20, 95%CI 0.75-1.91, Figure 4).

Figure 4. OS according to two categories according to the median survival value (10-year predicted OS: 60%) and the EORTC-STBSG 62931 study treatment arms.



Disease-free survival

The association between risk stratification according to Sarculator, effectiveness of chemotherapy and DFS of study participants was also examined to determine the value of chemotherapy for patients with lower predicted survival. This analysis confirmed a DFS benefit for chemotherapy in the low P-OS group (HR=0.46, 95%CI 0.24-0.89) but not in the intermediate (HR=0.74, 95%CI 0.41-1.34) and high P-OS (HR=0.90, 95%CI 0.54-1.50) groups, leading to a 21.0% 8-yr absolute risk reduction for adjuvant chemotherapy (8-yr DFS: 33.5% and 12.5% for Adj and Obs, respectively) and a NND of 4.76 (Figure 5). Consistently with the OS analysis, when patients were grouped according to the cut off of 60%, there was again a statistically significant reduction of risk of recurrence when adjuvant chemotherapy was used in the group at low predicted survival, i.e. <60% (HR = 0.49, 95%CI 0.28-0.85) while this difference was not detected in patients with high pr-OS (HR = 0.95, 95%CI 0.62-1.44).

Figure 5. DFS according to three pr-OS categories established in a previous study [24] and the EORTC-STBSG 62931 study treatment arms.

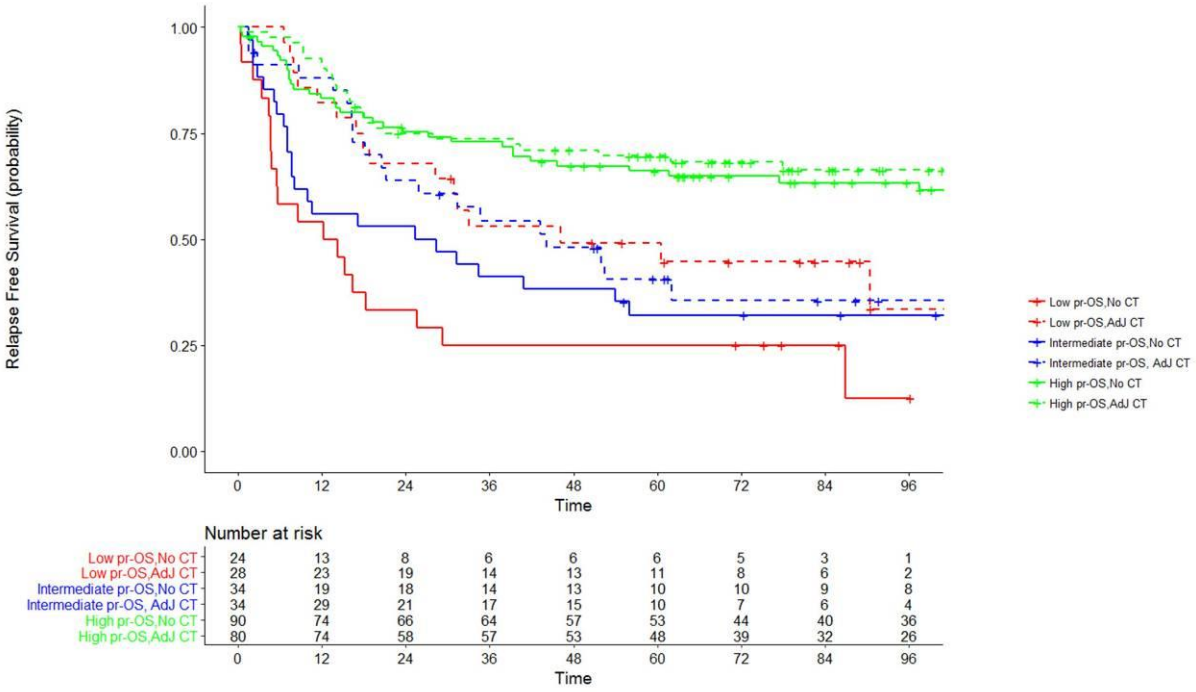


Table 3. Clinicopathological characteristics of 290 patients enrolled in EORTC-STBSG 62931 RCT and eligible for this analysis according to the study treatment arm and the two following Sarculator categories of probability of overall survival (pr-OS): pr-OS > 60% and pr-OS ≤ 60%; MFH: malignant fibrous histiocitoma; UPS: undifferentiated pleomorphic sarcoma; MPNST: malignant peripheral nerve sheath tumour.

Variables	pr-OS > 60%				pr-OS < 60%			
	Observation		Adjuvant chemotherapy		Observation		Adjuvant chemotherapy	
	N	%	N	%	N	%	N	%
Age, median (range) in years	45 (37-57)		46 (34-54)		52 (43-59)		55 (44-63)	
Tumour size, median (range) in cm	7 (4-10)		6 (4-9)		14 (10-16)		12 (9-17)	
Sex								
Female	51	46.8	41	40.6	12	30.8	19	46.4
Male	58	53.2	60	59.4	27	69.2	22	53.6
Tumour grade								
GRADE I	6	5.5	6	5.9	0	0.0	0	0.0
GRADE II	51	46.8	50	49.5	8	20.5	6	14.6
GRADE III	52	47.7	45	44.6	31	79.5	35	85.4
Tumour histology								

MFH/UPS	38	34.8	18	17.9	3	7.7	4	9.8
Fibrosarcoma	0	0.0	4	4.0	1	2.6	0	0.0
Liposarcoma	25	22.9	16	15.8	3	7.7	3	7.3
Leiomyosarcoma	9	8.4	16	15.8	7	17.9	16	39.0
Rhabdomyosarcoma	1	0.9	2	2.0	1	2.6	1	2.4
Angiosarcoma	1	0.9	0	0.0	2	5.1	0	0.0
Synovial sarcoma	17	15.6	15	14.8	6	15.4	10	24.4
MPNST	4	3.7	9	8.9	3	7.7	3	7.3
Unclassified sarcoma	3	2.8	15	14.8	9	23.1	2	4.9
Other	11	10.0	6	6.0	4	10.3	2	4.9

Discussion

This was an unplanned analysis conducted on individual data in a high-risk subgroup of patients enrolled into the EORTC-STBSG 62931 RCT. [14] That study had failed to detect any benefit for adjuvant chemotherapy over observation in the overall study population with primary localized STS. Within the high-risk subgroup, in contrast to the overall study conclusions, adjuvant chemotherapy was associated with a longer OS and DFS in comparison to the control arm. The subgroup was selected to overlap high-risk patients benefiting the most from a tumour response within the ISG-GEIS RCT on three vs five cycles of neoadjuvant chemotherapy with epirubicin plus ifosfamide in STS. [22-24]

The gain in long-term survival rate averaged 20%, a figure that is within the same range as that associated with epirubicin plus ifosfamide at the interim analysis of the last ISG-GEIS-FSG-1001 RCT. [17] This trial, which compared three cycles of epirubicin plus ifosfamide and three cycles of a histology-tailored regimen in the neoadjuvant setting, recruited 287 patients and was discontinued at a median follow-up of 12 months as the third futility analysis showed that patients treated with epirubicin plus ifosfamide had better prognosis (4-year DFS 62% vs 38%; 4-year OS 89% vs 64%). Indeed, such a difference contrasts with the negative results of the EORTC-STBSG 62931 RCT, [14] although two main discrepancies were that in one trial chemotherapy was administered pre-operatively, [17] while it was given as post-operative adjuvant in the other, [14] and that the two studies implemented different chemotherapy schedules. However, it is intriguing that when a high-risk subgroup of patients is singled out in this EORTC-SBTBSG trial, the results may look much less discordant. A major difference between the two studies lies in the eligibility criteria. The ISG-GEIS-FSG-1001 RCT, [17] following the previous ISG-GEIS study, [22-24] selected a higher-risk group of patients compared to the EORTC-STBSG 62931 trial, [14] who were affected by undifferentiated pleomorphic sarcoma, high-grade myxoid liposarcoma, synovial sarcoma, malignant peripheral nerve sheath tumours, and leiomyosarcoma of the extremities and trunk wall. The dose of ifosfamide is another difference among the two studies (9 g/m2 in the ISG-GEIS-FSG 1001 trial and 5 g/m2 in the EORTC

62931 trial). However, its effect may have been limited, especially in the light of what on the contrary was a major similarity of the two trials, i.e. the full dose of anthracycline.

This subgroup analysis exploits the value of prognostic risk stratification using the nomogram Sarculator in the EORTC-STBSG 62931 RCT. [14] Nomograms have been recently included in the 8th edition of the AJCC staging manual for several tumours, and they are expected to improve prognostic risk assessment as compared to the AJCC TNM stage. [21] Apparently Sarculator identifies patient risk very effectively, in a way that is likely to work for patient selection for adjuvant treatments, although this nomogram was not available when studies on adjuvant and neoadjuvant chemotherapy were designed. The inclusion criteria of these studies were predominantly based on malignancy grade, while Sarculator also includes tumour size as a continuous variable and histological sarcoma subtype as well as patient age. These are easy-to-obtain and reproducible clinical characteristics which also compare favourably with the conventional AJCC staging system. They should be incorporated in future studies for selecting homogenous populations of patients. In addition, recent data showed how the inclusion of biological signatures, such as CIN-SARC [28, 29] or genomic index [30] in sarcomas may improve further the prognostic accuracy of these tools. An effort to validate prospectively these biological signatures both as prognostic and predictive biomarkers is presently underway. If their value is proven they will be added to available nomograms, in order to refine them further.

Results from this subgroup analysis have some limitations. First, the selected subgroup of patients is relatively small (N=52), though, by using a different cut-off the number of patients was increased (N=80), and a difference was still observed. Second, this was an unplanned analysis of a RCT, though it used a validated prognostic tool, the Sarculator. These findings should be viewed as exploratory and hypothesis-generating. However, they may help reconcile the results of different randomised trials conducted in STS. Indeed, available meta-analyses on all published RCTs favoured adjuvant chemotherapy. [18, 19] This may be viewed as suggesting that a subgroup of patients may benefit from

chemotherapy and that this subgroup is defined by a high-risk of relapse. Clearly, a high-risk entails a higher absolute benefit for chemotherapy, under the assumption of the same proportional risk reduction. Probably, however, high-risk STS are also more sensitive to anthracycline-based chemotherapy, as long as their malignancy grade is higher on average. As a matter of fact, the same proportional reduction did not fully apply to patients with an intermediate or low predicted risk of recurrence.

In conclusion, this unplanned subgroup analysis of the EORTC-STBSG 62931 RCT may help interpreting conflicting results of available RCTs, especially if the final analysis of the ISG-GEIS-FSG-1001 RCT confirms the survival benefit for anthracycline-based chemotherapy detected at the interim analysis. They may also help to view results provided on neoadjuvant chemotherapy as less necessarily due to the placement of chemotherapy before surgery. These data emphasise that results of RCTs should always be interpreted and viewed in the light of all the available evidence.

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CHAPTER 5

Genetic insights of dedifferentiation in retroperitoneal liposarcoma: a comparison of rabdomioblastic and myogenic differentiation

Unpublished data

Introduction

Well differentiated and dedifferentiated liposarcomas (WD and DD) represent the commonest tumour of the retroperitoneal space and are among most common sarcomas [1]. WD and DD retroperitoneal liposarcoma (RLPS) classically pursue a different clinical course [2, 3]. WD RLPS are characterised by a 10-year survival of approximately 80%, although these tumours have a significant risk of local recurrence which can lead to patient death [4]. DD RLPS can behave either indolently or be rapidly fatal as they lead to spread of metastatic disease in up to 40-50% of patients [5]. Tumour differentiation, which plays a significant role in the determination of tumour grade in sarcoma and is embedded in prognostic models such as Sarculator (Chapters 3 and 4) [6, 7], has also prognostic implications according to different specific cell lineages [8, 9]. The presence of a specific dedifferentiated component towards a smooth muscle (i.e. myogenic) or skeletal muscle (i.e. rhabdomyoblastic) represents a powerful independent prognostic marker [5] with five-year overall survival rates of 42% and 29% for DD RLPS with myogenic and rhabdomyoblastic differentiation, respectively. Remarkably, patients with rhabdomyoblastic differentiation have little chances of showing any survival benefit to currently available treatment strategies. Genetic features underpinning these biological behaviours have not been investigated and have potential implications for patient treatment strategies.

In order to deeply understand the molecular determinants of rhabdomyoblastic aggressiveness and differences with myogenic differentation, we conducted a deep genome sequencing analysis in DD RLPS.

Materials and methods

Patients

This retrospective study was performed using tumour samples from patients who underwent surgery for a primary DD RLPS at Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, between 2008 and 2015. Patients were included if the following criteria were met: 1) surgery performed with curative intent (R0/R1 resections); 2) diagnosis of dedifferentiated liposarcoma showing either myogenic (M-DD), rhabdomyoblastic (R-DD), or neither myogenic nor rhabdomyoblastic dedifferentation (NM/NR-DD) and no other differentiation types; 3) MDM2 amplification assessed with in situ hybridization analysis; 4) availability of formalin fixed paraffin embedded specimen of tumour tissue; and 5) patient consented for having tumour specimen stored in the Institutional biobank and eventually analysed; 6) sufficient available tissue for RNA extraction. The following patient and tumour data were retrieved from a prospectively maintained database: patient sex and age at the index surgery, date of the index surgery, tumour malignancy grade, histology, and dedifferentiation, date of tumour recurrence classified as local or metastatic if any, date of last known with patient status. Pathological diagnosis were performed by pathologists with expertise in soft tissue tumours (S.L.R and P.C.)

Tumour samples.

All cases demonstrated *MDM2* amplification by in situ hybridization (ISH). The dedifferentiated component was graded according to the FNCLCC grading system [6, 7], as previously described [5]. All cases were stained for the following markers: 1) the myogenic markers smooth muscle actin-α (1A4), caldesmin, calponin, and desmin; and 2) the rhabdomioblastic marker myogenin. Definitions of

myogenic and rhabdomyoblastic differentiation were detailed elsewhere [5]. Briefly, myogenic DD was defined by the expression of one or more of the above mentioned markers in 10% of more tumour cells, and rhabdomyoblastic DD was defined by the presence of myogenin decorating any nucleus or neoplastic cells, as previously described. Cases which did not show neither myogenic markers nor myogenin as well as no other differentiation lineages were defined “non-rhabdomyoblastic and non-myogenic” (NRNM). Tumour slides were reviewed by a soft tissue pathologist (SLR) and areas from non-neoplastic tissue, WD component, and DD tumour component were selected for RNASeq analysis. An haematoxylin and eosin (H&E) control slide was performed in order to assess the quality of the material after cutting formalin fixed paraffin embedded (FFPE) blocks for RNASeq analysis.

Immunohistochemistry analysis and scoring

Unstained serial sections of 3 µm were cut from FFPE blocks to perform the immunohistochemical analysis described below. Slides were evaluated and scored by two sarcoma-dedicated pathologists in a blinded fashion (SR and APD). According to the RNAseq data and in order to explore the presence of immune infiltrate immunohistochemistry (IHC) staining was performed to identify lymphocytic (CD3, CD4, CD20), myelomonocitic (CD14, CD57, CD209) markers as well as CD34. Tumours which showed high positivity for CD3 were score also for Granzyme, Foxp3, CD8, and HLA1.

A quantitative and functional 5-class scoring was implemented. Presence of aggregates was assessed at scanning magnification (2,5x) as follow; positivity was considered “focal” if aggregates of immune cells were present in <50% of the specimen and“diffuse” when >50%. If present, the distribution pattern of aggregates was analyzed and recoded as diffuse (score 4) or focal (score 3). If no aggregates were visible at 2,5x the search of immune infiltrate proceeded at higher magnification and the presence and the distribution pattern of isolated immune cells was recorded as follow: present diffuse (score 2), present focal (score 1), and absent (score 0). To score CD34 neoplastic cell positivity, the 5 class scoring system used for the macrophagic markers was choosen.

RNA Extraction and whole transcriptome sequencing

Total RNA was isolated from Fresh-Frozen (FF) and Formalin Fixed, Paraffin-Embedded (FFPE) samples using the RNeasy FFPE Kit (Qiagen) according to the manufacturer's instructions.

Whole-transcriptome sequencing (RNA-seq) was performed on 18 FFPE DD liposarcomas (9 M-DD and 9 R-DD) together with the corresponding WD area (7 WD associated to M-DD and 5 WD to R-DD).

RNA-sequencing libraries were prepared as previously described [10] and sequenced on an Illumina HiSeq 1000 platform (Illumina, San Diego, CA, USA) to an average depth greater than 50 million paired-end reads per sample.

Data analysis

Distribution of IHC markers across M-DD and R-DD was analysed with Fisher's exact test.

Data from RNAseq analysis were evaluated for quality with the QoRts package [11] and aligned to the GRCh38.p2 genome assembly using STAR2.5.2a [12]. Htseq-count was used to assign reads to genes [13]. DESEq2 was used to exploratory data analysis and differential expression analysis of protein-coding genes. Gene enrichment and functional annotations were performed by using different suites: Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Redwood City, CA) ToppGene [14], GSEA [15], and Webgestalt [16].

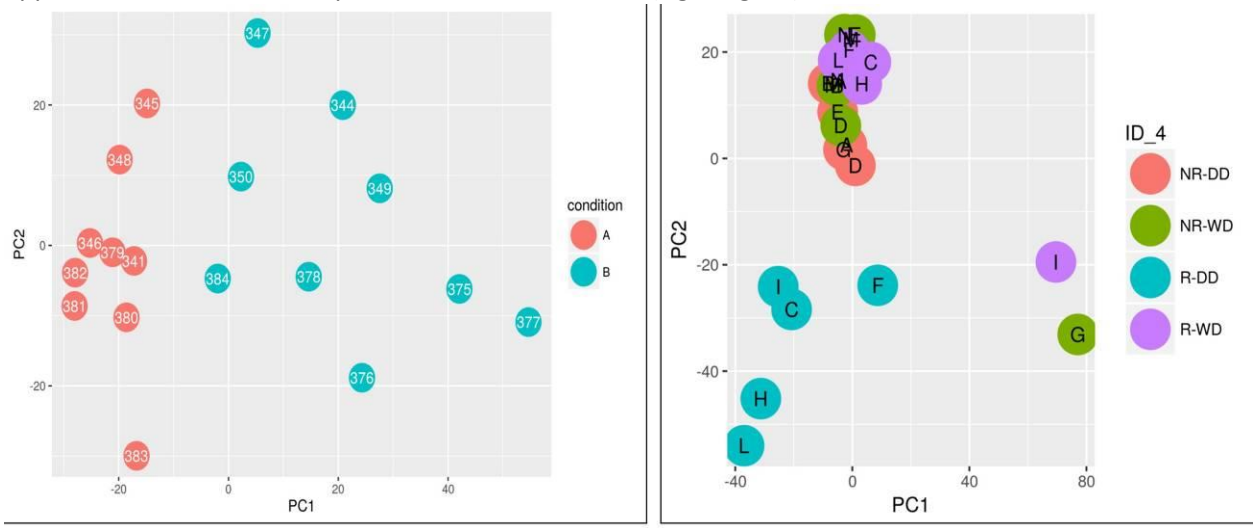
Results

Tumour samples from 22 patients met the inclusion criteria. There were 9 samples for each M-DD and R-DD tumours, while 4 samples were characterised by NM/NR-DD.

R-DD clustered separately from M-DD and WD component

Principal component analysis (PCA) of the transcriptional profile showed that R-DD and M-DD grouped separately. Moreover, when R-WD, M-WD, R-DD, and M-DD components were compared, both WD components clustered together with M-DD, while R-DD grouped separately (Figure 1).

Figure 1. Principal component analysis for M-DD and R-DD tumours (left figure). Same analysis has been applied also to the WD components of these tumours (right figure).



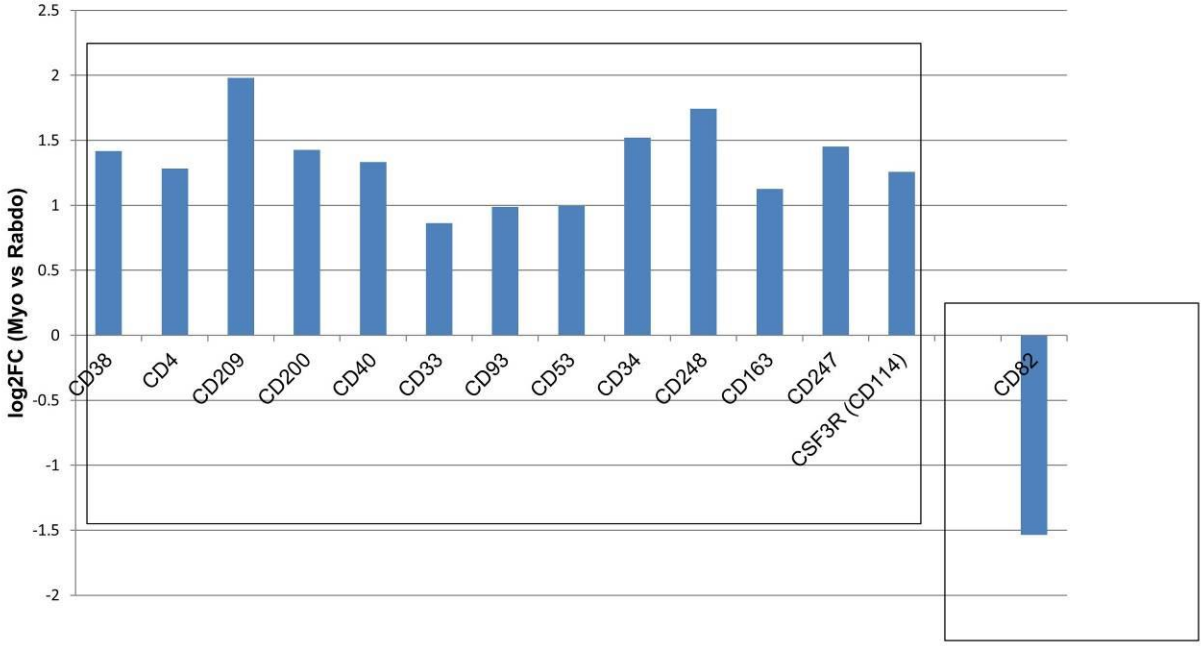
WD and DD tumours had different gene expression

The analysis of differentially expressed genes indicated that genes related to increased cell proliferation and reduced differentiation were strongly marked the transition from WD to DD.

Gene of 'immune' and 'inflammatory response' were over-expressed in M-compared to R-DD

An enrichment in genes belonging to skeletal muscle development was detected in R-DD as well as a marked augment of terms related to immune and inflammatory responses in M-DD subgroup at GO analysis and Gestalt. Specific antigens of immune system and inflammation were differentially expressed in the two subgroups with a generally higher expression in M-DD except for CD82 that was down-regulated in M-DD compared to R-DD (Figure 2).

Figure 2. Genes related to inflammation and immune system with different expression in M-DD compared to R-DD tumours.



Also, IPA and GSEA analyses revealed that genes induced by INF, TNF and IL2 were activated in M-DD. There was an activation, though to a lesser significant extent, also of the downstream of TGF-beta. Moreover, we found that genes belonging to angiogenesis pathways were more represented in M-DD compared to R-DD suggesting that lack of vessels may explain the underrepresented immune infiltrate in R-DD tumours.

Myelomonocytic cell markers are less represented in R-DD tumour

Lymphocytes were present in the vast majority of samples (8/9 in M-DD and 7/9 in R-DD) either as tertiary lymphoid structures (TLS, 0/9 in M-DD and 1/9 in R-DD), tumour infiltrating lymphocites (TILS, 3/9 in M-DD and 3/9 in R-DD) or both (5/9 in M-DD and 3/9 in R-DD).

Selected CD antigens (CD3 for T cell, CD57 for NK cell, CD14 for dendritic cell, CD209 for monocyte-derived dendritic cell and CD20 for B cell) were used for immunohystochemical analysis to explore the role of different immune cells populations. Staining for CD4, CD34 and CD163 was also performed to

validate the RNA seq results. In general, M-DD scored higher score (4) compared to R-DD. For this reason and in order to appreciate the potential difference we statistical analysis were performed using comparing score 4 and scores 0-3.

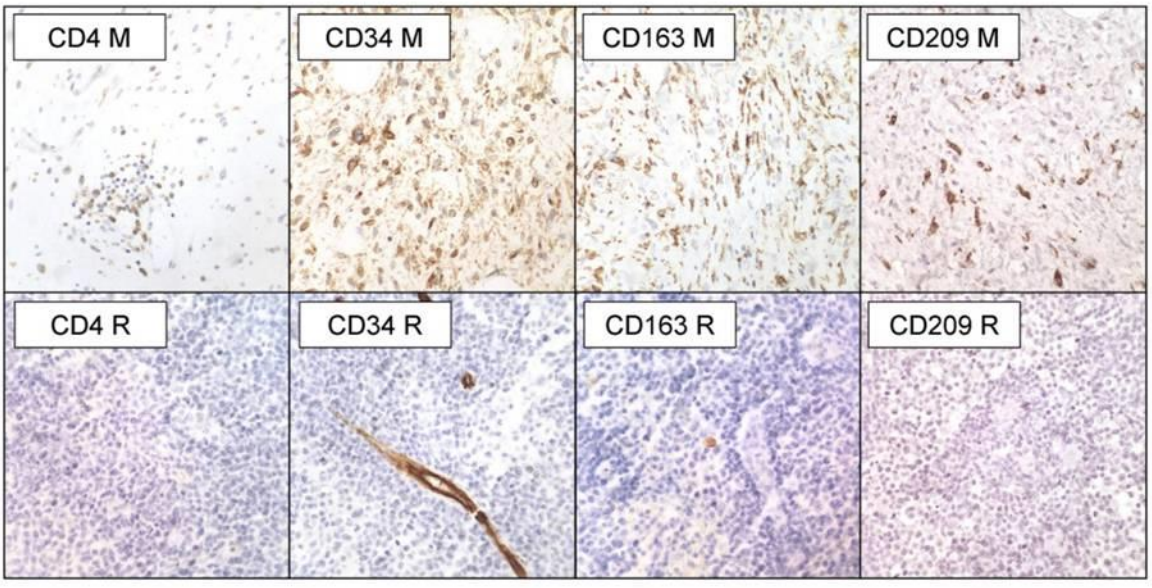
CD3 was expressed in 5/9 M-DD and 2/9 R-DD. CD20 was poorly represented (2/9 M-DD and 0/9 R-DD). Interestingly, this result was maintained also when scores 3 and 4 were grouped. CD57 was expressed in only one sample per group (1/9 M-DD and 1/9 R-DD). CD14 slightly higher in M-DD (8/9 M-DD and 6/9 R-DD), while CD209 was more expressed in M-DD (8/9) compared to R-DD (3/9) (*p*=X). CD4 was more frequently identified in M-DD (4/9) compared to R-DD (1/9). The same pattern was observed for CD34 (4/9 M-DD and 0/9 R-DD). We did not identify a difference in CD163 (3/9 M-DD and 3/9 R-DD). However, for CD34 and CD163 when scores 3 and 4 were grouped the trend for CD34 was more evident, and CD163 emerged as differently expressed (9/9 M-DD and 6/9 R-DD).

Table 1. Immunohistochemistry analysis of immune infiltrate according to differentiation type.

Variables		M-DD		R-DD		P-value
		N	%	N	%	
CD 3	0-3	4	44.4%	7	77.8%	0.335
	4	5	55.6%	2	22.2%	
CD 20	0-3	7	77.8%	9	100.0%	0.471
	4	2	22.2%	0	0.0%	
CD 57	0-3	9	100.0%	8	88.9%	1.0
	4	0	0.0%	1	11.1%	
CD14	0-3	0	0.0%	3	33.3%	0.206
	4	9	100.0%	6	66.7%	
CD4	0-3	5	55.6%	8	88.9%	0.294
	4	4	44.4%	1	11.1%	
CD34	0-3	5	55.6%	9	100.0%	0.082
	4	4	44.4%	0	0.0%	
CD163	0-3	6	66.7%	6	66.7%	1.0
	4	3	33.3%	3	33.3%	
CD209	0-3	0	0.0%	6	66.7%	0.009
	4	9	100.0%	3	33.3%	

In order to explore Tcells infiltrate, we scored those samples that showed a high expression of CD3 (score 4; *N* = 7, 5 M-DD and 2 R-DD) for CD8, FOXP3, Granzyme and HLA type I. CD8 was high in 4/5 M-DD and 1/2 R-DD. A superimposable pattern was observed for HLA type I (4/5 M-DD and 2/2 R-DD). FOXP3 did not show an higher expression in any samples. Staining for granzyme was high in one M-DD sample.

Figure 3. CD4, CD34, CD162 and CD209 staining in M-DD and R-DD tumours.



Discussion

This analysis revealed a profound transcriptional reprogramming and a resetting of the immune infiltrate in retroperitoneal liposarcoma harbouring rhabdomyoblastic dedifferentiation compared to those with myogenic differentiation.

Grade and type of differentiation in retroperitoneal liposarcoma are among the most meaningful prognostic factor for patient survival. Rhabdomyoblastic differentiation, a rare occurring event in these tumours, is associated with worse prognosis compared to other differentiation types with high grade primary rhabdomyoblastic liposarcoma leading to patients death within a year after surgery in virtually all patients. Conversely, myogeneic differentiation is associated with better patient outcomes. Here, we explored the potential differences between myogenic and rhabdomyoblastic DD which are the most frequently detected DD types in retroperitoneal liposarcoma. Analyses of 22 primary tumours treated with surgery showed a profoundly different genetic background of R-DD and M-DD RPLS. Such differences were not detected for the WD counterpart of these two differentiation types, leading to the hypothesis that R-DD can be acquired by RPLS later in the disease progression compared to both M-DD and WD components.

Intriguingly, M-DD was associated with a specific pattern of expression of genes belonging to inflammation and immune response, and we explored the expression of specific immune subpopulation with the aim of both validating results of RNA sequencing analysis and exploring the significance of immune infiltrate in RPLS.

Evidence from this analysis is limited by the relatively small number of analysed tumour samples. However, this study represents an unique source of information for the rare incidence of retroperitoneal sarcoma and the comparison two different differentiation tumour types. Also, results for immune infiltrate in retroperitoneal liposarcoma obtained at RNA sequecing analysis have been validated with IHC and not with functional experiments and therefore these findings are hypothesis generating and will require further analysis.

Other gene sequencing studies investigated the genomic landscape of liposarcomas, mainly aimed at identifying actionable mutations [17-21]. These studies showed high amplification/ deletion rate but a low frequency of recurrent mutations in both WD and DD liposarcomas. However, analyses were conducted merging together different liposarcoma histology, making clinical interpretation of the results and translation to clinical practice challenging. The current World Health Organization classification of soft tissue and bone tumours identified atypical lipomatous tumour/WD liposarcoma, DD liposarcoma, myxoid liposarcoma, and pleomorphic liposarcoma as different tumours characterised by distinctive clinical, histological and molecular features [22-24]. This is not to mention differences in these tumours according to their location, which has been acknowledged in the recent new AJCC staging manual where different staging system apply according to the primary tumour site [25]. Our study differs from these analysis in that it reported on a selected group of primary chemotherapy-untreated DD liposarcoma originating from the retroperitoneum, and specifically investigated different differentiation types.

The role of the immune system in inception and progression of mesenchymal tumours has been marginal for decades and believed to be restricted to specific sarcoma histologies characterised by specific immune infiltrate, such as myxoinflammatory fibroblastic sarcoma and dendritic cell tumours. The renaissance of immunotherapy observed in the last years in haematological and several solid tumours [26] has prompted further investigations in soft tissue sarcomas [27, 28]. Interestingly, early results showed that sarcomas harbouring tumour dedifferentiation may be more likely to develop a response to drugs targeting immune checkpoint inhibitors [29, 30]. For instance, the recent trial SARC-028 which has tested anti-PD1 agent pembrolizumab in metastatic sarcomas, showed anti-cancer activity in undifferentiated pleomorphic sarcoma and dedifferenetiated liposarcoma [31]. Consistently, combination of nivolumab, another PD-1 agent, and the first-in-class immune checkpoint inhibitor ipilimumab, an anti-CTLA4 agent, achieved better objective responses in dedifferentiated tumours in

another study [32]. The generalizability of these findings to patients with DD RPLS, either in the metastatic and in the adjuvant setting, is matter of speculation. Other promising immune therapy strategies, such as NY-ESO vaccination are not expected to be an option in DD liposarcoma, were only approcimately 5% of patients showed expression of this tumour antigen [33]. Few data are also available on immune infiltrate in these tumours. For instance, an interesting report on PD-1 and PD-L1 expression in sarcoma showed that these markers were positively and negatively expressed, respectively, in the majority of patients with WD and DD liposarcoma, which were considered together in this analysis with no tumour site characterization [34]. Tseng at al. showed the presence of tertiary lymphoid structures which were identified in 50% of FFPE DD retroperitoneal liposarcoma tumors treated with surgery and were associated to worse patient survival [35, 36]. This was a small study including patients with a wide spectrum of retroperitoneal liposarcoma. In our study lymphocytes either in TLS or TILS were present in approximately 50% of M-DD and 30% of R-DD. To the best of our knowledge, this is the first study on DD RPLPS accounting for myogenic and rhabdomyoblastic component, which in fact have markedly different biological aggressiveness.

In our study, although IHC analysis for expression of selected immune system markers did not reach statistical significance for all markers but CD209, sample size did not allow to reach robust conclusions. Obtained results generated the hypothesis that an enrichment of immune infiltrate existed in in M-DD compared to R-DD. Both in the RNA-seq and IHC analysis sustained a representation of both innate and adaptive immunity in M-DD. The functional role of this subgroups of cells remains unknown. We indirectly explored markers of T cells activation and found that FOXP3 and Granzyme were not highly expressed, suggesting low activity of CD8+ T cells. All these results, though exploratory, should be viewed together with clinical data describing outcomes of patients with DD RLPS. M-DD is associated with better patient survival, although it is not possible to draw a conclusion on the possible role of the immune system, these findings foster conduction of further research to investigate interactions

between DD component of these tumours and the immune system and the potential implications for disease progression and treatment strategies. In this regard, the upregulation of CD82, a marker of stemness, in R-DD lead to speculate that rhabdomioblastic dedifferentiation may represent a continuum with M-DD and that highly dedifferentiated RLPS can establish immune escape strategies that limit infiltrate. To further support this hypothesis we identified lessed angiogentic genes actiated in the R-DD tumours, which may lead to speculate abour the phenomenon of the so called 'immune desert' in thse tumours.

In conclusion this analysis supports considering myogenic and rabdomioblastic DD RLPS as two separate entities. We hypothesized a different role for the immune system in these tumours with potential implication for treatment strategies which will require further investigations. Implications for treatment strategies in these tumours are to be explored.

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CHAPTER 6

Controversies and perspectives in the staging and treatment of patients with lymph node metastasis from melanoma

Pasquali S, Spillane A. Contemporary controversies and perspectives in the staging and treatment of patients with lymph node metastasis from melanoma, especially with regards positive sentinel lymph node biopsy. Cancer Treat Rev 2014;40:893-9.

Introduction

Melanoma is one of the deadliest types of skin cancer. The incidence of skin melanoma has been increasing over the past 30 years worldwide at a pace greater than any other malignancy, which makes its management a key issue for national health care systems [1].

Melanoma is usually cured in the early stages with simple surgical removal of primary tumor [2, 3]. Conversely, when melanoma has spread such that there are lymph node (LN) metastasis it becomes a management challenge for surgical, medical, and radiation oncologists [4]. Performing sentinel LN biopsy (SLNB) and completion LN dissection (CLND) for SLNB-positive patients are both still debated, although the results of the Multicenter Selective Lymphadenectomy Trial (MSLT) suggests therapeutic value in patients with LN metastasis from intermediate thickness melanoma by earlier removal of the involved nodes [5-7]. After surgery, the survival benefit associated with the only approved adjuvant treatment, interferon alpha, is considered dubious by many medical oncologists [8], as it is the effectiveness of radiation therapy, which only adds benefit in the control of the regional LN field [9]. Important clinical trials of immune modulating drugs and targeted therapies are currently under way or due to report soon [10]. In this regard, the heterogeneous survival observed in patients with LN metastasis (90-13% after five years [11, 12]) exemplifies the challenge of accurately stratifying AJCC stage III patients for adjuvant therapy clinical trials. This review will pinpoint controversial issues regarding the staging and treatment of melanoma patients with LN metastasis, present a summary of important and potentially practice changing ongoing research and provide a commentary on what it all means at this point in time.

Search strategy and selection criteria

References for this Review were identified through searches of PubMed with the search terms "melanoma", "lymph node", "metastasis", "adjuvant" and "post-operative" and through searches of the authors' own files. Only papers published in English were reviewed. The final reference list was generated on the basis of originality and relevance to the broad scope of this Review.

Sentinel lymph node biopsy and completion lymphadenectomy

The MSLT

The presence of metastasis identified at SLNB has been recognized as the most important prognostic information for melanoma patients with no clinical evidence of metastatic disease and this has been included in the AJCC TNM staging system since the sixth edition in 2001 [13]. Conversely, the therapeutic effectiveness of SLNB has not been fully proven. Recently, the final results from the only randomized trial that has compared SLNB and nodal observation, the MSLT, have been published [6]. The trial, which was embraced by clinicians especially in Australia, was started in 1994 and enrolled 2,001 patients over eight years. The third interim analysis was published in 2006 and encompassed 1,296 patients with intermediate thickness melanoma (defined as 1·2-3·5mm thick primary) and showed that patients treated with SLNB had a better disease-free survival but similar overall survival compared to patients who underwent nodal observation [5]. That interim report assessed survival differences in patients with LN metastasis (either SLNB positive or having a recurrence in the regional LN field) showing that performance of an early completion lymphadenectomy was associated with a better survival compared to a delayed therapeutic lymphadenectomy performed for a regional LN recurrence. The significance of these results were widely debated and the effectiveness of SLNB for improving patient survival was

questioned on the basis of the main result of the trial, which is the lack of therapeutic value of SLNB in the whole group of intermediate thickness melanoma patients. [7]

The final analysis of the trial was expected to report on all the enrolled patients followed-up for a longer time, but it reported on 1,661 patients with intermediate and thick melanomas and excluded participants who had primary tumors <1·2mm [6]. Overall, results corroborated the findings of the previous analysis. SLNB was associated with a significantly longer disease-free interval in both patients with intermediate (absolute ten-year benefit: 7%) and thick (absolute ten-year benefit: 10%) primaries. Overall SLNB did not lead to a better prognosis for patients who have had it. However, when the analyses was performed only in the LN-positive participants, patients with intermediate thickness melanoma who have had a SLNB had a 21% better ten-year melanoma-specific survival rate (62·1% versus 41·5%), while no significant difference was detected among LN-positive participants with a thick primary.

These results are not going to completely remove the skepticism around SLNB because the statistically significant difference is seen in a non-randomized subgroup, as it was only shown when comparing the patients with positive LNs, and clearly there was no way of knowing this fact before they either had a SLNB or relapsed. Nevertheless there were almost exactly the same proportions of patients with LN metastasis in both groups suggesting that eventually all retained LNs will develop clinical disease, providing the patient does not die of systemic spread of disease in the interim. However, even in thick melanoma patients where there was no improvement in survival between the node positive groups, the commonest first site of relapse in the observation group in >90% of cases was the LN basin, with the median time to LN relapse around nine months [6].

It is most likely no co-incidence that previous non-randomized studies have also shown that SLNB can be associated with approximately 20% survival benefit over nodal observation for patients with involved

nodes. A meta-analysis of non-randomized studies encompassing 2,633 patients showed that SLNB was associated with a better survival and the results suggested that SLNB and CLND might prolong survival in one of five treated patients after five-year [14]. Another compelling source of evidence is a retrospective study investigating the influence of timing of surgery and LN tumor burden on the prognosis of 1,704 patients that compared the clinical scenarios of presentation with LN metastases and subsequent lymphadenectomy. This study showed that patients who had a CLND immediately after a positive SLNB had a better survival plateauing around 60% with very few events after seven or eight years compared to patients who did not have a SLNB and had a delayed lymphadenectomy for clinically positive LNs whose ten year survival was around 45%. This is despite patients in the SLNB group having worse primary tumor prognostic factors than the delayed lymphadenectomy group and having better survival until around three years [15]. Again, a consistent quantum of benefit was demonstrated.

The MSLT-2

Overall, the available evidence, though lacking conclusive evidence that comes from randomized patients, favors the performance of SLNB and CLND dissection. Without knowledge of this, the MSLT investigators started the MSLT-2 in 2004 [16] to investigate the therapeutic value of SLNB and CLND compared to SLNB and observation with CLND only if regional LN relapse occurs. The MSLT-2 study cohort is not diluted by lower risk patients that did not have any LN metastases, however there is very little stratification for factors that lead to the wide range of outcome for SLNB positive patients. The study has recently completed accrual and it has been anticipated that it will provide important information to standardize the treatment of melanoma patients with LN metastasis. However, there are concerns as well as limitations in the study design that may affect the acceptance and applicability of the final results. Some clinicians question the safety of conducting a trial where part of the therapy that led to a survival advantage is not given for the node positive patients in MSLT-1, resulting in anxiety that it

may not be safe to avoid lymphadenectomy in SLNB-positive patients, particularly in the high risk cases such as thick primary tumors and those with high sentinel LN (SLN) tumor burden. The MSLT-2 investigators would argue that it may be the SLNB alone that provides the survival advantage for node positive patients and not the addition of the lymphadenectomy. A recently published international survey and anecdote suggested that melanoma surgeons have been selective in which patients they offer the MSLT-2 [17]. The survey reported on 193 surgeons involved in melanoma treatment, of whom 78 (40·4%) were participating the MSLT-2 [17]. Only 56% of surgeons participating in the MSLT-2 offered virtually all patients randomization, whilst in the whole group of responders, which included non-MSLT-2 investigators, approximately one third thought the criteria for enrollment in MSLT-2 should be modified by considering predictors of non-SLN involvement at CLND and half the responders did not consider it appropriate to enroll patients with multiple positive SLNs in MSLT-2. This selection bias towards lower risk patients will firstly limit the power of the study to detect a meaningful differences and secondly if this factor is accurately reported when it comes to publication then the results should only apply to those patients fitting into the characterization of the typical MSLT-2 patient. There is a great similarity with the ACOSOG Z11 study testing the need for completion axillary lymphadenectomy after positive SLNB in breast cancer patients [18]. The Z11 trial was slow to recruit (and indeed never reached accrual target) and mainly involved low risk patients but despite this, at least in some parts of the world, has ended up changing practice for all LN-positive patients.

Considering these issues in more detail with regards MSLT-2, it should be noted that patients are not stratified according to the amount of melanoma in the SLN. In the last decade several studies have underlined the predictive and prognostic value of several measurements of melanoma metastasis in the SLN, such as the diameter of the largest metastasis [11], the location of the metastasis within the SLN [19], the penetrative depth of the metastasis in the SLN [20], the metastatic area [21], the presence of dendritic cells [22] and intra-lymphatic melanoma cells [23]. These parameters correlate not only with patient survival,

but also with the probability of having further metastasis in the non-SLN. Although the MSLT-2 is measuring the SLN tumor burden, this important predictive and prognostic information is not used for stratifying patients in the two arms of the trial.

After positive SLNB the presence of metastatic disease in non-SLN at CLND is now known to be of particular prognostic importance [24]. It is unclear whether MSLT-2 is powered to identify survival differences between patients who undergo immediate CLND and have involved non-SLN and those who recur and have a delayed CLND. There is a symmetry here with the criticisms leveled against MSLT-1 but in MSLT-2 the patients are all higher risk and more likely to fail with systemic metastases. Results from the aforementioned study on timing on lymphadenectomy showed a worse prognosis for SLNB-positive patients who had a delayed compared to an immediate CLND [15]. Other retrospective series did not identify survival differences between these two groups [25], but the results may be largely influenced by the presence of low risk patients in the group who did not undergo immediate CLND (i.e. the decision of not performing lymphadenectomy was based on the low risk of LN metastasis beyond the SLN).

Despite these criticisms, it must be acknowledge that most of the prognostic information derived from the histopathologic features of the SLN metastasis have become available after the design of the trial. MSLT-2 is expected to shed light on the management of melanoma patients with LN metastasis, but the existence of pitfalls in study design may burden the widespread acceptance of the results.

What constitutes an adequate lymphadenectomy?

National guidelines have few specific recommendations as to exactly what constitutes an adequate lymphadenectomy, with the common suggestion of performing a thorough anatomical procedure [26, 27]. Given this lack of guidance, it is not surprising that surgeons have heterogeneous opinions on how a lymphadenectomy should be performed. Whist in patients with bulky nodal disease a full regional

lymphadenectomy would most often be performed, more debate exists for SLNB-positive patients and those with localized lower volume clinical disease. In the aforementioned international survey, 35% of responders routinely performed a full-level one-five neck dissection, and 62% based the extent of neck dissection on the primary tumor site and lymphatic mapping [17]. Interestingly, recent research in head and neck patients has shown that after a positive SLN if there is later found to be non-SLN involvement, the majority of metastatic LNs are located in the same anatomic level as the SLN, supporting the idea of a less extensive lymphadenectomy may be adequate for SLNB-positive patients [28]. In the neck area, a randomized trial may be difficult to perform and further study aimed at localizing positive non-SLN or testing outcomes after less extensive procedures may be warranted. Conversely, axillary dissection represents a more standardized operation, as 81% of the survey responders routinely performed a three-level lymphadenectomy [17]. Recent reports suggested that level III of the axilla (i.e. beyond the minor pectoralis muscle) is rarely affected by metastasis with a reported incidence < 5% for SLNB-positive [29, 30]. Despite this, it is general opinion that dissection of the third level is not likely to increase the risk of lymphedema and that a recurrence in the third LN level can be difficult to diagnose before it is very difficult to manage surgically.

The management of the groin LN field is the most controversial. The appropriate extent of groin lymphadenectomy has been debated for many years, especially in case of a positive SLN [31, 32]. Inguinal lymphadenectomy is routinely performed in patients with groin metastasis, while pelvic dissection is recommended by several national clinical practice guidelines and by several authors in patients with clinically positive pelvic LNs, radiological imaging showing pelvic LN metastasis (on either ultrasound, CT or PET / CT scans), > three positive inguinal LNs and metastasis in the Cloquet's LN, which is considered the "sentinel node" of the pelvic field, even though it is well established that lymph flow more often enters the pelvis by other routes [26]. Other authors recommend performing an ilio-inguinal lymphadenectomy when any subclinical (SLNB-positive) or clinical LN metastasis is detected in the groin

[33]. The international survey on completion lymphadenectomy in SLNB-positive patients reported an inguinal dissection or an ilio-inguinal dissection are performed by 36% and 30% of surgeons, respectively, while the remaining 34% of responders select either inguinal or ilio-inguinal dissection according to the above mentioned criteria and also lymphatic drainage patterns [17].

A randomized trial comparing inguinal and ilio-inguinal dissection for any LN positive patients, the EAGLE FM Study from the Australia and New Zealand Melanoma Trial Group, is in the process of being initiated and will test whether it is safe to avoid doing pelvic lymphadenectomy if the preoperative PET/CT scan shows no evidence of pelvic LN involvement [34]. This will also be a proof-of-principle study of the potential impact of more extensive versus less extensive lymphadenectomy that might lead to similar studies in other LN regions.

Adjuvant treatments

After surgery, the only approved medical adjuvant therapy for patients with LN metastases is interferon alpha [10].

Several meta-analyses support the efficacy of adjuvant interferon alpha for the treatment of high-risk patients with cutaneous melanoma [35]. Interferon is effective for prolonging disease-free survival (recurrence risk reduction of 17%) and to a lesser extent overall survival (recurrence risk reduction 9%). The risk reduction for overall survival translates in a survival benefit at 5 years of approximately 3%. The most updated meta-analysis failed to identify the best treatment duration and dosages [35]. In this regard, a recent trial showed that clinical outcomes were better in patients who had the standard interferon regimen of one month of high dose treatment (five days a week) followed by 12 months (three days a week) with low dose treatment compared to one month of high dose interferon [36].

Although these results support the use of interferon for the adjuvant treatment of melanoma and its use as comparator in trials investigating new agents in the adjuvant setting, they cannot be considered optimal and if interferon is going to have a role then there may be at least three ways to improve its efficacy . Firstly, by improving the patient selection. About 40% of AJCC TNM stage II-III patients are likely to be cured with surgery alone [37]; thus, it is unnecessary to expose them to the toxicity of interferon therapy. Moreover, inclusion of these patients in interferon trials could represent a bias in evaluating interferon efficacy as they would never benefit from this treatment and thus their presence would only dilute the survival advantage associated with the administration of this cytokine to those who do harbor minimal residual disease after surgery. This issue can be addressed by developing effective biomarkers or methods to detect minimal residual disease after radical surgery, such as the detection of circulating tumor cells in the peripheral blood, which is advocated as a promising tool to select patients most likely to need adjuvant treatment [38, 39]. Some investigators have proposed LN micrometastasis (positive SLN) and ulceration as a histopathological biomarkers predictive of micrometastatic residual disease after surgery and an ongoing EORTC trial is evaluating whether or not interferon might be more effective in this subset of patients [40, 41]. Secondly, the understanding of molecular mechanisms underlying melanoma responsiveness to interferon would allow physicians to administer interferon selectively to people most likely to be responsive [4, 42]. Finally, the introduction of new drugs for the adjuvant treatment are hoped to be the major player for improving survival of patients after lymphadenectomy. The BRAF inhibitors induce immune response to melanoma cells in the metastatic setting and the effectiveness of the immune response is improved in case of surgically resected disease [43]. These observations along with the impressive anti-melanoma effectiveness of these targeted treatments has led to adjuvant trials testing the BRAF inhibitors (vemurafenib and dabrafeninb) and in combination with MEK inhibitors (e.g. tremetinib) in high risk node-negative patients and in those patients with LN metastasis removed [10].

The effectiveness of immunotherapeutic strategies based on the blockade of immune checkpoints with anti-CTLA-4, anti-PD-1, and anti-PDL-1 monoclonal antibodies demonstrates that lymphocytes need to be “unleashed” in order to act against melanoma cells [10, 44]. Despite the effectiveness and the long-term responses demonstrated in metastatic melanoma, these monoclonal antibodies may have detrimental side effects that can be non-reversible, such as bowel perforation and sometimes severe auto-immune responses like hypophysitis and or hepatitis [45]. Findings from EORTC Melanoma Group NCT00636168 trial, that has completed accrual, and from others that are ongoing or under design are awaited with great interest [10].

The other adjuvant therapy option that shows effectiveness in patients at high risk of regional relapse after lymphadenectomy is the irradiation of that area. The only randomized study assessing patients who underwent a therapeutic lymphadenectomy demonstrated a significant risk reduction for regional recurrence (44%) after adjuvant radiotherapy (48 Gy in 20 fractions) [46], however the study did not show a significant impact for radiotherapy on relapse-free and overall survival. Therefore systemic adjuvant therapy trials that may improve overall survival have priority but patients do not always meet trial entry criteria or decline trial entry and radiotherapy may be important in this setting. The decision algorithm is complicated by some of the systemic adjuvant therapy trials mandating dacarbazine or observation as the control arm.

Despite adjuvant radiotherapy benefits being limited to improved regional control [9], recent studies have suggested improved tumor response when radiotherapy was associated with BRAF inhibitors [47] and anti-CTLA-4 monoclonal antibodies [48]. Clinical trials combining these new treatments with radiotherapy are in progress.

Given the efficacy of BRAF inhibitors, MEK inhibitors, and immune modulating agents it is likely that novel applications of these agents in the neoadjuvant setting for stage III melanoma patients will occur

more often going forwards. Benefits of this approach include improved operability in locally advanced disease and as an indication of responsiveness to the agent. There is much work to be done in this area [49].

Quality assurance of regional lymph node surgery

The evaluation of the effectiveness of these new adjuvant treatments, alone or in combination, should be unbiased by standardized high quality surgical treatment and standardized pathological workup so that there is accurate staging and stratification as well as equivalent likelihood of surgery-induced regional control between investigating centers. For many solid tumors, such as gastro-intestinal cancers, the number of excised LNs is considered the major parameter to judge the quality of surgery for stage III disease [50]. For melanoma patients, there is less evidence to support the use of the number of excised LNs as quality assurance measure. The association between the number of excised LNs and patient survival has been demonstrated only recently [51], though no prospective data support it. For this reason, guidelines still suggest that there is insufficient evidence to support a minimum number of nodes to deem a dissection adequate [27]. However, the previously mentioned international survey showed that melanoma surgeons considered quality of surgery an important issue and most commonly used the number of excised LNs as preferred quality assurance measure [17]. Large studies from the Melanoma Institute Australia suggested that the desirable number of excised LNs should be greater than the 10th percentile of the number of LNs that had been excised in their practice (i.e. 90% of patients need to have a number of excised LNs > the tehnth percentile). The implication is that lymphadenectomies with a number of excised LNs < tenth percentile have to be assessed for explanation and whether inadequate surgery or inadequate pathological assessment has been performed [32, 33]. According to these results, at least ten, 20, eight, and 14 LNs are excised after axillary dissection, neck dissection involving $\geq$ four

anatomical levels, inguinal, and ilio-inguinal lymphadenectomy in >90% of cases, respectively. These values can be used for auditing the surgical performance and ensure high quality level of lymphadenectomy [33].

The issue of monitoring quality assurance might also be viewed from staging point view. Although there are still controversies around the therapeutic value of lymphadenectomy, this procedure offers the opportunity of staging patients according to the AJCC TNM manual, which indicates the number of positive LNs is one of the major determinants of patient survival. A recent study suggested that when less than 11 LNs are detected after dissection patient prognosis cannot be stratified according to the AJCC staging system [51]. Considering the number of excised LNs for each lymphatic field, at least 11, 14, ten, and 12 LNs were needed to stage patients accurately after a lymphadenectomy of the axilla, neck, inguinal, and ilio-inguinal LN fields, respectively.

Overall, differences exist in the proposed cut-off value for the minimum number of excised LNs for each field. Further analysis of prospective series, are warranted to provide reproducible minimal LN retrieval counts to monitor the quality of lymphadenectomy thus ensuring optimal control of disease, patient staging and stratification for randomized trials investigating new adjuvant therapies.

Improving patient staging

The current edition of the AJCC TNM staging manual for skin melanoma stratifies prognosis of patients with LN metastasis on the basis of the number of positive LN (one, two-three, and $\geq$ four) and the LN tumor burden (micrometastasis, that is the presence of metastasis in the SLN, and macrometastasis, that is the presence of clinically positive LNs) [52]. Increasing evidence suggests that the prognosis of

patients with micrometastasis and with macrometastasis needs to be evaluated using different sets of prognostic factors.

In the last decade several histopathologic prognostic factors have been described for SLN-positive patients [53]. Histopathologic characteristics of SLN metastasis, most of which have been mentioned above, have been shown to add meaningful staging information to the pre-existing staging features. Concerns have been raised around the reproducibility of these measures, not only because inter-observer differences may exist, but also because different pathological protocols may affect the accuracy of the micromorphometric characterization of tumor deposits [54]. A study on inter-observer variations in assessing the features of LN tumor burden demonstrated that the concordance between pathologists is generally high, though difficulties exist when continuous measurement are reported as a cut-off, as is the case for the largest diameter of the largest deposit of metastatic melanoma. When the cut-off value of 0.1mm was consider and patients classified as having a sub-micrometastasis ($\leq$0·1mm) or a micrometastasis (>0·1mm), a significant degree of discordance was observed, questioning the reproducibility of this cut-off to stratify prognosis [55]. Furthermore, some patients with sub-micrometastasis have adverse clinical outcomes, and this may be mainly due to an underestimation of extent of melanoma deposits with sampling error [56].

Another prognostic factor for patients with micrometastasis, that is easier to obtain and with less reproducibility issues than the micromorphometric features of the metastasis in the SLN, is the pathological status of the non-SLN [57]. A recent study, not only demonstrated that the adverse prognostic implications of non-SLN metastasis is independent from the number of positive LNs, but also that the non-SLN status identified two prognostic groups in the case of two or three microscopically positive LNs (AJCC TNM N2a disease) [24]. Remarkably, in cases with negative non-SLN (i.e. LN metastasis are limited to

the SLN) the number of positive LNs may no longer be a prognostic factor. If these results are confirmed in larger studies, the staging system for SLNB-positive patients with melanoma will need to be revised.

In both SLN-positive and clinically LN positive patients, several studies have reported the adverse prognostic significance of extra-nodal extension [58, 59]. Although the occurrence of metastasis outside the LN seems to strongly correlate with the occurrence of tumor progression and survival, independently of the timing of lymphadenectomy and the LN tumor burden [15], the inclusion of this important prognostic parameter in the routine staging of melanoma patients may be limited by the relatively low inter-observer reproducibility [55].

As discussed above, recent evidence support the prognostic value of the number of excised LNs, considered as a single factor [51] but also when it is used together with the number of positive LNs [60]. This is termed the LN ratio and is simply the number of positive LNs divided by the number of excised LNs. The significance of the prognostic value of the number of excised LNs can be explained by its value in accounting for removal of all the possible metastatic LN left in the regional lymphatic field at the time of surgery. Obviously, a more thorough dissection will allow detection of virtually all metastatic LNs, while a dissection including less retrieval of LNs may be at risk of missing some metastatic deposits and thus at risk of incorrect staging the patient. The other explanation is that removal of occult low volume disease, which is not detected by the standard histopathologic assessment done on lymphadenectomy specimens, improves outcome. According to the implications of LN ratio, it may be worth considering inclusion of the minimum number of LNs to be dissected in the staging of patients, as happens for other solid tumors, particularly gastro-intestinal cancers [50].

Beyond histopathologic prognostic information, increasing molecular information is available to define the process of tumor progression from primary melanoma through the SLN, the non-SLN and distant sites [61, 62]. For instance, polymerase chain reaction (PCR) has been tested in thousands of SLN-negative patients showing that PCR-based SLN ultra-staging correlates with survival [63], although results are not considered conclusive [64]. In this regard, the aforementioned MSLT-2 was expected to shed lights on the role of PCR in melanoma. The trial was originally designed also to test CLND in patients with histology negative and PCR-positive SLN, but this trial arm was closed prior to study conclusion.

Overall, SLN and non-SLN may be considered two different steps in the process of disease progression through the lymphatic vessels [57]. Several mechanism, such as lymphatic markers expression [65], tumor infiltrating lymphocytes [66], tumor associated macrophages [67], and dendritic cells [68], may act as regulator of this process.

In primary melanoma, the expression of lymphatic markers as well as immunohistochemistry-detected lymphatic vessels density and invasion have been associated with SLN metastasis [69, 70]. A high lymphatic vessels density in the peritumoral area and the presence of lymphatic vessels invasion increases the likelihood of harboring SLN metastasis [65]. A similar process may occur in the SLN, where the relationship between tumor and endothelial cells of the lymphatic vessels may lead to tumor progression to the non-SLNs.

On the other hand, the SLN is probably the preferred site for the development of the acquired immunity as auto-reactive lymphocytes encounter melanoma antigens through contact with dendritic cells, resulting in activation of tolerance [68]. Available evidence suggested that the complex relationship between lymphocytes (e.g. T-CD4+, T-CD8+, and T-regulatory cells), dendritic cells, CD56+ natural killer cells, and macrophages can have a meaningful role in creating and immunosuppressed environment in the SLN allowing tumor cells to evade immune surveillance and progress through the lymphatic vessels to the non-SLNs [71, 72]. Although the presence of immature dendritic cells in the SLN has been reported in

several studies [22, 61], the possibly of considering the implications of the immune status of the SLN for selecting patients for CLND as well as for adjuvant therapies and new immune-modulating drugs deserves further investigations.

The presence of mutations of BRAF in melanoma metastasis within the SLN may lead to a greater ability of melanoma to metastasize to the non-SLN and was the only independent prognostic factor along with the number of positive LNs in one study [73]. Similarly, patients with clinically positive LNs had poorer prognosis if they had BRAF or NRAS mutations and a lack of expression of genes involved in the immune response predicted poor survival [74].

These results show the multifaceted relationship between melanoma cells and immunity in the regional LN and underline the importance of improving the prognostic stratification as well as the treatment strategies for patients with LN metastasis from melanoma.

In conclusion, metastatic melanoma to LNs is a complex and controversial area in terms of diagnosis, therapeutic value of earlier removal, what constitutes adequate lymphadenectomy, understanding the role of the sentinel node in immune tolerance leading to worse prognosis and the place of new effective biological therapies in the adjuvant and neoadjuvant setting. It is an exciting time for surgeons and other oncologists managing these patients. Collaborative research and clinical trials with internationally recognized surgical standards is the way forward.

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CHAPTER 7

Non-sentinel lymph node status in patients with cutaneous melanoma: results from a multi-institution prognostic study (N=1,538)

Pasquali S, Mocellin S, Mozzillo N, Maurichi A, Quaglino P, Borgognoni L, Solari N, Piazzalunga D, Mascheroni L, Giudice G, Patuzzo R, Caracò C, Ribero S, Marone U, Santinami M, Rossi CR. Non-sentinel lymph node status in patients with cutaneous melanoma: results from a multi-institution prognostic study (N=1,538). J Clin Oncol 2014;32:935-41.

Introduction

The five years survival of melanoma patients with lymph node (LN) metastasis ranges from an average of 43% when patients present with clinically evident nodal disease to 67% when patients had their LN metastasis identified with sentinel lymph node biopsy (SLNB) [1]. Patients who have a completion LN dissection (CLND) for a positive SLNB show a wide heterogeneity in their prognosis, with five year survival rates ranging from 15% in case of multiple positive LNs to 90% in case of a small metastatic deposit in the sentinel lymph node (SLN) [2-13].

In these patients, the presence of melanoma metastasis in the non-SLNs (NSLNs) after CLND is an easy-to-obtain and reproducible prognostic factor, which suggests that not all LNs of a given lymphatic field share the same prognostic meaning, since melanoma LN metastasis limited to the SLN are associated with a better clinical outcome as compared to the metastasis that has reached the NSLNs [7-15]. It is still unclear whether the dismal prognosis of patients with NSLN metastasis merely reflects the prognostic value of a greater number of positive LNs or if it is related to a different biological behavior of LN metastasis beyond the SLN. Should the latter be the case, it is still unexplored how to include the NSLN status in the AJCC TNM staging system in order to improve patient risk stratification.

In this study we investigated whether the prognostic value of the NSLN status may improve the accuracy of the currently considered staging features in a large series of 1,538 patients; moreover, we formulated a proposal for including the NSLN status in the staging of SLNB-positive patients with two-three positive LNs and performed a literature meta-analysis to summarize the prognostic value of the NSLN status in these patients. Finally, we discussed the translational implications of having the LN disease limited to the SLN (i.e. negative NSLN at CLND).

Methods

The study protocol was approved by the Research Committee of the Italian Melanoma Intergroup (IMI), the Italian network for melanoma treatment and research (www.melanomaimi.it).

Retrospective data from SLNB-positive melanoma patients who underwent a CLND between 1993 and 2011 at nine IMI centers were gathered in a multi-center database.

Data were extracted according to the following selection criteria: 1) single primary melanoma; 2) performance of SLNB and CLND in a single lymphatic field (within 12 weeks from SLNB); 3) availability of information regarding tumor thickness, number of excised and positive LNs (including SLNB) and melanoma-specific survival. SLNB-positive patients who did not undergo CLND were excluded from the analysis.

CLND specimens were processed according to a standard protocol shared by all participating centers. LNs with a diameter <4 mm were totally embedded; LNs >4 mm in diameter were cut in 3 to 4 mm thick slices that were entirely embedded in paraffin blocks. From each paraffin block, two sections were obtained for H&E staining.

The following variables were considered for each patient: enrolling center ($\leq$150, >150 included patients), year of diagnosis (1993-2001, 2002-2010), patient age (at diagnosis of primary melanoma) and sex, primary tumor thickness, ulceration, Clark level of invasion, number of excised and positive SN(s) at SLNB, number of excised and positive lymph node(s) at CLND.

Variables ulceration and Clark level had missing values in 160 (10.4%) and 61 (3.9%) patients. The multiple imputation method was used to predict missing values, using variables with non-missing values as predictors (i.e. age, sex, tumor thickness and AJCC N stage) [16, 17].

The Fisher's exact test for categorical variables and the Mann-Whitney U test for continuous variables were used to investigate association between covariates and NSLNs status.

Melanoma-specific survival was calculated from the time of primary melanoma diagnosis to the time of melanoma death or last follow-up.

Survival analysis was fitted to data in 1) the overall patient population in order to investigate the prognostic value of the NSLN status, 2) patients with the same number of positive LNs (i.e. two positive LNs, that is the most common situation in which both SLN and NSLN can be positive) in order to further prove the prognostic independency of the NSLN status of the number of positive LNs, and 3) patients belonging to the same AJCC N stage (N2 and N3) in order to formulate a proposal for the inclusion of the NSLN status in the melanoma staging.

Univariate survival analysis was performed with the Cox proportional hazard model, which was then fitted to the data using a stepwise backward covariate selection procedure to study the association between significant covariates at univariate analysis (with special regard to NSLN status) and melanoma-specific survival. Survival curves were generated with the Kaplan-Meier limit method and compared with the log-rank test.

Finally, literature was searched to identify studies investigating the prognostic value of the NSLN status in patients with two-three positive lymph nodes with the standard method for systematic review [18] using the following key-words: “melanoma”, “sentinel lymph node”, “non-sentinel lymph node”. Meta-analysis methods, which we described in detail elsewhere [19-21], was applied to eligible studies to evaluate the overall prognostic value of the NSLN status in patients with two-three positive LNs. Briefly, summary hazard ratio (HR) was calculated as NSLN positive to negative ratio using the generic inverse-variance method; when HRs and their confidence interval (CI) were not available, they were estimated according to the method of Parmar et al. Meta-analysis was first performed with the fixed-effect model and consistency of the results was assessed with the Cochran *Q* test and the I^2 statistic (heterogeneity was considered significant in case of Cochran *Q* test P-value < 0.1 and I^2>50%).

Analysis were conducted setting the alpha level of significance at 0.05 and performed with STATA SE/11.0 (College Station, TX) Comprehensive Meta-Analysis (release version 2.2.046; Biostat, Englewood, NJ).

Results

1,538 SLNB-positive patients who underwent a CLND were eligible for the present study. Patient and tumor features according to the pathological status of the NSLNs are reported in **Table 1**.

Table 1. Clinical and pathological features of SLNB-positive 1,538 patients who had a CLND.

Variables		**Negative NSLN**		**Positive NSLN**		**P-value**
		N	**%**	**N**	**%**	
Age	median (IQR)	52 (40-65)		57 (41-67)		*0.005*
Sex	Male	642	54.2	192	54.4	0.952
	Female	543	45.8	161	45.6	
Breslow thickness	median (IQR)	2.5 (1.6-4)		3.5 (2.2-5.8)		*<0.001*
Ulceration	Absent	665	56.2	139	39.4	*<0.001*

	Present	519	43.8	214	60.6	
Clark level	II-III	312	26.3	72	20.4	*0.028*
	IV-V	873	73.7	281	79.6	
Mitotic rate	median (IQR)	1 (1-4)		1 (1-5)		0.274
Positive SLN(s)	median (IQR)	1 (1-1)		1 (1-1)		*<0.001*
	1	1,035	87.3	264	74.8	*<0.001*
	2	124	10.5	72	20.4	
	3	20	1.7	16	4.5	
	≥4	6	0.5	1	0.3	
Excised SLN(s)	median (IQR)	1 (1-2)		2 (1-3)		*0.002*
Positive LNs at SLNB+CLND	median (IQR)	1 (1-1)		1 (1-2)		*<0.001*
	1 (AJCC N1a)	1,035	87.3	0	0.0	
	2 - 3 (AJCC N2a)	144	12.2	243	68.8	*<0.001*
	> 3 (AJCC N3)	6	0.5	110	31.2	
Excised LNs at CLND	median (IQR)	18 (13-24)		20 (14-26)		*0.002*

The median follow-up was 45 months (interquartile range: 18-85 months). All the considered variables but enrolling center and year of diagnosis were significant prognostic factors for melanoma-specific survival at univariate analysis (**Table 2**).

Table 2. Univariate and multivariable analysis of the association between melanoma-specific survival and the considered clinico-pathological features, with special regards to the NSLN status.

Variables		**UNIVARIATE ANALYSIS**				**MULTIVARIABLE ANALYSIS**			
		HR	**95%CI**		***P-value***	**HR**	**95%CI**		***P-value***
Enrolling center	≤150 patients	1			*0.137*				
	>150 patients	0.84	0.66	1.05					
Year of diagnosis	1993-2001	1			*0.473*				
	2001-2010	1.06	0.75	1.49					
Age	Years	1.02	1.01	1.03	*<0.001*	1.02	1.01	1.03	*<0.001*
Sex	Female	1			*<0.001*	1			*<0.001*
	Male	1.65	1.29	2.09		1.55	1.22	1.99	
Breslow thickness	mm	1.04	1.03	1.06	*<0.001*	1.04	1.03	1.06	*<0.001*
Ulceration	Absent	1			*<0.001*	1			*<0.001*
	Present	2.00	1.58	2.54		1.84	1.45	2.35	
Clark level	II-III	1			*<0.001*				
	IV-V	1.42	1.19	1.70					
No. of excised LNs		0.98	0.97	0.99	*0.018*				
NSLN status	Negative	1			*<0.001*	1			*<0.001*

	Positive	1.40	1.23	1.59		1.34	1.18	1.52
AJCC N stage	N1 (1 pos LN)	1						
	N2 (2-3 pos LNs)	1.44	1.10	1.89	*0.007*			
	N3 (≥4 pos LNs)	2.59	1.83	3.68	*<0.001*			

Curves illustrating melanoma-specific survival estimates by NSLN status (log-rank test, P<0.001) are showed in **Figure 1A**.

At multivariable analysis the presence of metastasis in the NSLN was significantly and independently associated with worse prognosis (HR=1.34; 95%CI: 1.18-1.52; P<0.001), as did older age (P<0.001), male gender (P<0.001), thicker (P<0.001) and ulcerated primary tumors (P<0.001). Conversely, neither the AJCC TNM staging, nor the Clark level of invasion, nor the number of excised LNs was independent prognostic factors.

To rule out the possibility that the independent significance of the NSLN status merely reflected the prognostic influence of a higher number of positive LNs, the prognostic value of the NSLN status was tested separately in patients with the same number of positive LNs. In particular, we focused on patients with two positive LNs (*N*=294), which is the most common situation when both SLN and NSLN are positive (**Table 1**). Interestingly, patients with metastatic disease in one SLN and one NSLN (*N*=170) had a survival outcome significantly worse than that observed in patients with two metastatic SLNs (*N*=124, log-rank test, P=0.048, **Figure 1B**).

In order to formulate a proposal of a staging system that includes the prognostic value of NSLN, we tested our hypothesis separately in patients with AJCC N2 (two-three positive LNs) and N3 (≥ four positive LNs) stages. The presence of metastasis within the NSLNs was a negative independent prognostic factor for melanoma-specific survival in patients with two-three positive LNs (HR=1.39, 95%CI: 1.07-1.81, P=0.013, **Figure 1C**), along with older age (HR=1.02, 95%CI: 1.01-1.04; P=0.003), male

sex (HR=1.87, 95%CI: 1.17-3.01; P=0.009), greater tumor thickness (HR=1.08, 95%CI: 1.02-1.14; P=0.007) and fewer excised LNs (HR=0.97, 95%CI: 0.94-0.99; P=0.016). Conversely, the status of NSLNs was no longer a prognostic factor in patients with $\geq$ four positive LNs (AJCC N3, HR=0.75, 95%CI: 0.34-1.62, P=0.470) with tumor thickness being the only variable independently associated with survival (HR=1.16, 95%CI: 1.07-1.25, P<0.001).

Taking these findings together, we re-classified patients in four prognostic groups as follows:

- **Group-1**, one positive SLN after SLNB and negative NSLN after CLND (AJCC N1a**;** *N*=1,035);
- **Group-2**, two-three positive SLNs after SLNB and negative NSLN after CLND (AJCC N2a stage - NSLN negative; *N*=144);
- **Group-3,** two-three positive LNs including both sentinel and NSLNs (AJCC N2a stage - NSLN positive; *N*=243);
- and **Group-4**, $\geq$4 positive LNs including both sentinel and NSLNs, (AJCC N3 stage; *N*=116).

This classification had a significant prognostic value (log-rank test, P<0.001, **Figure 1D**).

After adjustment for conventional staging features, patients in Group-3 (HR=1.65, 95%CI: 1.23-2.23, P=0.001) and Group-4 (HR=2.24, 95%CI: 1.59-3.20, P<0.001) did worse than patients in Group-1. We did not observe a statistically significant difference between the prognosis of patients in Group-2 and that of patients in Group 1 (HR=0.89, 95%CI: 0.55-1.43, P=0.623). Older age (HR=1.02, 95%CI: 1.01-1.02, P<0.001), male sex (HR=1.67, 95%CI 1.32-2.13, P<0.001), thicker (HR=1.05, 95%CI: 1.03-1.06, P<0.001) and ulcerated primary tumor (HR=1.78, 95%CI: 1.40-2.27, P<0.001) were the other independent predictors of poor prognosis.

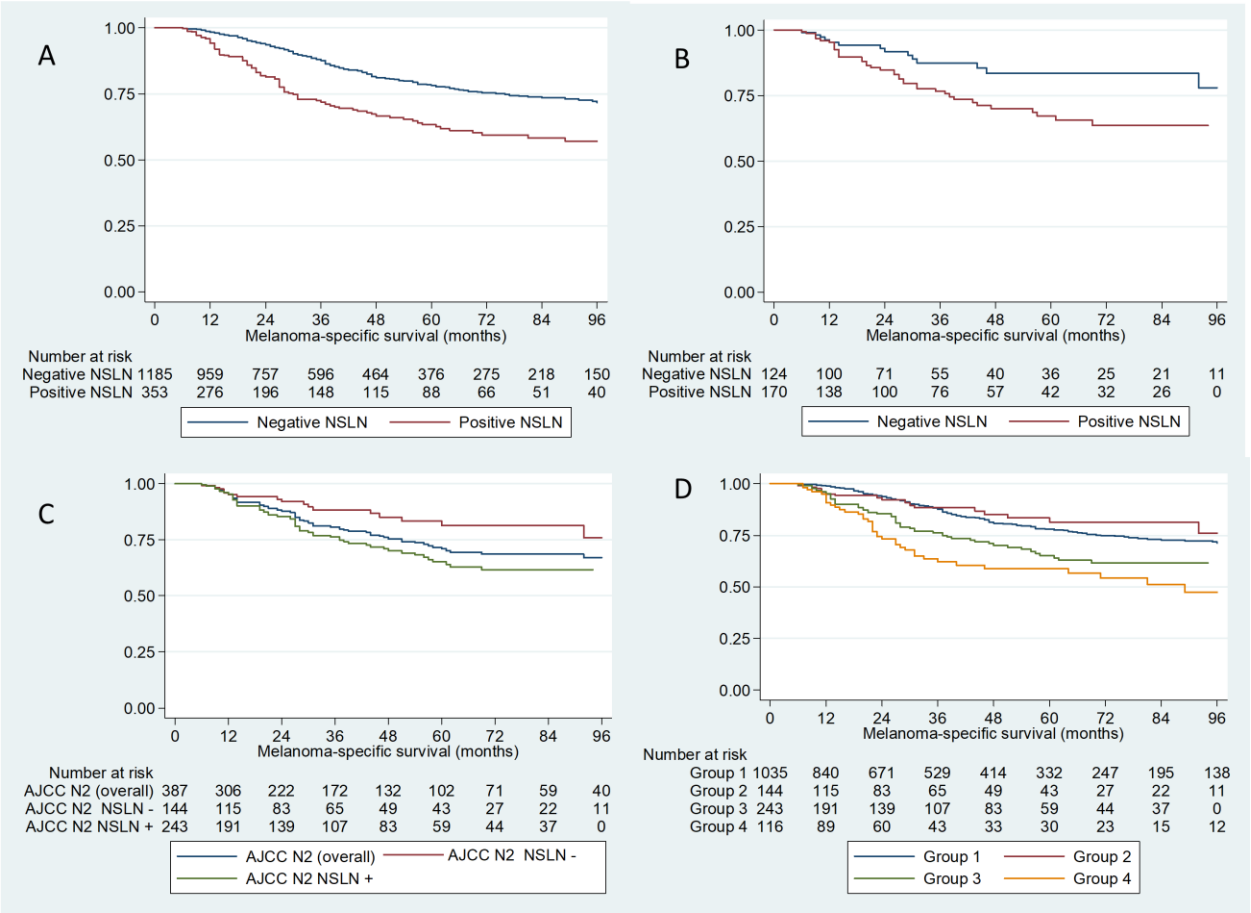

Figure 1. Melanoma-specific survival curves according to the pathological status of the NSLN in **(A)** all the 1,538 patients and **(B)** in patients with two positive lymph nodes after SLNB and CLND. Figure **C** reported survival of patients with two-three positive lymph nodes considered together and according to the NSLN status**.** Figure **D** reported melanoma-specific survival according to the following classification: **Group-1:** one positive SLN and negative NSLN (AJCC N1a stage); **Group-2**, two-three positive SLNs and negative NSLN (AJCC N2a stage – NSLN negative); **Group-3,** two-three positive lymph nodes including sentinel and NSLNs (AJCC N2a stage – NSLN positive); and **Group-4**, $\geq$ four positive lymph nodes including sentinel and NSLNs (AJCC N3).

To increase the clinical relevance of our work, we conducted a systematic-review of studies that investigated the prognostic value of the NSLN status in patients with two-three positive LNs and found three studies (**Table 3**) [8-10]. One report [10], which investigated only patient disease-free survival, was excluded from the analysis that was aimed at assessing the association of NSLN status and overall

survival. Therefore, we pooled our results with those from the two eligible studies. The meta-analysis, which included 620 patients (284 had negative NSLN and 336 had positive NSLN), showed that the NSLN status is a highly significant prognostic factor for patients with two-three positive LNs (summary HR=1.59, 95%CI 1.27-1.98, P<0.001, **Figure 2**). The lack of between-study heterogeneity (Cochrane *Q* test, P=0.15; I^2=48%) supports the consistency of the prognostic value of the NSLN status across studies.

Figure 2. *This Forest plot illustrates the summary hazard ratio (HR) of the prognostic value of the non-sentinel lymph node (NSLN) status for overall survival in patients with two-three positive lymph nodes from three studies (620 patients, Table 3).*

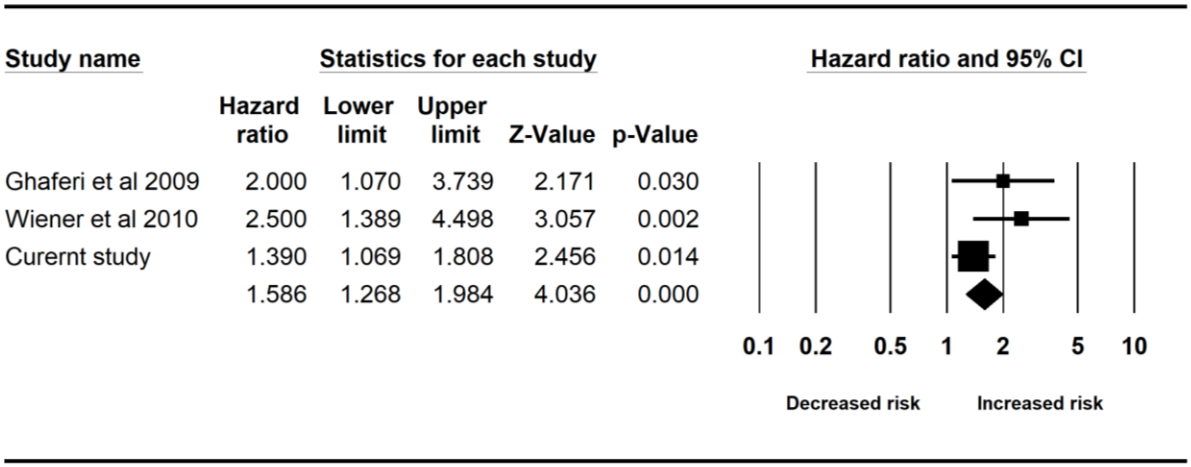

Table 3. Studies investigating the association between the pathological status of the non-sentinel lymph node (NSLN) and patient prognosis in case of two-three positive lymph nodes (AJCC N2 melanomas).

Study, year	Overall no. of pts	No. of pts with AJCC N2 melanomas	NSLN+ / NSLN-	Survival	Notes
Ghaferi et al, 2009 [9]	429	131 (30.5%)	41 / 90	**DMFS:** *P<0.02* **OS:** *P<0.001*	Univariate analysis only
Wiener et al, 2010 [8]	323	102 (31.6%)	50 / 52	**OS:** *P=0.04*	Multivariable analysis (HR not provided)
Reintgen et al, 2013 [10]	331	93 (28.1%)	31 / 62	**DFS:** *P=0.0019*	Multivariable analysis (HR=2.7)
Current study	1,517	387 (26.5%)	151 / 256	**OS:** *P=0.013*	Multivariable analysis (HR=1.39)

Discussion

This study investigated the hypothesis that the presence of melanoma metastasis in the NSLN of patients who underwent a CLND for a positive SLNB has an independent prognostic value. In the largest series analyzed for this purpose (*N*=1,538) we demonstrated the association between the presence of melanoma metastasis beyond the SLN (i.e. in the NSLN) and patient survival, independently of the number of positive LNs. Patients with metastatic disease in their NSLNs (i.e. beyond the SN) had a 36% increase of melanoma death risk as compared to NSLN-negative patients with metastatic disease confined to the SLN (HR=1.36, P<0.001).

At a first glance the negative prognostic role of the NSLN metastasis might simply reflect the worse prognosis of patients with multiple positive nodes. Focusing on patients with the same number of positive LNs, we confirmed that the survival rate of patients with negative NSLN was higher than that observed in patients with positive NSLN (**Figure 1B**), which further strengthens the evidence on the role of the presence of melanoma metastasis in the LNs beyond the SLN.

Then, we analyzed the prognostic value of the NSLN status in patients currently considered as having a homogeneous prognosis, that is, with two-three (AJCC N2a stage) or $\geq$ four (AJCC N3 stage) positive LNs [1, 5]. In this subgroup analysis, the NSLN status was an independent prognostic factor for patients with two-three positive LNs (AJCC N2a, P=0.013, **Figure 1C**) but not for patients with $\geq$ four positive LNs (AJCC N3, P=0.470), suggesting that risk stratification can be improved by exploiting the information on NSLN status specifically in patients with two-three positive LNs.

Following our findings, patients were re-classified into four groups (**Figure 1D**): *Group-1*, one positive SLN after SLNB and negative NSLN after CLND (AJCC N1a); *Group-2*, two-three positive SLNs after SLNB and negative NSLN after CLND (AJCC N2a stage - NSLN negative); *Group-3***,** two-three positive LNs including both sentinel and NSLNs (AJCC N2a stage - NSLN positive); and *Group-4*, $\geq$ four positive LNs including both sentinel and NSLNs

(AJCC N3 stage). This classification had prognostic significance (P<0.001) and stratified survival of patients with two-three positive LNs (AJCC N2a stage) in two new sub-groups with significantly different prognosis (Group-3 versus Group-2, HR=1.39, P=0.013).

These results demonstrated that in SLNB-positive patients both the number of LNs harboring melanoma metastasis and the NSLN status are independent determinants of patient survival, especially in case of two-three positive LNs. Three previous reports studied the prognostic value of NSLN status in patients with two-three positive LNs (**Table 3**). Reintgen *et al* investigated the association of NSLN status with disease-free survival (but not overall survival) in 93 patients, and reported an independent 2.7-fold increase in risk of recurrence for patients with metastatic NSLN [10]. Ghaferi *et al* analyzed a single institution series of 131 patients and showed that NSLN status was a significant prognostic factor for metastasis-free and overall survival, but did not perform a multivariable analysis to adjust the prognostic effect of the NSLN status for other staging features. Differently, Wiener *et al* performed a multivariable analysis of 102 patients showing a borderline significance difference in overall survival according to NSLN status (P=0.04, HR not reported) [8].

In order to summarize the available evidence on this topic, we pooled our results with those from the two studies that had investigated the prognostic value for overall survival of NSLN status in patients with two-three positive LNs (Ghaferi *et al* [9] and Wiener *et al* [8]): the meta-analysis, which encompassed 620 patients (284 had negative NSLN and 336 had positive NSLN, **Figure 2**), showed that the NSLN status is a highly significant prognostic factor for patients with two-three positive LNs (summary HR=1.59, P<0.001).

The present study did not show a significùant difference in survival between patients in Group-1 and Group-2, which suggests that these two categories of our prognostic model might be combined into a single prognostic category. However, we believe that more research is needed to draw definitive conclusions on this aspect due

to the larger sample size required to address this specific issue with an adequate number of events and thus with a sufficient statistical power.

The original findings from our series as well the summary result of the meta-analysis add information to the existing literature and foster the implementation of the NSLN status into the TNM staging system to improve the risk stratification of patients with two-three positive LNs.

Although there is still lack of evidence about the therapeutic value of performing SLNB followed by CLND [2], this study strengthens the importance of SLNB as a source of prognostic information for melanoma patients. In fact, the prognostic value of the SLNB is not limited to the definition of the absence (AJCC stage I and II) or presence (AJCC stage III) of metastasis in the SLN, but offers other criteria to stratify patient risk [22, 23], such as the NSLN status (which can be assessed by performing CLND after a positive SLNB). Waiting for definitive results regarding the therapeutic value of performing CLND in SLNB-positive patients from the ongoing Multicenter Selective Lymphadenectomy Trial-2 (MSLT-II, which is comparing lymphadenectomy and observation after a positive SLNB) [24], the findings of the present study underscore the prognostic role of merging the information derived from both SLNB and CLND, supporting the use of these procedures as a two-step prognostic tool [13].

These findings not only have a prognostic relevance but could also have some implications for characterizing the biology of melanoma progression from the primary tumor through the regional lymph nodes. In fact, our data support the hypothesis that the SLN may act as a physiological barrier to melanoma spreading through regional LNs. In particular, melanoma metastasis within the SLN might be better controlled by the immune system, while the presence of melanoma cells in NSLNs would witness the failure of the SLN barrier and herald a faster disease progression through the lymphatic system. Several mechanisms, such as lymphatic markers expression [25-27], tumor infiltrating lymphocytes [28-30], tumor associated macrophages [31-34] and dendritic cells [35-38]

may act as regulators of this process. In primary melanoma, the expression of lymphatic markers [e.g. the vascular endothelial growth factor-C] as well as immunohistochemistry-detected lymphatic vessels density and invasion have been associated with regional LN metastasis [25, 39]. A high lymphatic vessels density in the peritumoral area and the presence of lymphatic vessels invasion increase the likelihood of harboring SN metastasis [40]. A similar process may occur in the SLN, where the relationship between tumor and endothelial cells of the lymphatic vessels may lead to tumor progression to the NSLNs, as observed in breast cancer [41]. Dedicated translational research on this topic is needed to understand the molecular mechanisms underlying this hypothesis, which might open a new avenue also in the therapeutic setting.

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CHAPTER 8

Assessment of lymphatic and blood vasculature in primary cutaneous melanomas of scalp and neck

Pasquali S, Montesco MC, Ginanneschi C, Baroni G, Miracco C, Urso C, Mele F, Lombardi AR, Quaglino P, Cattaneo L, Staibano S, Botti G, Visca P, Zannoni M, Soda G, Corti B, Pilloni L, Anselmi L, Lissia A, Vannucchi M, Manieli C, Massi D. Lymphatic and blood vasculature in primary cutaneous melanomas of the scalp and neck. Head Neck 2015;37:1596-602

Introduction

Although cutaneous melanoma is not the most common form of skin cancer, it is recognized as the most life-threating [1]. The seventh edition of the AJCC staging system considers features of primary tumor, the presence of lymph node and distant metastases as essential staging criteria, however other factors, such as patient age and sex as well as primary tumor location showed prognostic value [2]. Melanoma on the head and neck region represents approximately 20% of all cutaneous melanomas and shows a greater risk of disease progression and melanoma death than other tumor [3-8] locations. Among these tumors, scalp and neck location, which represents roughly 5% of all melanomas and 35% of those arising on the head and neck region, accounts for 10% of all melanoma deaths with a 10-year survival rate of 63%, a significantly poorer prognosis compared to that of patients with melanomas of the face and ear region (10-year survival of 80%) [5, 9, 10].

A greater incidence of brain metastasis [11] and pitfalls in primary tumor treatment, such as narrower excision margin [12] and difficulties in the prediction of the lymphatic drainage (which lower the accuracy of sentinel lymph node biopsy [13]) are possible explanation for the risk of disease progression observed in these patients. In addition, the higher risk of disease progression observed in these patients may be also associated with a higher tumor vascularity [14, 15]. However, measurement of lymphovascular invasion and density has not been accurately assed in large series of scalp and neck melanomas yet.

Due to the rarity of this tumor presentation, we retrospectively collected tumor samples and follow-up data from 156 patients with a primary invasive melanoma of the scalp and neck region from 16 institutions and performed double immunostaining with D2-40/CD34 antibodies. The aim of the present study was two-fold. First, we compared diagnostic effectiveness of double D2-40/CD34 immunohistochemical (IHC) staining with conventional H&E morphology in identifying lymphovascular invasion. Afterwards, we analyzed the association of IHC-detected lymphovascular invasion and lymphatic and blood vessel density with common patient and tumor features as well as with patient survival.

Patients and methods

The study protocol was approved by the Research Committee of the Italian Melanoma Intergroup (IMI), the Italian network for melanoma treatment and research (www.melanomaimi.it), and the Italian Association of Anatomic Pathology and Diagnostic Cytopathology (SIAPEC, www.siapec.it), the Italian branch of the International Academy of Pathology. Retrospective data from patients with invasive melanoma of the scalp and neck diagnosed between January 1995 and January 2012 at 16 centers were gathered in a multi-center database. Data were extracted according to the following selection criteria: 1) single invasive primary melanoma; 2) paraffin-embedded tissue available for IHC analysis. In situ melanomas and lentigo maligna melanomas were excluded from the analysis.

IHC analysis was performed on representative sections 4µm in thickness of formalin-fixed, paraffin-embedded tumor tissues. Primary antibodies anti-podoplanin (mouse monoclonal clone D2-40, ready to use, Ventana, Tucson, AZ) and anti-CD34 (mouse monoclonal clone QBEnd/10, ready to use, Ventana) were placed on the same slide for a double staining, incubated according to the IHC DS uDAB-uRED protocol suggested by Ventana automated stainer BenchMark Ultra. Diaminobenzidine (DAB) and fast red were used as chromogens for podoplanin and CD34, respectively. Upon completion of the staining run, tissue sections were removed from the stainer and counterstained with Mayer's haematoxylin. Sections of lymphangioma and tonsil were used as positive controls for podoplanin and CD34, respectively. Negative controls were performed by substituting the primary antibody with a non-immune serum at the same concentration. The control sections were treated in parallel with the samples. All sections were dehydrated and mounted with Permount.

IHC double-stained slides were reviewed independently by three pathologists (CG, MCM, DM), who were blinded to clinical outcome. LVI and BVI (present or absent) was defined as the presence anywhere within the

primary tumor of neoplastic cell(s) with morphologic features of melanoma cells in lumens highlighted by D2-40 (LVI) and CD34 staining (BVI), respectively. Questionable instances were discussed and disagreements were resolved by consensus reading. The location of vessel invasion was also topographically assessed as within the intratumoural or peritumoural areas. Peritumoral vessels were defined as D2-40-positive or CD34-positive vessels within an area of 500μm from the tumor border. Intratumoral vessels were defined as D2-40-positive or CD34-positive vessels located within the tumor mass and not confined by invagination of normal tissue. Double stained sections were digitally scanned using a D-Sight scanner (Menarini, Italy) for computer-assisted morphometric analyses. Upon identification of the "hot spot", LVD and BVD were quantified in 5 adjacent fields at x40 (corresponding to $1mm^2$), both in intratumoral and peritumoral location.

The following variables were considered for each patient: patient age and sex, primary tumor site (neck, scalp), thickness, ulceration, Clark level of invasion, tumor histotype (superficial spreading melanoma, SSM, versus others), tumor infiltrating lymphocytes (TIL), microscopic satellitosis and H&E-detected lymphovascular invasion.

Variables Clark level, tumor infiltrating lymphocytes, lymphovascular invasion and satellitosis had missing values in 1 (0.6%), 8 (5.1%), 5 (3.2) and 4 (2.5%) patients. The multiple imputation method was used to predict missing values, using variables with non-missing values as predictors (i.e. age, sex, tumor thickness, ulceration and mitotic rate) [16, 17].

Diagnostic effectiveness of IHC for detecting LVI/BVI was studied with the McNemar's test. The Fisher's exact test and the chi-square test were used to investigate association between LVI/BVI and covariates, while the Mann-Whitney U test and the analysis of variance were fitted to data to investigate association between intratumor/peritumoral LVD/BVD and covariates. Significant variables were tested at multivariable analysis (the logistic regression for LVI/BVI and the multivariate regression for LVD/BVD).

Disease-free survival (DFS) and melanoma-specific survival (MSS) were calculated from the time of primary melanoma diagnosis to the time of melanoma recurrence and death, respectively, or last follow-up. Univariate and multivariable survival analysis was performed with the Cox proportional hazard model (stepwise backward procedure) to study the association of covariates with DFS and MSS.

Analyses were conducted setting the alpha level of significance at 0.05 and performed with STATA SE/11.0 (College Station, TX).

Results

A total of 156 patients met the inclusion criteria of this study (Table 1).

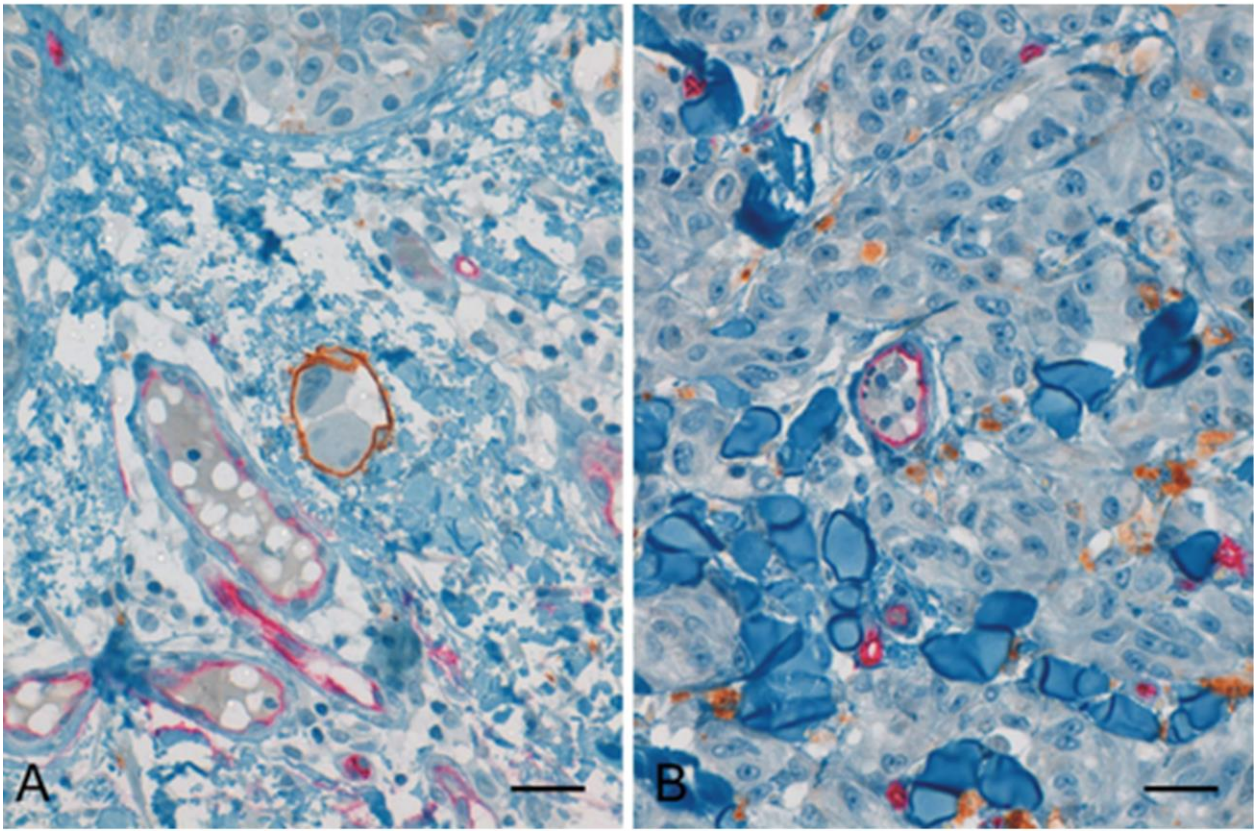
Table 1. Association of IHC-detected LVI/BVI with clinical and pathological features of 156 patients with scalp and neck cutaneous melanomas.

Variables		**LVI/BVI Absent**		**LVI/BVI Present**		***P-value***
		N	**%**	**N**	**%**	
Age (IQR) years		64 (46-75)		64 (47-75)		*0.531*
Sex	*Female*	23	24.7	22	34.9	*0.208*
	Male	70	75.3	41	65.1	
Primary tumor site	*Scalp*	61	65.6	43	68.3	*0.863*
	Neck	32	34.4	20	31.7	
Tumor thickness	*Median (IQR) mm*	1.15 (0.7-2.8)		3.5 (1.6-6-6)		*<0.001*
	<1.01mm	39	41.9	10	15.8	*<0.001*
	1.01-2.00mm	20	21.5	11	17.4	
	2.01-4.00mm	23	24.7	13	19.0	
	>4.00mm	11	11.9	29	47.8	
Ulceration	*Absent*	73	78.5	30	47.6	*<0.001*
	Present	20	21.5	33	52.4	
Mitotic rate	*<1/mm2*	34	36.5	4	6.3	*<0.001*
	≥1/mm2	59	63.5	59	93.7	
Clark level of invasion	*II-III*	81	87.1	48	76.2	*0.088*
	IV-V	12	12.9	15	23.8	
Tumor histotype	*SSM*	57	61.3	26	41.3	*0.015*
	Others	36	38.7	37	58.7	

TILs	*Absent/Nonbrisk*	83	89.2	56	88.8	*1.000*
	Brisk	10	10.8	7	11.2	
Satellitosis	*Absent*	89	95.7	56	88.8	*0.120*
	Present	4	4.3	7	11.2	

IHC markers increased the detection of LVI/BVI (Figure 1) compared to H&E (63 patients, 40.4%, with IHC versus 26 patients, 16.6%, with H&E, P<0.001). IHC-detected LVI/BVI was located in the intratumoral and peritumoral areas in 18 (33.3%) and 14 (25.4%) patients, respectively. Strikingly, while IHC-detected LVI was identified in 54 (34.6%) patients, BVI was diagnosed in 21 cases (13.5%).

Figure 1. D2-40/CD34 double immunostaining in melanoma tissues: A) A tumour embolus is observed within a D2-40 positive lymphatic channel (brown staining). Note adjacent capillary blood vessels (red staining) containing red blood cells (original magnification x40). B) A dilated CD34 positive blood vessel (red staining) shows melanoma cells inside its lumen (original magnification x40).



IHC-detected LVI and BVI

The presence of both IHC-detected LVI and BVI was associated with primary tumors showing greater thickness (P<0.001), ulceration (P<0.001), mitotic rate ≥ 1/mm2 (P<0.001) and histotype other than SSM (P=0.015) (Table 1). Upon multivariable analysis, greater tumor thickness [odds ratio (OR)=1.21, 95% confidence interval (CI) 1.07-1.37, P=0.003) and mitotic rate ≥ 1/mm2 (OR=7.35, 95%CI 2.18-24.8, P=0.001) were independently predictors of LVI/BVI.

Predictors of IHC-detected LVI and BVI were also separately investigated. Upon univariate analysis, LVI was associated with tumors showing greater thickness (P=0.002), ulceration (P=0.001), mitotic rate ≥ 1/mm2 (P<0.001), histotype other than SSM (P=0.029) and satellitosis (P=0.049). Upon multivariable analysis, ulceration (OR=2.34, 95%CI 1.13-4.88, P=0.023) and mitotic rate ≥ 1/mm2 (OR=2.34, 95%CI 1.45-12.15, P=0.009) were the independent predictors of LVI. Upon univariate analysis, BVI was associated with tumors showing greater thickness (P<0.001), mitotic rate ≥ 1/mm2 (P=0.002), Clark level IV-V (P=0.012) and histotype other than SSM (P=0.019). Upon multivariable analysis, greater tumor thickness (OR=1.17, 95%CI 1.04-1.34, P=0.011) was the only independent predictor of BVI.

LVD and BVD

In the intratumoral area, BVD (median 25/mm2, IQR 7-38/mm2) was greater than LVD (median 6/mm2, IQR 0-13/mm2). Similarly findings were detected in the peritumoral area, where BVD (median 35/mm2, IQR 15-50/mm2) was greater than the LVD (median 10/mm2, IQR 2-19/mm2).

Table 2 reported the association of patient and tumor features with LVD and BVD considering their intratumoral or peritumoral location. LVD in the intratumoral area showed only a borderline correlation with the SSM histotype (P=0.045), while in the peritumoral area it was associated with mitotic rate ≥ 1/mm2 (P=0.001) and Clark level of invasion IV-V (P=0.044). The presence of IHC-detected LVI was associated with LVD

surrounding (P=0.001) but not within the primary tumor (P=0.192). Upon multivariable analysis, peritumoral LVD was associated with Clark level II-III [Correlation Coefficient (CC)=-4.83, 95% confidence interval (CI) -8.34 - -0.76, P=0.007], mitotic rate $\geq$ 1/mm2 (CC=4.57, 95%CI 1.44-7.69, P=0.004) and LVI (CC=4.11, 95%CI 1.32-6.91, P=0.004).

Interestingly, BVD in the intratumoral area was associated with ulceration (P<0.001) and mitotic rate $\geq$ 1/mm2 (P=0.001). Furthermore, the same pathologic features were associated also with peritumoral BVD (ulceration, P=0.002; mitotic rate, P=0.002) along with tumor thickness (P=0.002). Remarkably, IHC-detected BVI was associated with both intratumoral (P=0.020) and peritumoral (P=0.018) BVD. Strikingly, upon multivariable analysis, ulceration was the only factor independently associated with greater intratumoral (CC 8.43, 95%CI 0.87-16.00, P=0.029) and peritumoral (CC 7.16, 95%CI 0.10-14.22, P=0.047) BVD.

Table 2. Association of IHC-detected peritumoral and intratumoral LVD and BVD with clinical and pathological features.

		Intratumoral LVD		Peritumoral LVD		Intratumoral BVD		Peritumoral BVD	
		Median (IQR)	***P-value***	**Median (IQR)**	***P-value***	**Median (IQR)**	***P-value***	**Median (IQR)**	***P-value***
Age	*≤64 years*	9 (2-14)	*0.166*	10 (6-19)	*0.364*	23 (15-37)	*0.572*	32 (22-46)	*0.098*
	>64 years	5 (2-10)		10 (4-18)		26 (13-42)		39 (50-32)	
Sex	*Female*	7 (2-12)	*0.753*	9 (4-20)	*0.682*	25 (13-44)	*0.740*	36.5 (26-49)	*0.696*
	Male	6 (2-13)		11 (5-18)		26 (13-36)		35 (22-50)	
Primary tumor site	*Scalp*	7 (2-14)	*0.753*	12 (8-20)	*0.080*	30 (11-42)	*0.544*	36.5 (24-52)	*0.642*
	Neck	6 (2-12)		10 (4-18)		24 (15-35)		34 (25-49)	
Breslow thickness	*continuous variable*	R2=0.02	*0.086*	R2=0.003	*0.510*	R2=0.02	*0.064*	R2=0.06	*0.002*
Ulceration	*Absent*	6 (3-12)	*0.631*	10 (5-17)	*0.124*	21 (11-33)	*<0.001*	34 (21-44)	*0.002*
	Present	4 (1-17)		13 (4-20.5)		31 (21-48)		41 (29-58.5)	
Mitotic rate	*<1/mm2*	5 (1.5-9)	*0.139*	7.5 (3.5-10.5)	*0.001*	18 (8-23)	*0.001*	26 (15.5-39.5)	*0.002*
	≥1/mm2	7 (2-14)		12 (5-20)		27.5 (15.5-42)		37 (29-53)	
Clark level of invasion	*II-III*	7 (2-14)	*0.082*	11 (5-19)	*0.044*	23 (13-36)	*0.136*	34 (24-50)	*0.423*
	IV-V	4 (1-9)		6.5 (3-14)		31 (17-48)		39 (29.5-49.5)	
Tumor histotype	*SSM*	8 (3-14)	*0.045*	10 (5-16)	*0.492*	23 (11-35)	*0.208*	34 (24-46)	*0.368*
	Others	3 (1-10)		11.5 (4-20)		26 (16-42)		38 (26.5-52)	
TILs	*Absent/Nonbrisk*	6.5 (2-13)	*0.716*	10 (5-18)	*0.757*	26 (14-40)	*0.404*	36 (25-50)	*0.062*
	Brisk	5.5 (1.5-12)		12 (6-19.5)		21.5 (12-30.5)		25.5 (15.5-39.5)	
Satellitosis	*Absent*	7 (2-13)	*0.144*	10 (5-19)	*0.676*	24 (13-39)	*0.635*	35 (24-50)	*0.844*
	Present	2 (0-13)		11 (4-18)		27 (17-36)		37 (28-50)	
IHC-detected LVI	*Absent*	5.5 (1.5-12)	*0.192*	9 (4-16)	*0.001*	NA		NA	
	Present	7 (2.5-17)		14 (8-23)		NA		NA	
IHC-detected BVI	*Absent*	NA		NA		23 (12.5-35.5)	*0.020*	34 (24-46)	*0.018*
	Present	NA		NA		37.5 (22-59.5)		50 (31-55)	

Survival analysis

The median follow-up was 44 months (interquartile range, IQR 18-80).

At univariate analysis for DFS, the presence of HIC-detected LVI/BVI was associated with shorter time to recurrence [hazard ratio (HR=2.34), 95%CI 1.31-4.21, P=0.004] along with scalp primary tumor (HR=2.48, 95%CI 1.23-5.00), thicker (HR 1.07, 95%CI 1.03-1.10, P<0.001) and ulcerated tumors (HR=1.89, 95%CI 1.06-3.39, P=0.032) as well as those showing Clark level IV-V (HR 2.61, 95%CI 1.37-4.98, P=0.004), mitotic rate $\geq$ 1/mm2 (HR=3.68, 95%CI 1.43-9.60, P=0.007) and satellitosis (HR=5.17, 95%CI 2.48-10.78, P<0.0001). LVD (intratumoral, P=0.07; peritumoral, P=0.993) and BVD (intratumoral, P=0.607; peritumoral, P=0.799) did not correlate with DFS. Upon multivariate Cox regression analysis, thicker tumors (HR=1.06, 95%CI 1.02-1.09, P=0.001) and mitotic rate $\geq$ 1/mm2 (HR=3.11, 95%CI 1.19-8.13, P=0.02) were independently associated with a shorter DFS, while IHC-detected LVI/BVI was no longer significant.

At univariate analysis for MSS, neither the presence of IHC-detected LVI/BVI (P=0.217) nor LVD (intratumoral, P=0.184; peritumoral, P=0.576) nor BVD (intratumoral, P=0.382; peritumoral, P=0.794) were prognostic factor for patient survival. Upon multivariate Cox regression analysis, tumor thickness was the only significant predictor of MSS (HR=1.07, 95%CI 1.03-1.12, P=0.002).

Discussion

To our knowledge this is the first study examining the clinical relevance of IHC-detected vascular invasion and lymphangiogenesis in a large cohort of patients with scalp and neck melanomas. We found that the use of a dual D2-40/CD34 immunostaining identified roughly 25% more tumors showing LVI/BVI than conventional H&E staining (40.4% vs. 16.6%, P<0.001). This observation confirms previous findings [22, 24-30] that lymphovascular invasion is more accurately detected by IHC than in H&E-stained tissues [18-25]. In our study, LVI was demonstrated in the 34.6% of the primary tumors. Reported LVI incidence for cutaneous melanoma, irrespective of the anatomic site, ranges from 16% to 37%, while it has been reported in 15%-23% of cases with head and neck melanomas [18-25]. Accurate comparison with previous studies is

complicated by the fact that scalp and neck melanomas are rarely evaluated as a separate subset, being more frequently grouped together with melanoma of the face under the category of “head and neck” melanomas.

Here, IHC-detected BVI was observed in 13.5% of melanomas of the scalp and neck region, approximately four times higher than the 2-4% reported in previous studies, irrespective of primary tumor site [19, 20, 26]. In the head and neck area, Storr et al reported a BVI incidence of 5.8%. [20] We also showed that the density of blood vessels was greater than the density of lymphatic vessels both in the intratumoral and peritumoral areas. Overall, these data suggest that scalp and neck melanomas have peculiar vascular characteristics in terms of higher blood vessel invasion and density, which may stimulate tumor growth and represent a potential source of early blood-borne metastases. These results may also lead to formulate the hypothesis that this more aggressive subgroup of melanomas may preferentially benefit from anti-angiogenetic therapies.

We also found a strong association of primary melanoma features with vessel invasion and density. The presence of ulceration significantly correlated with LVI and was the only independent predictor of intratumoral and peritumoral BVD. The association between H&E-detected [27, 28] or IHC-detected LVI [20, 29] and ulceration has been previously taken into account to explain the higher propensity of ulcerated tumors to develop sentinel lymph node and distant metastasis. It was shown that the presence of both ulceration and LVI increased the risk of sentinel lymph node metastasis up to 45% and 61% depending upon patient age. [29] In our study, due to the limited number of patients submitted to sentinel lymph node biopsy (data not reported), the ability of D2-40/CD34 double IHC to predict sentinel lymph node status cannot be assessed.

The relationship between ulceration and LVI is a matter of speculation. It has been suggested that ulceration in melanoma is indicative of a hypoxic state that promotes lymphangiogenesis, which in turn results in a larger vessel area that facilitates dissemination of melanoma cells to the sentinel lymph node and distant sites. Recent evidence showed the activation of genes involved in cell adhesion and

extracellular matrix interactions in ulcerated melanomas, including osteopontin, a strong stimulator of lymphatic endothelial cell migration and lymphangiogenesis.[39-41]

Finally, we analyzed the prognostic relevance of LVI/BVI and vessel density in scalp and neck melanoma. Although univariate analysis of IHC-detected LVI/BVI data showed a significant association with disease-free survival, this association was no longer significant at multivariable analysis. Furthermore, in a multivariable model for melanoma-specific survival, where thickness remained the only independent predictor of survival, nor lymphovascular invasion nor density were prognostic factors. A recent systematic review [22] summarized previous studies addressing the prognostic impact of lymphatic biomarkers in melanoma (though not strictly confined to the scalp and neck area) and highlighted heterogeneous results. There is evidence that IHC-detected LVI is a predictor of sentinel lymph node metastasis and poorer survival [14, 30] but these observations are still controversial [20, 26]. In addition, though the presence of greater density of lymphatic vessels in the peritumoral area appears to be associated with melanoma spread to regional lymph nodes and distant sites [31], the prognostic significance of LVD remains unclear [20]. Wide methodological variations, including endothelial cell markers for blood and lymphatic vessels identification, differences in study design and sample size, have been advocated to explain these discrepancies. It has also been suggested that the functionality of vessels rather than absolute vessel density may determine disease prognosis in melanoma as in other cancers [32, 33].

In conclusion, IHC increases the detection of lymphovascular invasion in scalp and neck melanomas. Peculiar vascular characteristics in terms of higher blood vessel invasion and density may account for the greater risk of dissemination through the haematogenous route, though vascular invasion and vessel density did not add prognostic information to the common staging features. Among them, ulceration was associated with LVI, peritumoral and intratumoral BVD, supporting the invasion of lymphatic vessels and the blood vessels sprouting as potential mechanisms beyond the negative prognostic value of ulceration. Nevertheless, higher blood vessel supply offers a strong biological rationale to explore therapeutic strategies targeting the angiogenic axis in this subgroup of melanoma patients.

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CHAPTER 9

IMMUNOHISTOCHEMISTRY-DETECTED ANGIOGENESIS AND LYMPHANGIOGENESIS IN PRIMARY MELANOMA AND SENTINEL LYMPH NODE METASTASIS: A PREDICTIVE AND PROGNOSTIC STUDY

Unpublished data

Background

Sentinel lymph node (SLN) biopsy is a widely recognized prognostic tool for patients with early stage melanoma [1, 2]. Patients with SLN metastasis are considered at high-risk of harbouring distant disease and have approximately 60% 10-year survival [3-5]. Tumor cells are thought to acquire motility and invasiveness, to detach from the primary tumor, to infiltrate the extracellular matrix, to penetrate in the lymphatic vessels, and, finally, to reach the SLN. Lymphatic vessels play a pivotal role in the metastatic process and a growing body of evidence supports the hypothesis that they are not only passive participants providing the channels through which tumor cells move, but that they are actively involved in cancer cell recruitment to lymph nodes, modulation of immune response, and maintenance of cancer stem cell [6-12]. Tumor cells actively participate in this process through releasing growth factors, such as vascular endothelial growth factor (VEGF), to induce lymphangiogenesis and increase the number of lymphatic vessels both at primary and metastatic sites [6-8]. Observational studies investigated both markers of angiogenesis and lymphangiogenesis in primary melanoma and found a correlation with SLN metastasis as well as distant metastasis, though conflicting results have been reported [13]. Limited evidence exist on the role of blood and lymphatic vessels markers when detected in the SLN, which seems to be associated with progression of melanoma cells to non-SLN (NSLN) through lymphatics [14]. NSLN metastases are detected only in approximately 20% of patients with positive SLN biopsy and are a determinant of patient outcomes [15-17]. It has been hypothesized that the SLN can act as a barrier against the spread of melanoma cells through the regional lymphatic and to distant sites and that the interaction between melanoma cells and endothelial cells of lymphatic and blood vessels determined the occurrence of NSLN metastasis, as already reported in breast cancer [18].

The aim of this study was to assess angiogenesis and lymphangiogenesis in both primary melanoma and metastatic SLN to investigate possible association with SLN and NSLN metastasis, respectively, and patient prognosis.

Materials and Methods

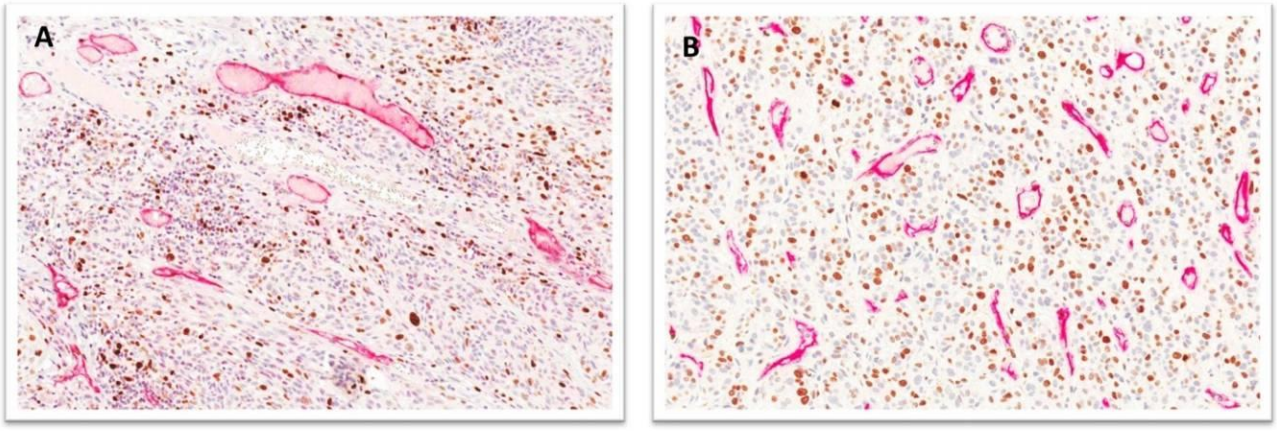
Case series

Data for patients with primary cutaneous melanomas treated between 1994 and 2014 at the Surgery Branch of the Department of Surgery, Oncology and Gastroenterology of the University of Padova were extracted from a prospectively maintained database using the following selection criteria: (1) patients presenting with a single primary cutaneous melanoma; (2) wide excision and SLN biopsy performed at the University of Padova; (3) primary melanoma and SLN biopsy specimen assessed at the Pathological Anatomy and Histology Unit of the Veneto Institute of Oncology IOV- I.R.C.C.S. and of the Pathological Anatomy Unit of the University of Padova; (4) both specimen from primary tumour and SLN available; (5) 6 months or more of follow-up.

Immunohistochemistry

Double immunohistochemical staining to detect proliferating endothelial cells in blood and lymphatic vessels was automatically performed using the BOND-MAX system (Leica Biosystems, Newcastle upon Tyne, UK) on 4 μm-thick sections from each FFPE primary MM and SLN. Tissue sections were subjected to heat-induced antigen retrieval in citrate buffer for 30 minutes. The first immunoreaction was performed with the mouse primary antibody for Ki-67 (clone MIB-1; Agilent Technologies, Santa Clara, USA; working dilution 1:100) using the Bond Polymer Refine Detection kit (Leica Biosystems) with the 3,3'-diaminobenzidine (DAB) chromogen as substrate. The second one was achieved with the mouse primary antibodies for podoplanin (clone D2-40; Agilent Technologies; working dilution 1:25, 20 min, citrate buffer, Figure 1A) or for CD34 (clone QBEND-10; Thermo Fisher Scientific, Waltham, USA; working dilution 1:800, 20 min, citrate buffer, Figure 1B) using the Bond Polymer Refine Red Detection kit (Leica Biosystems) with Fast Red chromogen as substrate.

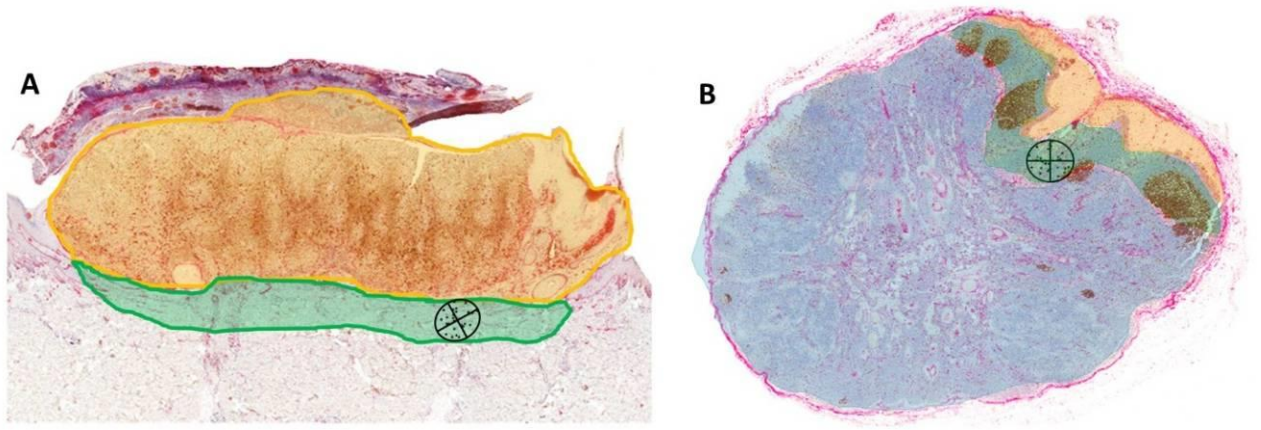
Figure 1. Double staining for D2-40 (red) and Mib1 (brown) for lymphatic vessels (A) and double staining for CD34 (red) and Mib1 (brown) for blood vessels (B)



Sections were finally counterstained with haematoxylin. Appropriate positive and negative controls were immunostained concurrently. All the immunostained slides were evaluated by two pathologists (M.C.M. and R.C.) unaware of any clinical information.

For the analyses, primary melanomas were divided in two zones (Figure 2A): intratumoral zone, namely the area inside the tumor mass, and peritumoral zone, namely the area between the invasive margin of the tumor mass and a distance of a Chalkley grid diameter at 200x magnification. In the SLN three zones were identified (Figure 3A): intratumoral zone, namely the area inside the metastasis (considered only if greater than a Chalkley grid at 200x magnification), peritumoral zone, namely the lymph node area between the margin of the metastasis and a distance of a Chalkley grid diameter at 200x magnification, and extratumoral zone, namely the lymph node area farther away than a Chalkley grid diameter at 200x magnification from the metastasis. Each of these zones was scanned at 40x magnification to find the three (or at least one in small metastases) zones with the highest number of immunostained vessels (hot-spots) avoiding areas with ulceration, regression, and necrosis. All the evaluations were performed in these hot-spots.

Figure 2. Primary melanoma (A) and SLN (B) were divided in two and three areas, respectively. An intratumoral and a peritumoral area were identified in primary melanoma, while SLN had also an extratumoral area identified.



Blood and lymphatic vessel densities were defined as the absolute number of CD34 or podoplanin immunostained vessels, respectively, in a 400x magnification field in the hot-spot area [19]. Blood and lymphatic Chalkley scores were calculated using a 25-point Chalkley eyepiece grid (a round grid containing 25 randomly positioned dots) at 200x magnification. In brief, the grid was rotated until the maximum number of dots were superimposed on CD34 or podoplanin immunostained vessels in the hot-spot area and this number was recorded [20]. Blood and lymphatic vessel Proliferation Indexes were assessed by counting the number of Ki-67 positive CD34 or podoplanin immunostained cells in a 400x magnification field in the hot-spot area.

Figure 3. Chalkley method for scoring D2-40 positive lymphatic (A) and CD34 positive blood (B) vessels.

Statistical analysis

Normality was assessed both by graphical (box-plot and qq-plot) and formal methods (Shapiro-Wilk test). In the absence of normality, Wilcoxon rank-sum test with continuity correction was used for continuous variables. As for categorical variables, χ^2 test or Fisher's Exact test were applied as appropriate. DFS was defined as time from the MM diagnosis to recurrence or death from any cause, whereas OS was defined as time from the MM diagnosis to death from any cause. Kaplan-Meier curves and Log-rank test were used for the survival analyses. Multivariate logistic regression analysis was used to assess the independent contribution of different variables. All the statistical analyses were performed with the R software (version 3.2.3; R Foundation for Statistical Computing, Wien, Austria). A *p* value <0.05 was considered statistically significant.

Results

Case series

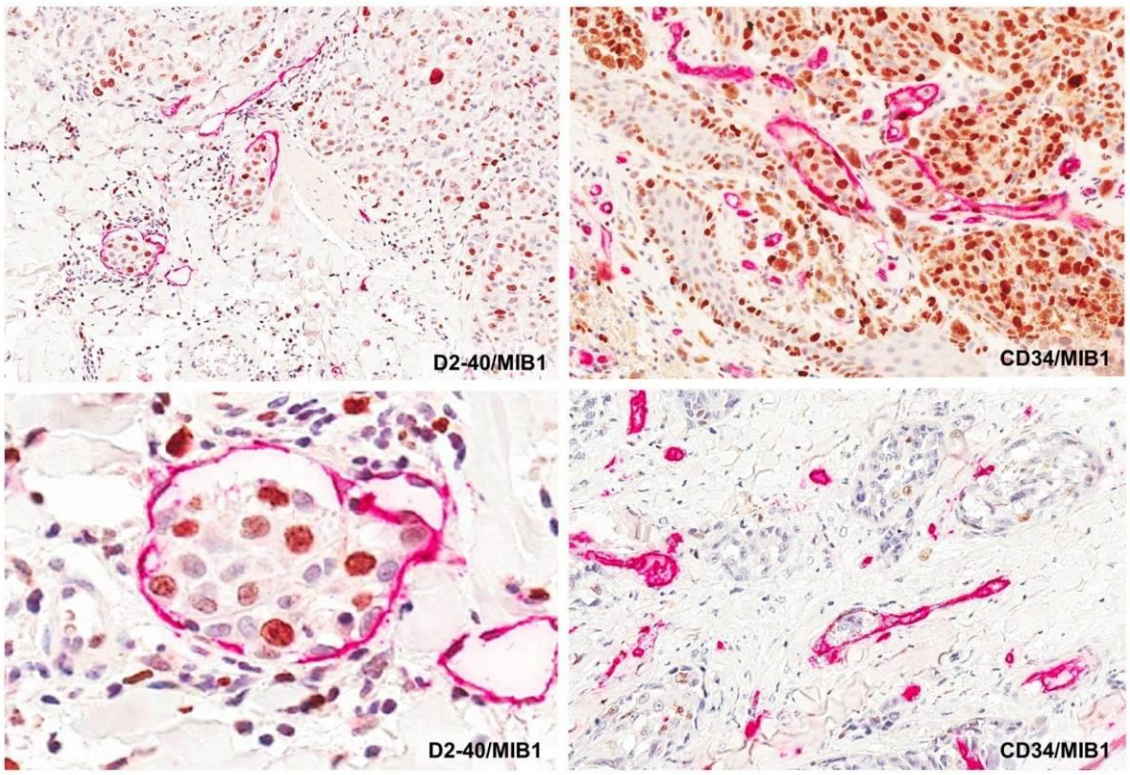
There were 122 patients eligible for this analysis. SLN was positive in 49 patients (40.1%) and a completion lymph node dissection (CLND) was performed in 40 patients with 15 patients harbouring further metastasis in their NSLN (37.5%).

Category	**n**	**%**
Age at diagnosis (yrs)		
- Mean ± SD	60.0 ± 15.2	
- Range	20-86	
Gender		
- Male	68	55.7
- Female	54	44.3
Primary site		
- Head and neck	17	14.9
- Trunk	54	44.3
- Extremity	51	41.8
Histotype		
- Superficial spreading	79	64.5
- Nodular	27	22.1
- Other	16	13.4
Ulceration		
- Present	43	35.2
- Absent	80	64.8
Breslow thickness		
- ≤ 1.00 mm	18	14.7
- 1.01-2.00 mm	38	31.1
- 2.01-4.00 mm	43	35.3
- > 4.00 mm	23	18.9
Clark level		
- III	9	7.4
- IV	102	83.6
- V	11	9.0
Mitotic Index		
- < 1	5	4.1
- 1-6	74	60.6
- > 6	43	35.3

Intra-Tumoral Lymphocytes		
- Absent	20	16.4
- Nonbrisk	89	72.9
- Brisk	13	10.7
Regression		
- Present	30	24.6
- Absent	92	75.4
Vascular invasion		
- Present	15	12.3
- Absent	107	87.7
Sentinel lymph node status		
- Positive	49	40.1
- Negative	73	59.9
Nonsentinel lymph node status		
- Positive	15	37.5
- Negative	25	62.5
- CLND not performed	9	-
Follow-up (mos)		
- Mean ± SD	32.0 ± 16.6	
- Range	1-102	

In primary tumour, standard H&E detected lymphovascular invasion in 15 patients (12.3%). Peritumoral and intratumoral lymphatic vessel invasion (LVI) was detected in 15 (12.3) and 22 (18.2%) patients, respectively (Figure 4). Similarly, peritumoral and intratumoral blood vessel invasion (BVI) was detected in 3 (2.5%) and 5 (4.1%) patients, respectively (Figure 4).

Figure 4. This panel figure depicted double staining for either D2-40 and Mib1 or CD34 and Mib1 to highlight the presence of melanoma cells in lymphatic (left side) or blood vessels (right side).



Primary melanoma

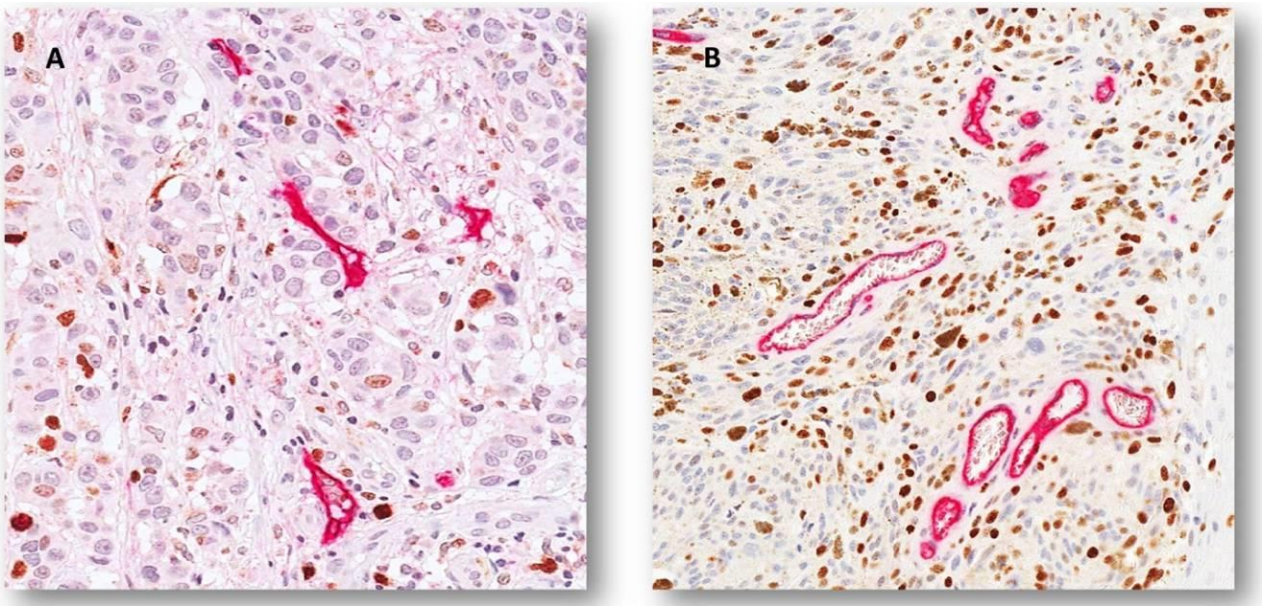
SLN and NSLN metastasis

SLN metastasis were associated with currently used predictive factors of primary melanoma, including presence of primary tumour ulceration (P=0.0223), greater Breslow thickness (P=0.0001), higher Clark level of invasion (P=0.0178), and increased mitotic index (P=0.0016). Also, absence of intratumoral lymphocytes (P=0.0052) in the primary tumour was associated with greater likelihood of SLN metastasis.

Interestingly, lymphatic vessel invasion (P=0.0045) was associated with SLN metastasis. This association was limited to peritumoral lymphatic vessel invasion (P=0.0112), as there was no statistically significant association between intratumoral lymphatic vessel invasion. This evidence is also supported by the association of mean peritumoral (P=0.0263), but not intratumoral, lymphatic vessel density and SLN metastasis (Figure 5). Conversely, although blood vessel invasion was not significantly associated with SLN

metastasis, mean intratumoral blood vessel density did show an association with SLN metastasis (P=0.0191).

Figure 5. Lymphatic (A) and blood (B) vessel density.



Lymphatic and vascular measurements showing statistically significant association with SLN metastasis were adjusted for primary melanoma staging features Breslow thickness and ulceration with multivariable logistic regression analysis. In order to limit collinearity between variables, each lymphatc and vascular variables was associated with prognostic features singularly.

Intratumoral lymphatic vessel density did not show independent predictive value for SLN metastasis when adjusted for Breslow thickness (P=0.310 and P=0.002, respectively) or primary tumour ulceration (P=0.173 and P=0.079, respectively). Conversely, peritumoral lymphatic vessel density was associated with SLN metastasis when adjusted for Breslow thickness (P=0.004 and P<0.001, respectively) or ulceration (P=0.002 and P=0.005, respectively). Also, lymphatic vessel invasion retained independent association with SLN metastasis when adjusted for Breslow thickness (P=0.010 and P=0.002, respectively) or primary tumour ulceration (P=0.005 and P=0.058, respectively).

NSLN metastasis were only associated with melanoma seated in the extremity (P=0.0285) and absence of intratumoural lymphocytes (P=0.0069). Lymphatic and blood markers of primary tumour did not correlate with NSLN metastasis.

DFS and OS

DFS and OS were associated with thicker primary (P<0.0001 for both outcomes) and increased mitotic index (P=0.0019 and P=0.0062, respectively). Lymphatic vessel invasion was associated with both DFS (P=0.0009) and OS (P=0.0024). Blood vessel invasion of primary tumour was associated with both shorter DFS (P=0.0127) and OS (P=0.0038). In particular, there was an association for intratumoral vessel invasion with both DFS (P=0.0014) and OS (P=0.0007) but not for peritumoral vessel invasion. Lymphatic vessel invasion, either intratumoral or peritumoral, did not correlate with survival.

Multivariable Cox regression analysis was performed to adjust effect on DFS and OS of the variables showing a statistically significant association at univariate analysis considering each variable alone to avoid issues of collinearity. Blood vessel invasion and intratumoral blood vessel invasion were both independent prognostic factor for DFS and OS (HR=8.61; 95%CI 2.26-32.75, P=0.002 and HR=10.59, 95%CI 2.65-42.26 P=0.001, respectively). Breslow thickness maintained its prognostic significance in all models for DFS and OS.

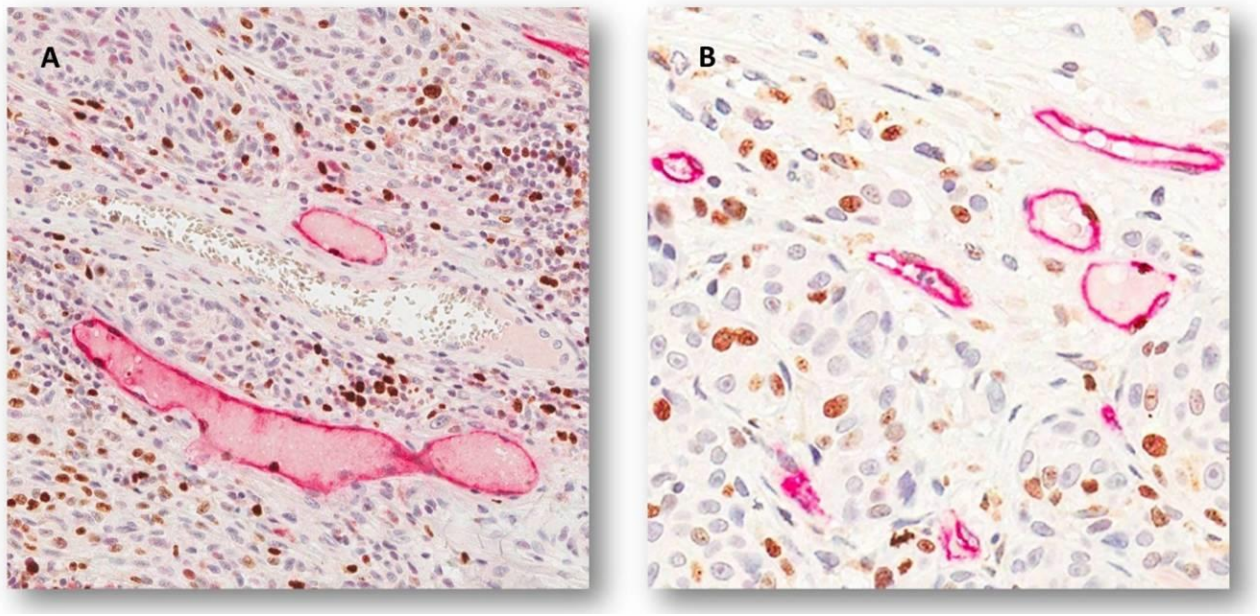
Metastatic SLN

NSLS metastasis

Univariate analysis are reported in Table S1 available as supplementary material. SLN metastasis size (P=0.0002) was associated with NLSN metastasis in patients who underwent CLND. Interestingly, performed measures of lymphatic and blood vessel density and invasion in the SLN did not correlate with NSLN metastasis. Intriguingly, the progression of melanoma cells through lymphatics was associated with

peritumoral (P=0.0138) and extratumoral (P=0.0485) lymphatic Proliferation Index (Figure 6). When the predictive value of these variables was adjusted for the SLN metastasis size, only peritumoral lymphatic proliferation index showed an independent association with NLSN metastasis, though to a borderline extent (P=0.055), while metastasis size lost its independent association (P=0.278).

Figure 6. Proliferation index in D2-40 positive lymphatic (red, A) and CD34 positive blood (red, B) vessels.



DFS and OS

Upon univariate analysis both measurements of lymphatic and blood vessel density in the SLN did correlate with survival. Mean intratumoral blood vessel density was associated with OS (P=0.0396), mean intratumoral lymphatic vessel density was associated with DFS (P=0.0342), and mean peritumoral lymphatic vessel density predicted both DFS (P=0.0107) and OS (P=0.0143). However, SLN lymphangiogenetic features parameters were adjusted for histopathological staging features their association with patient prognosis was no longer statistically significant.

Discussion

This study extensively investigated immunohistochemistry-detected lymphatic and blood vessel markers in both primary cutaneous melanoma and SLN of 122 patients with primary cutaneous melanoma. In the primary tumours, lymphatic invasion and density, especially when located in the peritumoural area, were associated with SLN metastasis. Conversely, prognosis was determined by blood vessel invasion, with a stronger case for intratumoral location. In the SLN, lymphatic proliferation index predicted progression of

melanoma cells through lymphatics to the NSLN, while lymphangiogenetic features within the SLN did not correlate with patient prognosis.

These results have been achieved through in-depth analysis of lymphatic and blood vessels using immunoistochemical markers. Precise count of immunostained vessels was performed under the microscope with the naked eye and with the aid of a Chalkley grid to assess density and absolute number of blood and lymphatic vessels in hot-spot areas. In our opinion Chalkley method is much quicker but may led to bias when facing with dilated vessels. Indeed, several dots may be superimposed on the same vessel altering the results. Moreover, Proliferation Indexes were calculated for both blood and lymphatic vessels. The applicability of the evidence from this study in routine pathological practice is limited by i) the technical complexity in carrying out double immunostaining in a pigmented tumour such as melanoma, ii) the subjectivity in the selection of the hot-spot areas, and iii) the extremely time-consuming methodologies needed to evaluate lymphatic and blood vessels immunoistochemical markers in primary melanoma and sentinel lymph node.

Several studies have investigated lymphatic vessel density leading to different results [13]. Density of lymphatic vessels have been both correlated with presence [7, 8, 21-23] or absence [24-28] of lymph node metastasis. However, these studies were highly heterogeneous in their patients selection, for instance not all patients underwent SLNB, lymphatic markers used, and methodology to assess angiogenesis and lymphangiogenesis [13]. Storr et al [27], who conducted a high qualitative study when guidelines of lymphatic marker assessment were considered [19] found similar lymphatic density, expressed as number of lymph node vessels, both in the intratumoral and peritumoral area of SLN negative and positive patients. Shayan et al, who defined lymphatic vessel density considering the degree of patency of lymphatic vessel lumina confirmed this lack of difference while they showed a difference in the intratumoral and peritumoral area [23]. Massi et al instead considered the number and area of lymphatic vessels both intratumoral and peritumoral and did show a correlation with SLN metastasis [22]. The present study differs from the previously reported as it has used a strict and reproducible methodology, with evaluation conducted blindly

by two experienced pathologists in skin tumours. However, the inverse correlation between increased lymphatic density and negative SLN is counterintuitive at first glance and deserve further investigations. We observed that presence of tumour regression or extensive tumour infiltrate can influence the presence of lymphatic vessel density as well the ability of pathologists to detect it. For instance, peritumoural lymphatic density, outside the areas with regressive features, is higher in tumours showing regression compared to those that do not. Tumour regression has been considered a protective factor for SLN metastasis [29-32] and this evidence was confirmed in this analysis.

Pastushenko et al investigated 44 SLN-positive melanoma patients to assess association with NSLN metastasis and prognosis [14]. NLSN metastasis developed more frequently when peritumoral lymphatic and blood Proliferation Indexes and intratumoral Chalkley scores were high [14]. This is in line with our finding that high peritumoral and extratumoral lymphatic Proliferation Indexes were associated with NSLN metastasis, despite the differences in the populations (a consecutive cohort of patients in this paper and a cohort of patients with large-sized SLN metastasis in the study by Pastushenko et al) [14]. These results can have implications for the management of patients with lymph node metastasis. Firstly, markers of proliferation of lymphatic vessel can be add to existing predicting models for NSLN metastasis to enhance the predictive accuracy of currently used clinical and pathological variables [33-35]. This is particularly timely since two RCT has demonstrated lack of effectiveness of routine immediate CLND for SLN-positive patients [36, 37]. New guidelines from American Society of Clinical Oncology and Society of Surgical Oncology suggest that high risk SLN-positive melanoma patients, that includes those who are likely to harbour NSLN metastasis, need to be thoroughly counselled about the potential risks and benefits of foregoing CLND and been monitored [38, 39]. Following this approach roughly 20% of patients with SLN melanoma metastasis will develop regional lymph node recurrence and will undergo delayed regional lymphadenectomy, with significant implication for the burden of post-operative complications, especially lymphedema [40]. Also, the impact of a regional lymph node recurrence after a positive SLN on patient prognosis is poorly understood, although there is evidence that this specific subgroup of patients may be characterised by a negative prognosis [41]. The identification of patients who may harbour NSLN metastasis after a positive SLNB can have implications

also to optimize the selection of the adjuvant regimens. For instance, the anti-CTLA4 monoclonal antibody ipilimumab [42] and the combination of this drug with the anti-PD1 monoclonal antibody nivolumab [43] has showed greater activity in patients with multiple positive lymph nodes, that represents patients with both sentinel and non sentinel positive lymph nodes.

In conclusions, this study highlighted the predictive value of primary melanoma lymphatic vessel invasion and density, when detected in the peritumoral area, for SLN metastasis as well as that of the lymphatic proliferation index for NSLN metastasis. These findings have implication for prediction of patients with SLN and NSLN metastasis and thus for patient selection for SLNB and CLND. The association of peritumoral lymphatic invasion and vascular invasion in the SLN and patient survival suggest these features as potential biomarkers for investigating efficacy of anti-VEGF agents in melanoma patients with lymph node metastasis.

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CHAPTER 10

Overall conclusions and future perspectives

This PhD project has offered insights into the stratification of patients with high-risk CM and STS with possible implications for perioperative therapies. It encompassed a brunch of analysis on patient outcomes and translational data, which stemmed from clinical observations.

Issues for treatment of patients with high-risk STS have been discussed. A prognostic tool based on commonly available easy-to-obtain and reproducible staging clinic-pathological features was used to identify patients likely to benefit from perioperative anthracycline based chemotherapy. Data on sarcoma differentiation, with hints on the role of immune system in these tumours, which is almost unexplored, have been provided focusing on a single STS, that is dedifferentiated liposarcoma. Future studies that exploit sarcoma tumour models and deep genome sequencing for identifying optimal treatment strategies for these patients are ongoing.

Prognosis of patients with high-risk skin melanoma was also analysed generating the hypothesis that the process of melanoma progression through lymphatics is driven, at least in part, by lymphangiogenesis. Lymphatic and vascular progression of primary melanoma through lymph nodes have been extensively

examined. Further evaluations using image analysis are in progress to determine whether assessment of lymphangenetic markers will have a role for melanoma patients with lymph node metastasis.

Implementing and enriching translational study is the way forward to stratify patient outcomes, selecting patients for the best available therapy, and identifying new treatment options.

Future projects

A new international multicentre randomised controlled trial (RCT), the STRASS-2 study, will investigate preoperative chemotherapy for primary retroperitoneal high grade dedifferentiated liposarcoma (DDLPS) and leiomyosarcoma (LMS) and will be launched in 2020. STRASS-2 will randomised 230 patients over 3-5 years to receive either surgery alone or neoadjuvant chemotherapy with epirubicin plus ifosfamide or adriamicin plus dacarbazine for retroperitoneal liposarcoma or leiomyosarcoma, respectively, followed by surgery.

Co-clinical trials employing tumour models such as patient-derived xenografts (PDXs) have implications for stratifying patient risk and identifying those who will benefit from a specific treatment regimen. PDXs are generated by the implantation of surgically resected tumours into immuno-compromised mice and retain the main molecular characteristics for several passages, closely recapitulating the original heterogeneity. An advantage over cell line-derived xenografts relies on their ability to better predict tumour response to specific treatments, to test new therapeutic agents, and to provide insights useful for drug scheduling and understanding mechanism of tumour resistance or response.

The development of predictive preclinical models could accelerate the evaluation of anticancer agents and therapeutic combinations for retroperitoneal sarcoma. The enrollment of PDX models in a co-clinical trial that mirrors an ongoing human RCT allows real-time integration of preclinical and clinical data finalized to identify predictive genomic biomarkers, design rational treatment strategies for relapsing patients, and generate hypotheses for future clinical trials.

We therefore plan to: 1) generate retroperitoneal high grade DDLPS and leiomyosarcoma PDX models for testing the activity of several drugs already available for sarcomas or other tumours; 2) test effectiveness of epirubicin plus ifosfamide and adriamicin plus dacarbazine for high grade DDLPS and LMS, respectively, in a co-clinical study on PDX models derived from patients enrolled in the above mentioned STRASS-2 trial; 3) generate PDXs from recurrent tumours of patients in the STRASS-2 trial and assess the efficacy of treatment strategies, which have been deemed effective in Aim1, in resistant models.

To date, 17 consecutive patients with untreated primary DDLPS, harbouring a spectrum of tumour differentiation including rhabdomioblastic, myogenic, or "homologous" lipoblastic differentiation underwent surgery and had their tumour sampled for model generation. Six PDXs models (35%) representing all the above mentioned type of tumour differentiation have been successfully established, based on three mouse-to-mouse passages and growth characteristics. The histopathology and the main molecular characteristics have been already compared with the corresponding clinical tumours and their origin was confirmed. We already established PDXs for epithelioid sarcoma (unpublished data) with the same methodology which were also used for in-vivo evaluation of standard chemotherapeutics (e.g. epirubicin, ifosfamide, and dacarbazine), targeted drugs (e.g. pazopanib), and epigenetic agents (e.g. tazemetostat) alone or in combination.

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